NEUROMODULATION OF ACTIVITY-DEPENDENT SYNAPTIC PLASTICITY BY SEROTONIN AT CRAYFISH NEUROMUSCULAR JUNCTION

by

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Neuromodulation of Activity-dependent Synaptic Plasticity by Serotonin at Crayfish Neuromuscular Junction

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Abstract

Recovery of activity-dependent synaptic enhancement (ADSE) after moderate trains of action potentials (30 secs, 15 Hz) exhibits characteristic rapid (τ<1 sec, facilitation) and slow (τ $\approx$10 secs at 15.5°C; augmentation) phases at crayfish neuromuscular junction (nmj). Serotonin (5-HT) enhances the amplitude of individual excitatory junctional potentials (EJPs) by 2-5 fold by increasing the release of transmitter (e.g., Dixon and Atwood 1985; 1989). In the course of this study, a previously unknown neuromodulatory effect of serotonin was discovered. 5-HT (1μM) slows the recovery of the slow phase of ADSE by 3 fold (3.2±0.4, mean±SEM; n=12) without changing the initial magnitude of slow ADSE measured immediately after the train. Reducing extracellular [Ca^{2+}] by 50% reduces transmitter release but has little or no effect on the prolongation of ADSE by 5-HT, suggesting the prolongation is not an artifact of saturation of transmitter release. The prolongation of ADSE and enhancement of EJP amplitudes by 5-HT reverse with a similar time course during several hours of washing (slope=0.9, r=0.8). Action potentials recorded during and shortly after stimulus trains show no additional 5-HT-associated changes in width or height. As well in 5-HT-modified saline, the hyperpolarization of presynaptic membrane potential which builds up during tetanic stimulation recovers to pre-tetanus levels at the same rate as that in normal saline. Imaging presynaptic terminal with the fluorescent Ca^{2+} indicator fura-2 revealed that serotonin does not significantly alter intracellular calcium dynamics. The effects of 5-HT on the slow phase of ADSE, thus, do not result from increased intracellular Ca^{2+} buffering, by changing either the amount or the affinity of endogenous Ca^{2+} buffer, nor are they consistent with a slowing of Ca^{2+} removal by pumps. Injection of exogenous Ca^{2+} buffer (EDTA) may further augment the prolongation of ADSE caused by 5-HT, suggesting an effect of residual calcium in this phenomenon. A computer simulation of a first order kinetic model suggests that reducing the K_d (dissociation constant) of a calcium-driven reaction can not account for both the prolongation of recovery and the lack of change of the initial amplitude of slow ADSE in a simple manner. Neuromodulation of the time course of decay of ADSE may be important for

the implementation of time-integrative properties of neural networks particularly for storage of short-term memory "traces".

Dedication

This work is dedicated to my grandma for her great love

Acknowledgments

This graduate work was made possible through the valued contributions of several individuals to whom I am indebted:

- Dr. Kerry Delaney, who guided me into this wonderland of Neuroscience, providing the expertise, funds and time throughout this project. His energy and dedication to work have always given me the impetus to progress and perfection.

- Dr. Glen Tibbits, whom I feel lucky to have had on my supervisory committee. Beyond his valuable insights into my research, he helped me achieve scientific growth with his inspiring approaches, and did so in a kind, thoughtful manner.

- Dr. Norbert Haunerland, who taught me how to understand biochemistry; Dr. Gerald Sholomenko, who patiently "educated" me in the basics of the neurophysiology lab. I am also thankful for the generous help offered by Dr. Thor Borgford on calcium-binding reactions.

Thanks go to my fellow students: Ian, Ben, Sean (my lab-mates), Curtis (a nice neighbor) and Kenny (a helpful buddy), who created such a friendly and warm atmosphere around me and spent lengthy time proofreading my manuscripts. I also wish to thank Department of Biosciences for having friendly financial policies supporting international graduate students.

I would like to express my gratitude to my grandparents, parents and extended family for their firm support throughout these years. Especially, I would like to thank my grandma for her wonderful love, her extraordinary open-mindedness and her personal strength she showed during difficult times, which will prove to be three most precious gifts I can ever receive for my life. My heartfelt thanks go to my parents. They are the persons who elicited my scientific interest and painstakingly nurtured it so that it could grow. I also deeply appreciate their constant encouragement over the years which really helped mold my internal drive for excellence.

Last, but not the least, many thanks go to hui, for his remarkable patience, caring and support, and most of all, for always being there whenever I needed during these eight hundred and two days and nights since we met on that "gloomy" day.

Table of Contents

List of Tables

List of Figures

Chapter I
Introduction

1.1 Theoretical background of synaptic plasticity

1.1.1 General classification of synaptic plasticity

Neural connections are not static. The strength of synaptic connections between nerve cells is subject to modulation that depends upon the history of activity and the chemical environment of the nervous system. This remarkable feature of synapses, commonly termed synaptic plasticity, is thought to underlie the adaptive capability of various neural circuits (Jessel and Kandel 1993). Since the processes of learning and memory exemplify how the complex circuitry of the brain can be adapted to the changing environment and self-modified by experience, neurobiologists have long believed that the elementary aspects of these two essential brain functions, are likely to be interpreted in terms of synaptic plasticity (review see Hawkins et al. 1993). Consequently, synaptic plasticity has been a main focus of study during the past decades.

Many forms of synaptic plasticity have been identified (e.g., Dudel and Kuffler 1961; Kandel and Tauc 1965a, b; Magleby 1987). Modification of synaptic strength could occur either presynaptically or postsynaptically. Plasticity with a presynaptic origin is ultimately expressed as a change in the amount of transmitter released from the presynaptic neuron, whereas postsynaptic regulation of synaptic efficacy could result from the alteration of sensitivity of postsynaptic receptors responding to the transmitter released from presynaptic terminals. Synaptic transmission provides a major framework for communication between nerve cells. Neuromodulation of synaptic transmission, thus, imparts special importance on many aspects of synaptic function. Neuroactive substances, either from an adjacent neuron or from a remote endocrine organ, can diffuse through the extracellular fluid to reach the receptors on either the presynaptic or postsynaptic neurons. By coupling with membrane receptors and initiating biochemical cascades in neurons, these neuromodulators regulate synaptic transmission by greatly diversified means. In addition to modulation from external sources, synaptic strength can also be changed by the activity

within the pre- and postsynaptic neurons themselves through a process called activity-dependent synaptic plasticity (see Figure 1).

This project is designed to study serotonergic modulation of a form of activity-dependent synaptic plasticity called augmentation. Both forms of plasticity have been suggested to increase the probability of transmitter release from the presynaptic neuron (Dudel 1988; Mintz and Korn 1991; Zucker 1993).

1.1.2 Modulation of transmitter release

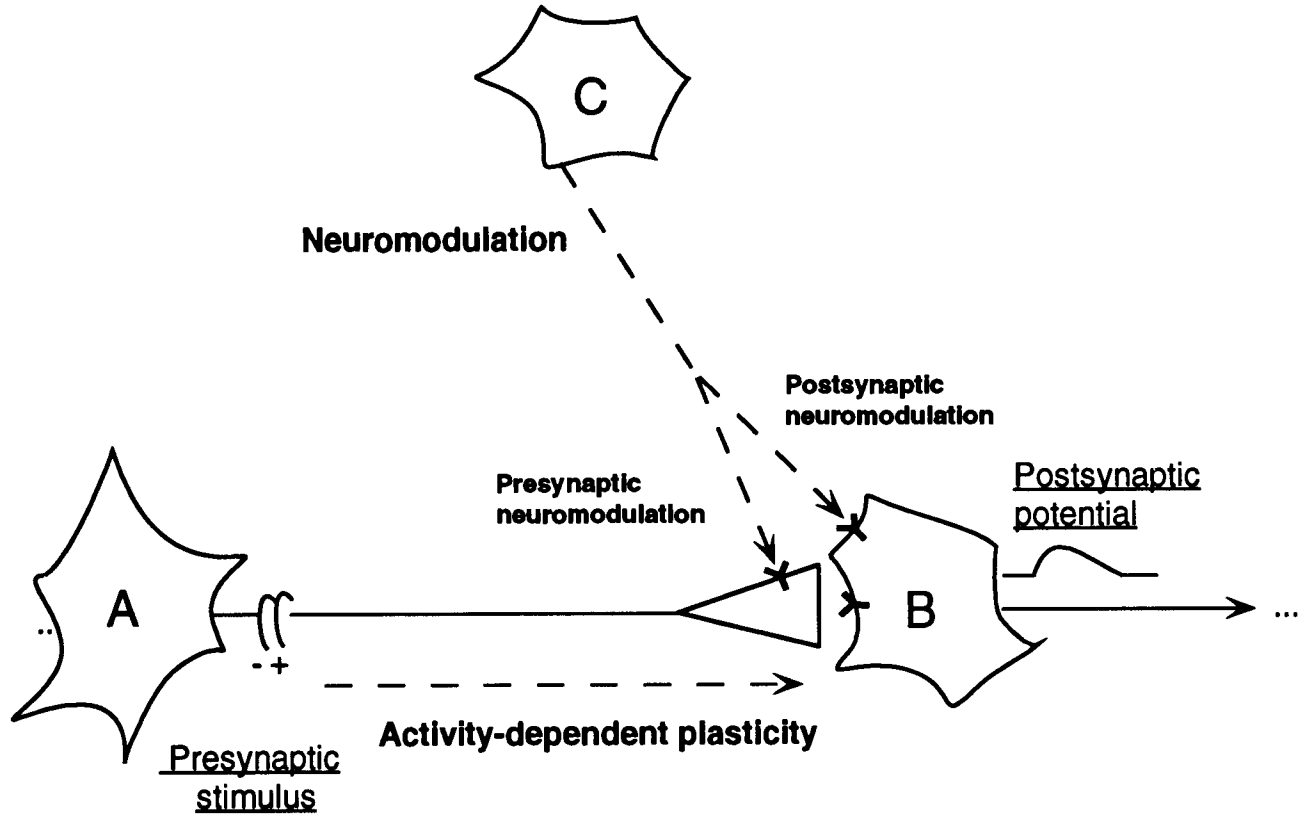
The basic steps of transmitter release are well established. Each synaptic vesicle, which is thought to contain an approximately equal number of neurotransmitter molecules (Fatt and Katz 1952), provides the anatomical basis of quantal release (Ceccarelli and Hurlbut 1980; Del Castillo and Katz 1956). These vesicles dock at specialized release sites located at active zones along the membrane, and subsequently fuse with the membrane to release their transmitter molecules. The release of each quantum is probabilistic. In the absence of action potentials, there are only a few quantal packets of transmitter occasionally released (spontaneous release), giving rise to miniature postsynaptic potentials (Kuffler et al. 1984). When an action potential arrives at the terminal, voltage-gated calcium channels are opened, resulting in a transient local elevation of cytosolic calcium concentration (Llinas et al. 1992). This elevation greatly enhances the release probability of synaptic vesicles, which results in massive transmitter release.

Once transmitter is released, it diffuses through the synaptic cleft between the presynaptic terminal and postsynaptic target sites, and then binds to the receptors on the postsynaptic membrane. The binding consequently allows specific ions to flow across the postsynaptic membrane, thus generating the postsynaptic potential (PSP). At invertebrate neuromuscular junctions (nmjs), an excitatory PSP is often called excitatory junctional potential (EJP). The amplitude of the PSP is often used as a measure of the efficacy of synaptic transmission.

Many steps in the release process could be regulated such as the mobilization of vesicles from a reserve pool into a releasable pool (discussed in more detail in 1.2.1). Modulation of each

Figure 1. A schematic illustration of types of synaptic plasticity.

Changes in synaptic strength could take place in either the presynaptic neuron (A) or postsynaptic neuron (B). Synaptic plasticity can be subdivided into two major groups based on the source of the modulation: neuromodulation by modulatory substance released from other neurons (C) or remote excretory organs, and activity-dependent synaptic plasticity as a result of the neural activity in neuron A or neuron B. Presynaptic neuromodulation by serotonin and presynaptic activity-dependent augmentation are the two types of plasticity involved in this project.


C
Neuromodulation
Postsynaptic
neuromodulation
Presynaptic
neuromodulation
Postsynaptic
potential
A
B
...
- +
Activity-dependent plasticity
Presynaptic
stimulus
PRESYNAPTIC NEURON
POSTSYNAPTIC NEURON

step during synaptic transmission provides physiological substrates for alteration of transmitter release. For decades, researchers have been trying to resolve synaptic plasticity into identifiable molecular events. While considerable progress has been made, especially in simpler invertebrate systems (e.g., Atwood and Wojtowicz 1986; Castellucci and Kandel 1976; Delaney and Tank 1991b; Klein et al. 1986; Magleby 1987; Regehr et al. 1994), there are still a lot of uncertainties concerning the fundamental mechanisms which operate at various levels of the neural hierarchy. Current research is conducted mainly at two different levels. At the cellular level, extensive efforts are being made to elucidate the cellular and molecular events underlying synaptic plasticity. From a behavioral perspective, researchers are attempting to fill the significant gaps between synaptic plasticity and behavioral modification. The aim of this study is to provide evidence that could contribute to a better understanding of nervous systems at both levels.

1.2 Previous studies

1.2.1 Augmentation

Postsynaptic potentials can grow or decline as a function of presynaptic activity due to changes in the amount of transmitter released. Practically all synapses, including neuromuscular junctions, exhibit some activity-dependent modification of transmitter release following repetitive stimulation of presynaptic neurons (review see Magleby 1987).

Activity-dependent synaptic enhancement (ADSE) builds up during a stimulus train and gradually decays afterwards. At crayfish neuromuscular junctions, several components of enhancement have been conventionally defined based on their rates of recovery after repetitive stimulation (Atwood et al. 1989; Atwood and Wojtowicz 1986). These include short-term facilitation (also called paired-pulse facilitation, composed of two components with time constants of about 50 msecs and 300 msecs), augmentation (with a time constant around 10 secs at 15.5°C), and posttetanic potentiation or PTP (with a time constant 1 min or more). Different frequencies and durations of stimulation may lead to different amounts of enhancement, but the time constants

for those components of enhancement remain unchanged, except that of PTP (Magleby 1987), which persists longer after either a longer or higher frequency stimulation.

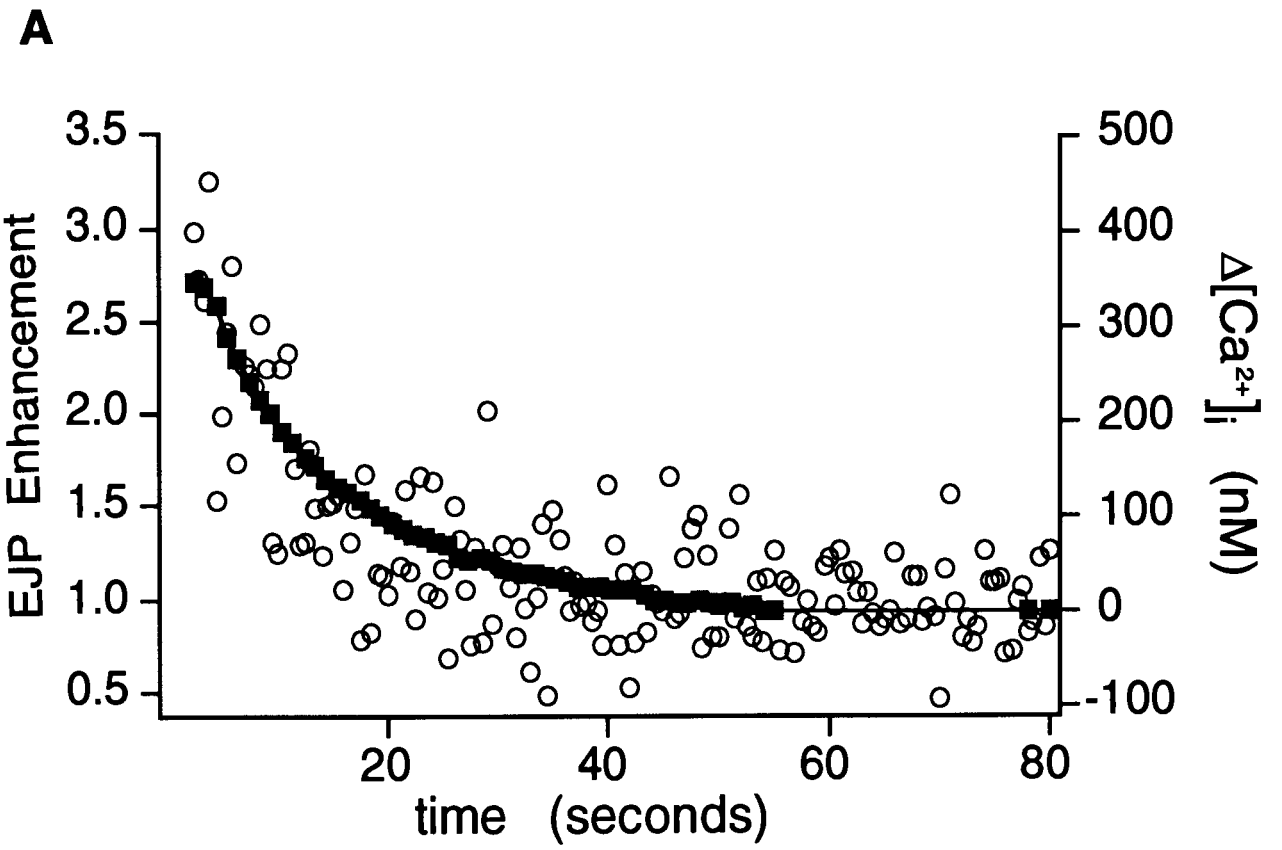
During repetitive stimulation, calcium ions accumulate in the presynaptic terminal because the rate of Ca^{2+} removal from cytoplasm initially lags behind the rate of Ca^{2+} influx via voltage-gated calcium channels (Charlton et al. 1982; Delaney and Tank 1991b; Tank et al. 1995). This accumulated free Ca^{2+} is termed residual calcium. A residual calcium model has been proposed to account for some or all forms of presynaptic activity-dependent synaptic enhancement (Katz and Miledi 1968). In this classical model, the accumulation of intracellular Ca^{2+} during a train of action potentials is responsible for buildup of synaptic enhancement. The recovery of calcium concentration back to resting levels somehow determines the decay phase of synaptic enhancement.

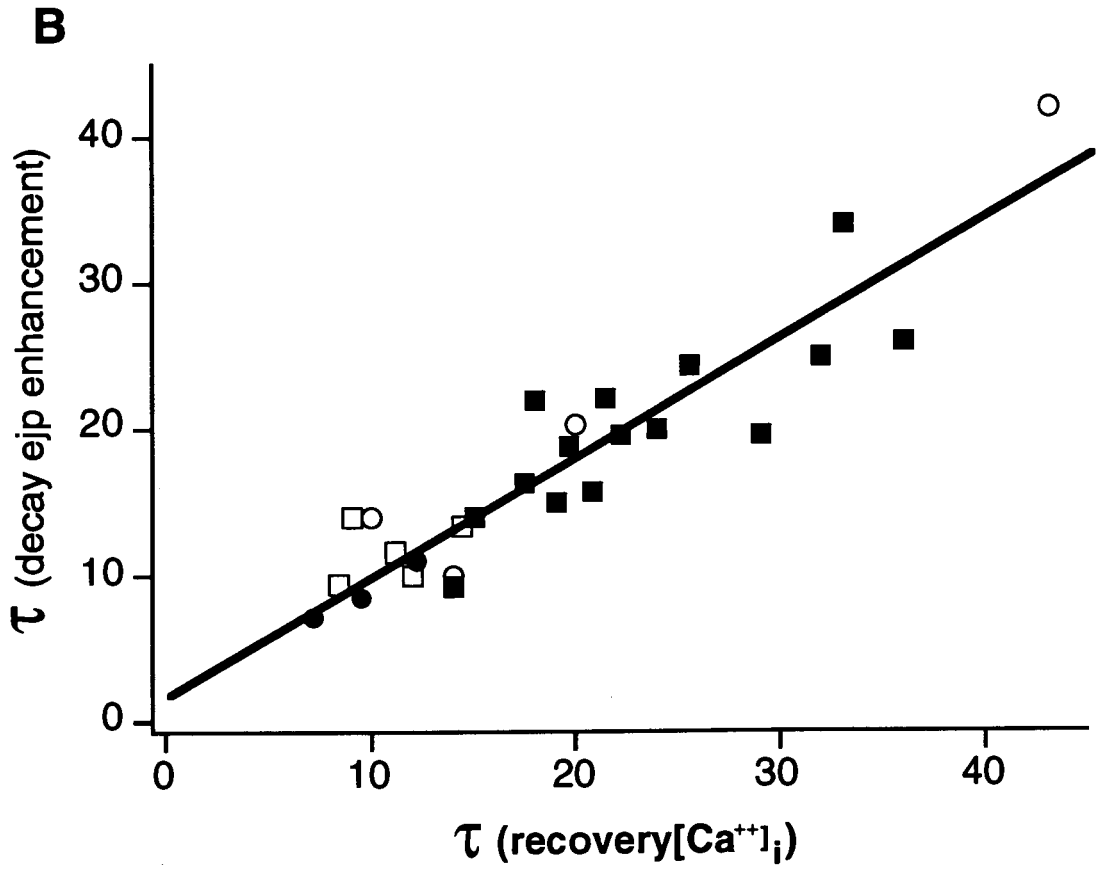
A considerable body of evidence suggests that residual calcium contributes directly to some forms of synaptic enhancement (e.g., Augustine et al. 1987; Connor et al. 1986; Delaney et al. 1989; Erulkar and Rahamimoff 1978; Kamiya and Zucker 1994; Katz and Miledi 1968). Elevation of free Ca^{2+} in presynaptic terminals enhances both spontaneous and evoked-transmitter release (Charlton et al. 1982; Delaney et al. 1990; Erulkar and Rahamimoff 1978; Mulkey and Zucker 1991). In contrast to evoked transmitter release which occurs quickly after calcium influx (< 1 millisecond, Cope and Mendell, 1982; Llinas et al. 1981), the relatively slow process of synaptic enhancement seems to allow adequate time for more biochemical reactions to participate in regulating transmitter release. Thus, transient calcium influx not only causes immediate transmitter release, but could also provide an opportunity to modulate transmitter release by contributing to buildup of the longer-lasting residual calcium.

However, the precise role that Ca^{2+} plays in the modulation of transmitter release is still an open issue. Previous study using a Ca^{2+}-sensitive indicator (fura-2) to measure presynaptic calcium concentration shows that the time course of augmentation following tetanic stimulation is normally dictated by the time course of $[Ca^{2+}]_i$ decay at crayfish neuromuscular junctions (Delaney and Tank 1994) (see Figure 2). This result implies that, after a train, the removal of intracellular Ca^{2+} is the rate-limiting step in the recovery of this component of synaptic enhancement. Since

Figure 2. The recovery of synaptic enhancement and the recovery of residual calcium follow a similar time course.

(A) $\Delta[Ca^{2+}]_i$ (solid squares) and EJP enhancement (open circles) after a 4 sec 50 Hz train. Data for EJPs are averages of five trials with 1 Hz stimulation beginning 0.5 sec after the train and five trials beginning 1 sec after the train. The recovery of synaptic enhancement and the recovery of residual calcium follow a similar time course. (B) Comparison of the rate of decay of synaptic enhancement to the rate of decay of presynaptic calcium. Time constants were estimated using an exponential fitting function beginning 1.5-3 seconds following the termination of 50 Hz train. Different sets of symbols stand for the data obtained in different experiments under various conditions, e.g., difference in temperature or difference in the amount of exogenously injected Ca^{2+} buffer. *Solid line* is a linear fit to the data (slope = 0.84, r = 0.91, intercept = 1.98). There is a linear correlation between the recovery of residual calcium and the recovery of augmentation (figure and caption adapted from Delaney and Tank 1994).


A
EJP Enhancement
3.5
3.0
2.5
2.0
1.5
1.0
0.5
500
400
300
200
100
0
-100
Δ[Ca2+]i (nM)
20
40
60
80
time (seconds)


B
τ (decay ejp enhancement)
40
30
20
10
0
0
10
20
30
40
τ (recovery[Ca++]i)

augmentation can be driven by rather low and slowly changing $[Ca^{2+}]_i$ (600 nM, compared to the 100 μM needed to trigger fusion, Simon and Llinas 1985), it could be dominated by Ca^{2+}-mediated processes occurring some distance away from the membrane surface where fusion takes place. Ca^{2+}/calmodulin was suggested to activate CaM kinase II which phosphorylates synapsin I, leading to the mobilization of vesicles in the process of augmentation (Delaney and Tank 1994, see Figure 3). Identification of the target proteins of calcium, however, awaits the development of more selective molecular probes.

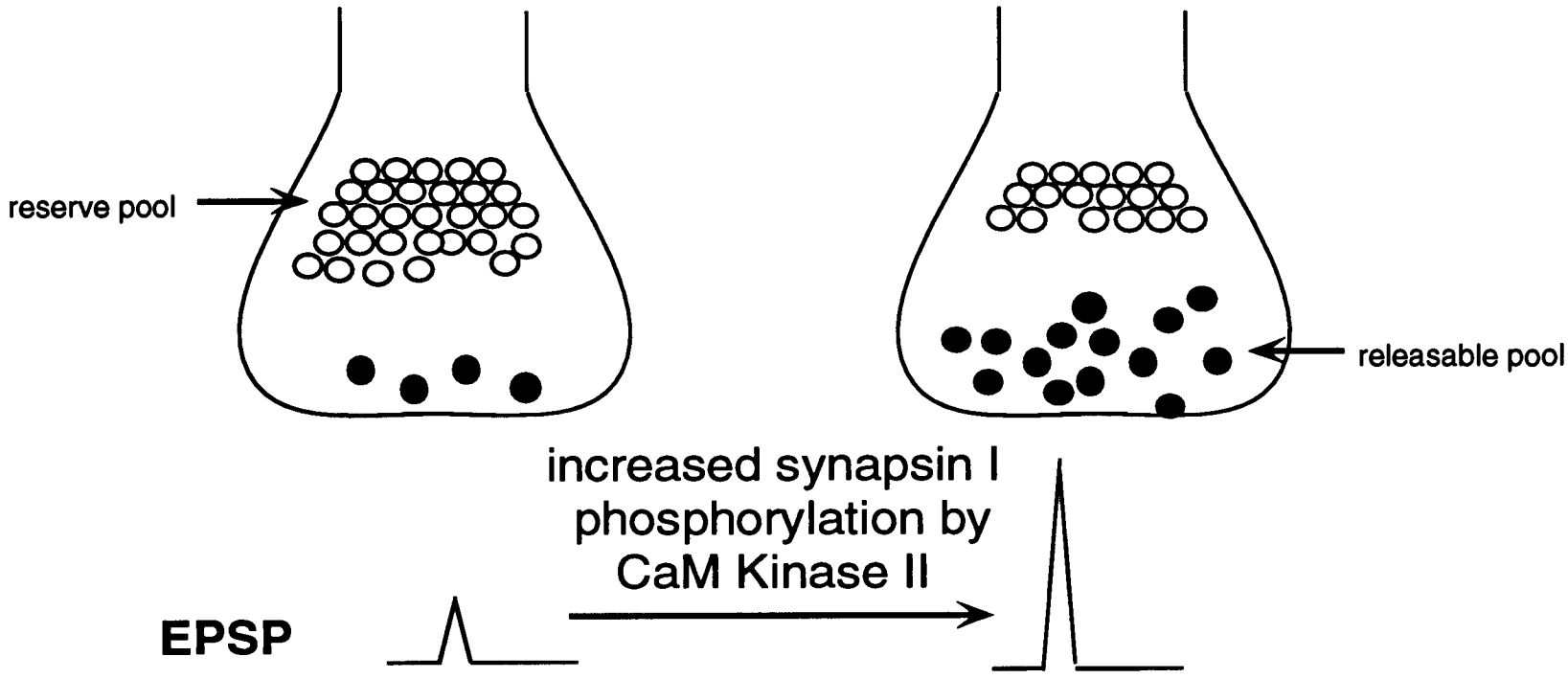
1.2.2 Serotonergic enhancement

Serotonin (5-hydroxytryptamine, 5-HT) acts as an excitatory neurotransmitter or facilitatory neuromodulator in vertebrate (Green 1985) and many invertebrate (Welsh and Moorhead 1960) nervous systems. A growing body of evidence suggests that serotonin plays an important role in a wide range of neural functions and defects in serotonin-mediated synaptic function underlie many diseases of the nervous system including schizophrenia, aggression and anxiety state (review see Leonard 1994). As many as ten 5-HT receptor subtypes have been identified in rat brain. While there is some controversy over the functional importance of many of these receptor subtypes, there is evidence that they fall into two major groups according to the nature of their coupling to secondary messengers or ion channels. One major group including the 5-HT1 and 5-HT2 receptor subtypes) appears to occupy the G protein receptor subfamily which may be coupled either to adenylate cyclase (via G_S, most 5-HT1 subtypes) or phosphatidyl inositol (via G_O, 5-HT2 subtypes). Serotonin, by binding these receptors, triggers a variety of second messengers which act on ion channels and other substrate proteins, leading to the modification of synaptic behavior.

Serotonin-induced behavioral sensitization at sensory-motor synapses of *Aplysia* (Castellucci and Kandel 1976; Castellucci et al. 1970; Hawkins et al. 1981; Pinsker et al. 1970) is a well-studied phenomenon whose mechanism links serotonin-induced synaptic plasticity with behavioral modifications. The cellular actions of serotonin, in this instance, seem to be exerted

Figure 3. Mobilization of synaptic vesicles from a reserve pool to a releasable pool.

Phosphorylation of synapsin I is proposed to play a functional role in the transition of vesicles from a reserve pool (open circles) to a releasable pool (solid circles). The amount of transmitter release (as represented by the size of EPSPs) could be regulated through this mechanism. This figure is adapted from Greengard et al. (1993).


reserve pool
releasable pool
increased synapsin I
phosphorylation by
CaM Kinase II
EPSP

through broadening of the action potential as a result of decreased K^+ efflux. This broadened action potential in turn results in an increased Ca^{2+} influx via calcium channels (Edmonds et al. 1990; Eliot et al. 1993), thereby enhancing evoked transmitter release from the sensory neuron. The molecular mechanisms underlying these changes involve both protein kinase A and protein kinase C pathways (Bernier et al. 1982; Ghirardi et al. 1992; Klein et al. 1986, 1993). Serotonin may broaden the action potential by activating adenylate cyclase to close K channels. Meanwhile, the activation of protein kinase C is thought to increase the availability of synaptic vesicles by mobilizing them to release sites. The second process is reflected in a substantial increase in spontaneous release which does not result from changes in intracellular free Ca^{2+}.

Enhancement of EJP amplitude by serotonin has also been observed in many crustacean neuromuscular junctions (e.g., Dudel 1965; Fisher and Florey 1983; Glusman and Kravitz 1982). Serotonin, as a circulating neurohormone in crustacea (Breen and Atwood 1983; Kravitz et al. 1985), exerts extensive modulatory effects on synaptic transmission through a wide range of presynaptic and postsynaptic actions (Atwood and Wojtowicz 1986; Harris-Warrick and Kravitz 1984). Brief exposure to serotonin leads to the enhancement of excitatory synaptic transmission, which involves both evoked (Dudel 1965) and spontaneous (Glusman and Kravitz 1982) transmitter release from motor nerve terminals. This serotonergic enhancement lasts about 30 minutes to 1.5 hours, decaying in two phases from an initial peak. Presently, the cellular and molecular mechanisms that underlie serotonin's facilitatory effect on crustacean neuromuscular junctions are not fully understood. Quantal analysis indicates that the action of serotonin is mostly presynaptic in origin (Dudel 1988). At the crayfish opener neuromuscular junction, neither increased calcium influx (Delaney et al. 1991a) nor spike broadening (Dixon and Atwood 1985) was observed during the application of serotonin. Thus, serotonergic enhancement at the crayfish nmj is clearly not induced by raising calcium influx, the mechanism well supported in *Aplysia.*

Nevertheless, the involvement of cyclic AMP and phospholipase C systems seems to be common in both *Aplysia* sensory-motor synapses and crustacean nmjs (Dixon and Atwood 1989a, b; Goy et al. 1984). An adenylate cyclase activator, forskolin (Seamon and Daly 1983) can partly

duplicate the physiological effects of serotonin in lobster (Goy et al. 1984). Brief application of forskolin to crayfish nmj also increases quantal release in a manner similar to that caused by serotonin (Dudel 1988). On the other hand, pharmacological manipulations which block the phosphatidylinositol system completely eliminate serotonergic EJP enhancement, while injection of inositol 1,4,5-trisphosphate (IP_3) into the terminal mimics the early phase of this facilitation. Thus, phospholipase C seems necessary to initiate the facilitatory effect induced by serotonin (Dixon and Atwood 1989a). According to these experimental observations, Dixon and Atwood (1989b) proposed a sequential scheme, in which the early phase of serotonin-induced facilitation is attributed to a serotonin-dependent activation of phospholipase C and the slower phase of the facilitation is dependent upon a slower activation of adenylate cyclase by protein kinase C. In this model, subsequent production of cAMP triggers a cAMP-dependent enzyme cascade to phosphorylate various target proteins, leading to the longer-lasting phase of enhancement.

In summary, the involvement of adenylate cyclase and phosphatidylinositol systems in the action of serotonin appears to emerge as a common theme. However, the target proteins of protein kinase A and protein kinase C, as well as the detailed intermediate biochemistry, seem distinct for different species, or even different body regions in the same species.

1.2.3 Interaction between augmentation and serotonergic enhancement?

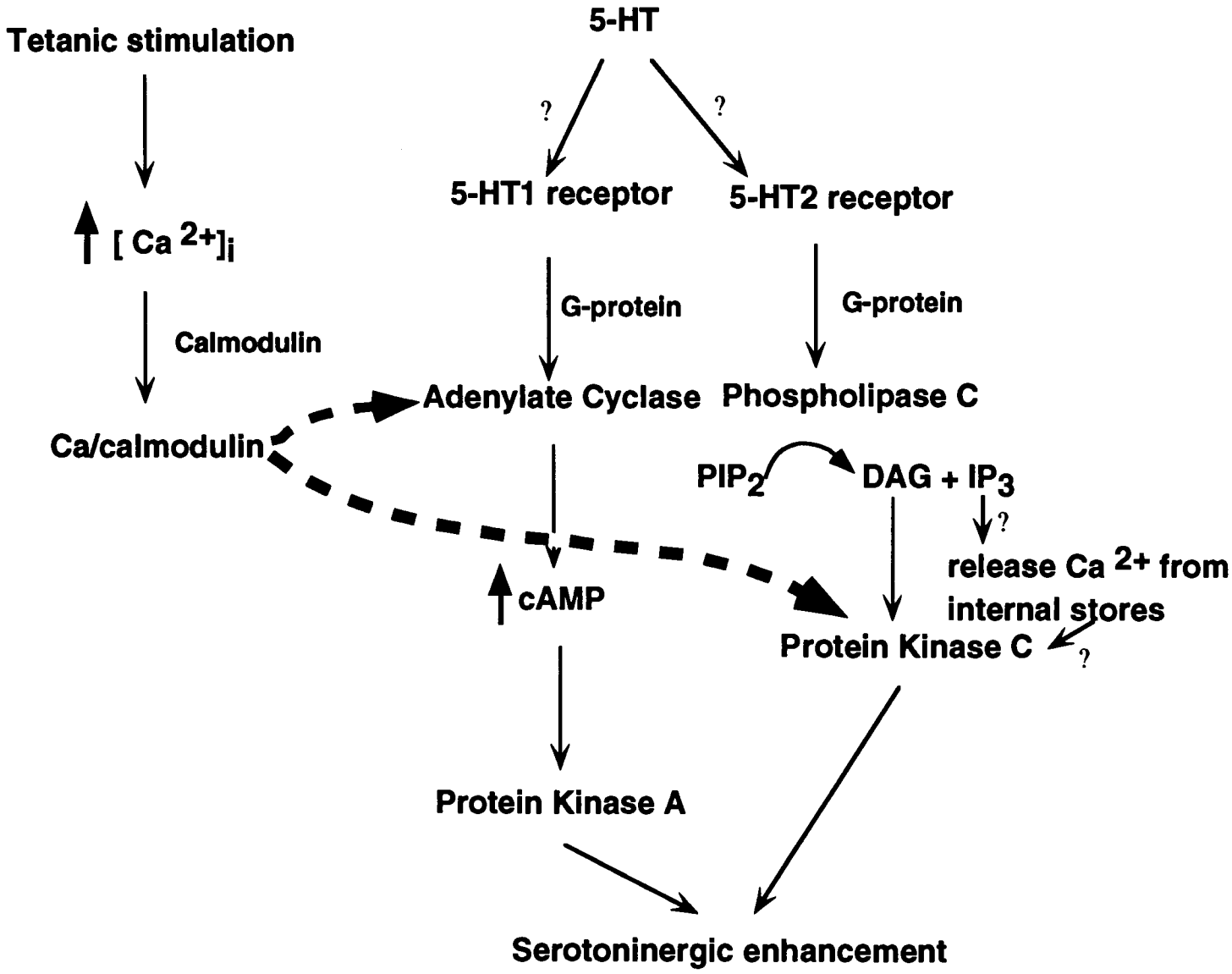
Different biochemical pathways can interact with each other in many different ways (review see Bourne et al. 1993). A specific example is the interaction of the serotonin-activated pathway and the Ca^{2+}-mediated pathway (presumably activated by presynaptic activity) in the classical conditioning of the defensive withdrawal reflex of *Aplysia* (Abrams et al. 1991; Hawkins et al. 1983; Walter and Byrne 1983). In this case, the combination of serotonin application and Ca^{2+} influx during a train of action potentials greatly enhances the production of adenylate cyclase by the interaction of these two separate pathways. The Ca^{2+}-mediated pathway has a synergistic effect on the serotonin-activated pathway, thus escalating the amount of transmitter release. An attractive feature of this activity-dependent presynaptic neuromodulation is that it could be a type of general

mechanism underlying all sorts of associative learning.

Calcium-dependent augmentation and serotonin-induced facilitation were chosen for this specific study, because these two components of enhancement have already been separately demonstrated and intensively studied at the crayfish neuromuscular junctions (e.g., Delaney and Tank 1994; Dixon and Atwood 1985). These previous studies have provided a basis for further investigation of interaction between the two forms. Augmentation, along with posttetanic potentiation (PTP), is closely linked to presynaptic calcium dynamics (Delaney et al. 1989; Delaney and Tank 1994). The calcium ions that accumulate in the terminal during tetanic stimulation may bind calmodulin to form a CaM complex. This complex can, in turn, activate various target proteins and eventually lead to the modification of transmitter release (Delaney and Tank 1994). Although there is one previous study using a calmodulin blocker suggests that calmodulin is not involved in augmentation (Kamiya and Tank 1994), one could also argue that the calmodulin blocker they used may not be able to block the activation of all calmodulin molecules since there are various types of calmodulin in the cell. On the other hand, serotonin has been suggested to ultimately act downstream of calcium influx in the process of transmitter release (Zucker 1993) through cAMP and phospholipase C pathways. There would be potential biochemical interactions between tetanic stimulaton that produces augmentation and serotonergic enhancement (see Figure 4) since it is known that both adenylate cyclase and protein kinase C can be regulated by Ca^{2+} via calmodulin (review see Casey 1995). Further studies on the interaction of these two processes instead of studying them separately, might bring us valuable insights into the molecular events underlying these two forms of enhancement from a fresh angle.

Figure 4. A conceptual biochemical scheme showing two examples of potential interactions between tetanic stimulation that produces augmentation and serotonergic enhancement.

Tetanic stimulation that produces augmentation, via calmodulin, can potentially exert an effect on PKA and PKC pathways.


Tetanic stimulation
5-HT
?
?
5-HT1 receptor
5-HT2 receptor
[Ca 2+]i
G-protein
G-protein
Calmodulin
Adenylate Cyclase
Phospholipase C
Ca/calmodulin
PIP2
DAG + IP3
?
release Ca 2+ from
internal stores
cAMP
Protein Kinase C
?
Protein Kinase A
Serotoninergic enhancement

Chapter II
General Materials and Methods

2.1 Crayfish neuromuscular junctions

2.1.1 Advantages

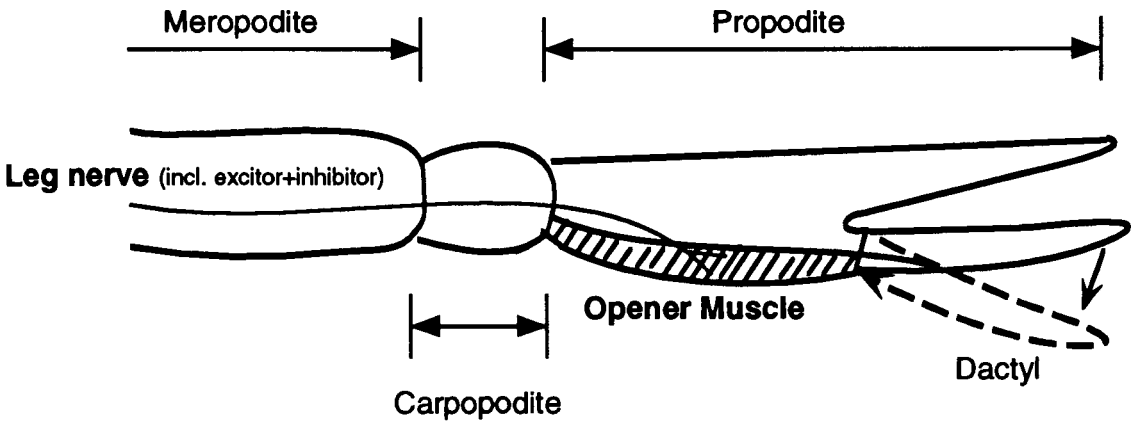
The complex circuitry of central nervous systems (even in invertebrates) presents difficulties for studying the cellular mechanisms of synaptic plasticity. The inaccessibility of individual neurons and the difficulty of penetrating pairs of pre- and postsynaptic neurons simultaneously can limit the level of analysis. Many crustacean neuromuscular junctions, on the other hand, provide accessible nerve terminals whose synapses show robust short-term (lasting ms to hours) and long-term (lasting hours to days or months) synaptic plasticity. Crayfish dactyl opener muscles (see Figure 5a) are innervated by both excitatory neurons which release L-glutamate (Onodera and Takeuchi 1976; Shank and Freeman 1976; Takeuchi and Takeuchi 1964) and inhibitory neurons releasing gamma-aminobutyric acid (GABA, Takeuchi and Takeuchi 1965) as transmitter compounds. This neuromuscular junction exhibits enormous facilitatory capability with little or no synaptic depression. Furthermore, the relatively small amount of transmitter released in response to a single nerve impulse potentially leaves a wide margin for synaptic enhancement at this synapse. These properties make the crayfish opener nmjs an ideal system for the study of synaptic plasticity.

Crustacean nmjs also share many features with central synapses. Their polyneuronal and multiterminal innervations are distinct from those at vertebrate mononeuronal endplates, and are more comparable to those of central synapses. Therefore, crayfish nmjs offer researchers interested in synaptic plasticity "central-type" synapses which are not easily accessible in most other animals.

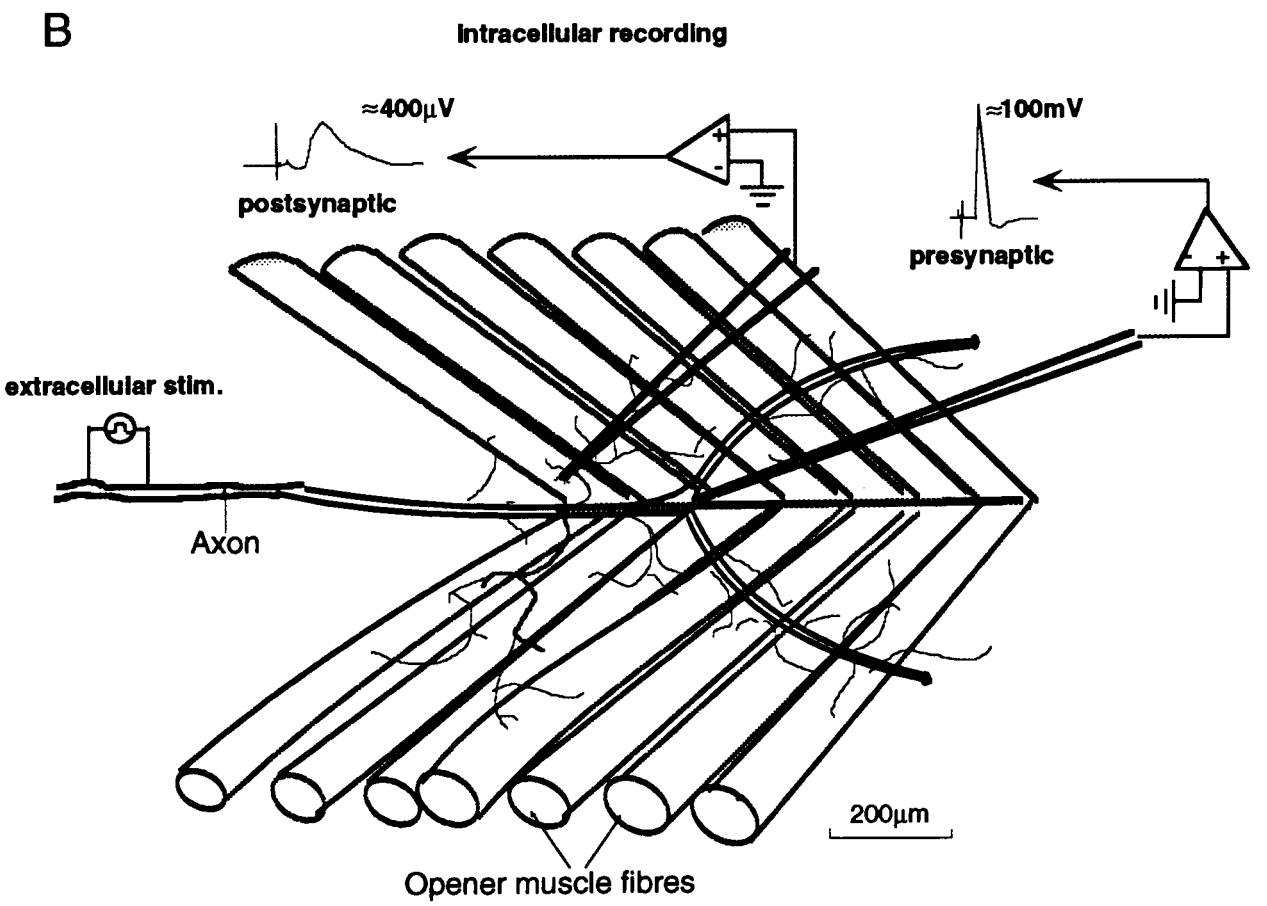
Figure 5. Schematic representations of (A) a crayfish claw and (B) an opener muscle preparation.

Stimulation of the excitor axon in meropodite results in an action potential (amplitude ≈ 100 mV) which can be recorded by an intracellular electrode in the axon. This electrode can also be used to inject EDTA and fura-2 by passing continuous hyperpolarizing current. Another intracellular electrode is placed in the postsynaptic muscle fibre, recording EJPs (around 400μV is a typical response to a single isolated action potential).

A


Meropodite
Propodite
Leg nerve (incl. excitor+inhibitor)
Opener Muscle
Dactyl
Carpopodite

B


Intracellular recording
≈400μV
postsynaptic
≈100mV
presynaptic
extracellular stim.
Axon
200μm
Opener muscle fibres

2.1.2 Preparation

All experiments were performed on the claw opener muscle of the first walking leg of the fresh water crayfish *Procambrus clarkii*. Animals were kept in shallow trays maintained at approximately 16°C. The first walking legs of medium-sized specimens (around 15 cm in length) were used. These legs, from the tip of the dactyl to the proximal end of the meropodite (also see Figure 5), were typically about 30-50 mm in length.

The two distal segments of the leg were pinned to the Sylgard-lined bottom of a 60 mm-diameter plastic petri dish. A small amount of Krazy Glue™ helped further stabilize the shell on the Sylgard surface. These procedures reduced the movement of the claw during high frequency stimulation which might otherwise dislodge the microelectrode from muscle fibres or axons.

By carefully removing the closer muscle and sensory nerve bundles, the dorsal surface of the opener muscles was exposed. The nerve bundle containing the opener excitor axon was then gently isolated from other nerve bundles in the meropodite (one of them contains the inhibitor axon which also innervates these muscle fibres). The bundle was subsequently sucked into a small plastic suction electrode to selectively stimulate the excitor axon.

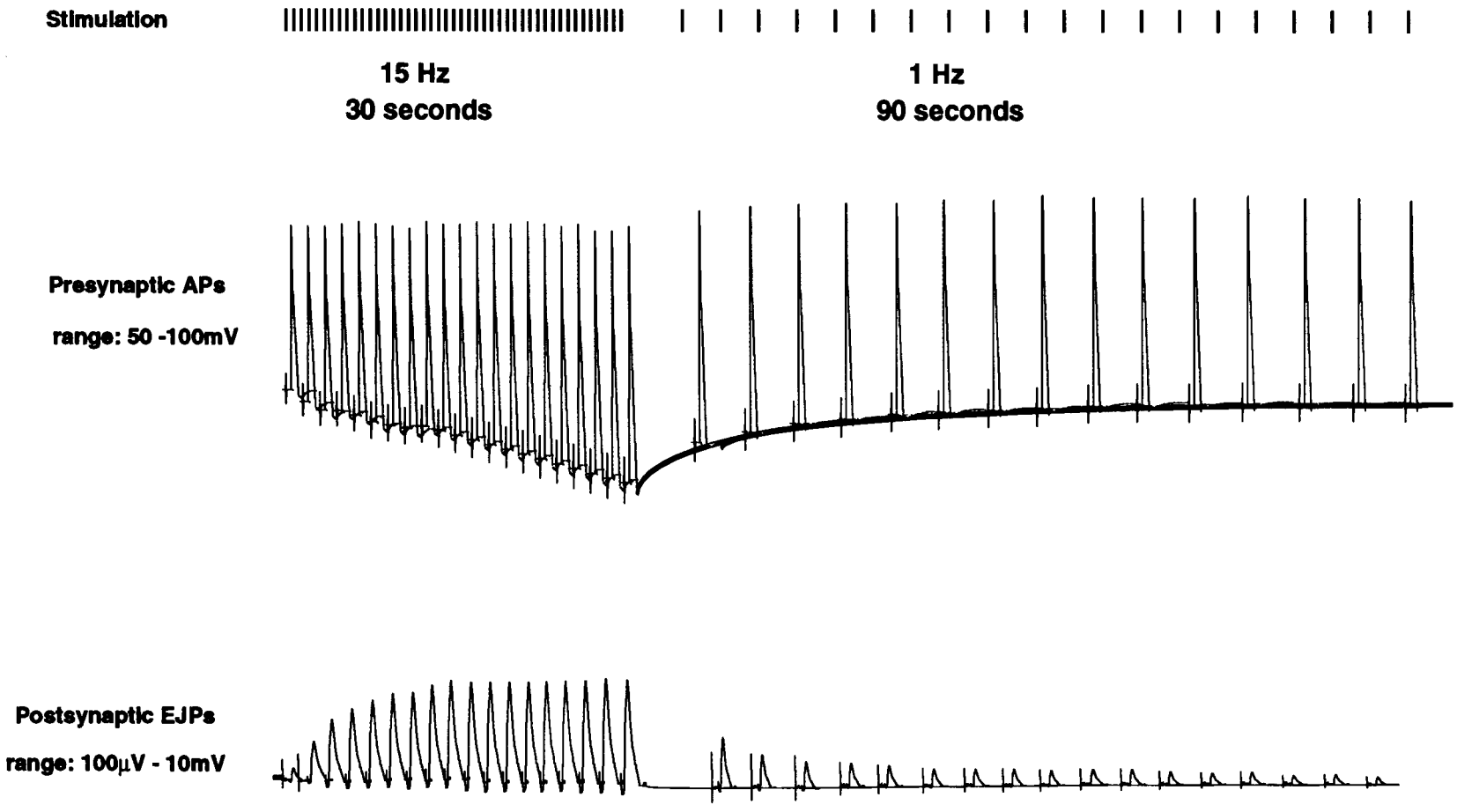
2.2 Stimulation paradigms and electrophysiological recording

2.2.1 Stimulation

A digital stimulator (PG4000, Neural Data, New York, NY) produced stimulation signals which were then delivered to the suction electrode via a constant current-stimulus isolation unit (SIU90, Neuro Data). The standard experimental protocol consisted of a 15 Hz 30 sec conditioning train to build up augmentation (without significant production of PTP), followed by a 1 Hz 90 sec train to map out the decay phase of augmentation (see Figure 6). The 1 Hz testing impulses were arranged to start approximately 1.5 secs after the termination of the 15 Hz train so that most short-term facilitation (F1, F2) had decayed before the onset of the testing trials. This

Figure 6. Schematic illustration of presynaptic and postsynaptic responses to the standard stimulation paradigm used for testing augmentation.

A 15 Hz 30 seconds conditioning train is delivered to build up synaptic enhancement. 1.5 seconds after the tetanus, 1 Hz testing impulses are used to map out the decay phase of synaptic enhancement. It is noted that presynaptic membrane potential has a slow component of hyperpolarization which gradually recovers after the tetanus.


Stimulation
15 Hz
30 seconds
1 Hz
90 seconds
Presynaptic APs
range: 50 -100mV
Postsynaptic EJPs
range: 100μV - 10mV

protocol allowed measurement of augmentation without significant contribution from short-term facilitation or PTP.

2.2.2 Electrophysiological recording

EJPs in the muscle fiber were monitored using an intracellular microelectrode filled with 3 M KCl (resistance 8-20 MΩ). For the purpose of this study, the muscle fibers chosen for impalement were within 1-4 mm from the very proximal end of the propodite. The amplitudes of unenhanced EJPs recorded from these individual muscle fibres ranged from 100μV to 400μV. Release probabilities are normally low from these synapses so usually 4 to 12 sets of responses (typically 12) were averaged in order to obtain a reasonable estimate of the time course of augmentation.

Intracellular electrodes were also used to detect action potentials from the excitor axon, although the resistance of the electrode was much higher than that used in recording EJPs (25-50 MΩ when filled with 3 M KCl and over 100 MΩ when filled with EDTA solutions). In order to penetrate the membrane of the excitor without doing serious damage to the axon, the electrode was advanced at an angle of slightly less than 30° relative to the horizontal level and tapped it lightly to aid penetration. As opposed to EJP recording, averaging is not needed for measuring action potentials.

An amplifier suitable for recording intracellular potentials (Neuroprobe Amplifier, Model 1600, A-M Systems, Everett, WA or Dual Channel Intracellular Recording Amplifier, Model IR-283, Neuro Data Instruments, New York, NY) was used to record EJPs and APs. The EJP signals were band-pass filtered (0.1-300 Hz) and amplified (x1000 gain). For recording action potentials, a x100 operational amplifier replaced this conditioner. Conditioned signals were subsequently digitized with a 12-bit A/D board (MacADIO II, GW instruments, Somerville, MA), and acquired on a Macintosh computer (Quadra 800 or 950). In some cases, an additional DC offset was added to the signals to avoid the saturation of the A/D board. Specialized software (Superscope II™, Cambridge, MA) displayed, averaged, and recorded the electrophysiological

signals for further off-line analysis. The exponential fitting of decay phases of enhanced EJPs was implemented with standard routines of a commercial program (Igor™, Wavemetrics, Eugene, OR). The general form of the exponential function used to fit the decay phase is $K_0 + K_1 * e^{(-t/\tau)}$. The goodness of fit was assessed by 1) the fitted line passed through the middle of the data; 2) the data were not better fitted by a double exponential function. The EJP amplitude at $t = \infty$ (K_0) estimated from the fitted function was not different from the average EJP amplitude before the tetanus. Therefore, for the sake of convenience, we used K_0 as a good estimate of the EJP amplitude before the tetanus when normalizing synaptic enhancement. For calculating the magnitude of slow ADSE at the end of the train, the value at $t = 0$, i. e., the sum of K_0+K_1 is, therefore, an approximation of the initial EJP amplitude at the end of the tetanus. The initial magnitude of synaptic enhancement A_0 (initial EJP amplitude/EJP control amplitude) was thus estimated by $(K_0+K_1)/ K_0$ in data analysis.

2.3 Control of temperature and perfusion

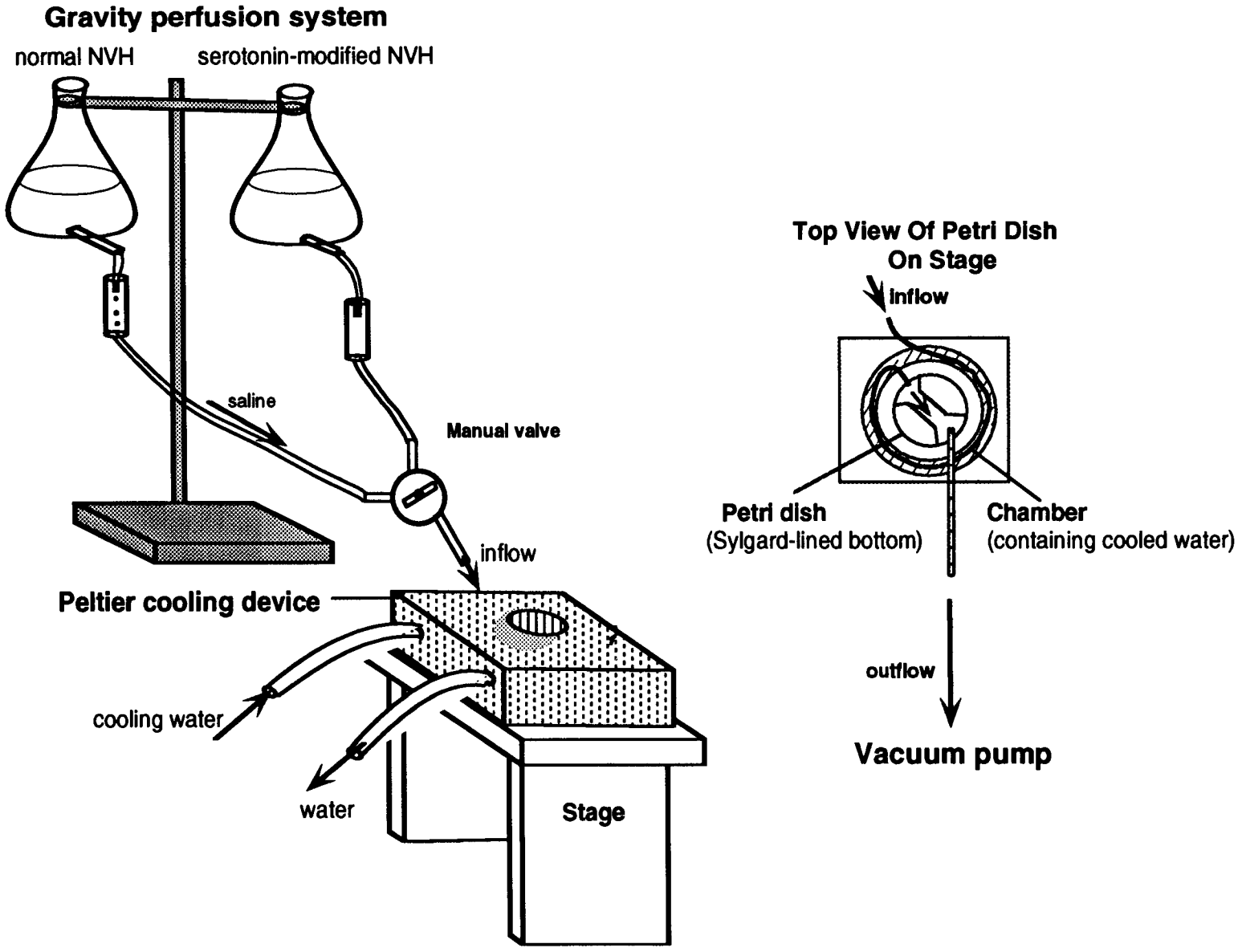
2.3.1 Cooling system

A Peltier heat transfer device was used to cool the preparation (see Figure 7). The temperature was monitored with a 1 mm thermister (Fluke, model 51K/J) probe submerged in the saline beside the claw. The temperature differed from one experiment to another in the range of 14°C-18°C. However, during each experiment, the temperature was held to ±1°C.

2.3.2 Perfusion

A gravity system was employed to perfuse normal and serotonin-modified salines (see Figure 7). A manual valve located near the preparation was used to switch between different salines. The rate of perfusion was approximately 1 ml per minute so that it generally took less than two minutes to fill the volume of the dish. The saline was precooled before entering the preparation chamber by flowing through a loop of tubing submerged in chilled water. An outflow

Figure 7. **A schematic diagram of the experimental setup for perfusion and cooling.**


Gravity perfusion system
normal NVH
serotonin-modified NVH
saline
Manual valve
inflow
Peltier cooling device
cooling water
water
Stage
Top View Of Petri Dish
On Stage
inflow
Petri dish
(Sylgard-lined bottom)
Chamber
(containing cooled water)
outflow
Vacuum pump

tube was placed at the opposite end of the dish. A series of small holes in the side of the tube near its tip helped make the rate of outflow self-regulated with changes in the bath level. A vacuum pump performed the removal of the saline.

2.3.3 Salines

The normal van Harrevald's saline (NVH) contained 195 mM Na^+, 13.5 mM Ca^{2+}, 5.4 mM K^+, and 2.6 mM Mg^{2+} with 10 mM HEPES and pH adjusted to 7.3-7.4. In order to reduce muscle movement and the possible saturation of transmitter release (as explained in Results below), salines containing 50% of normal calcium concentration were applied in some experiments. Serotonin solution (1 μM) was prepared fresh by diluting a 100μM stock serotonin solution (creatinine sulfate complex, Sigma, St. Louis, MO) into NVH saline. The stock serotonin remained stable for at least one month when frozen at -20°C.

Chapter III

Effect of Serotonin on Augmentation

3.1 Purpose

Initial experiments were designed to examine whether there was any effect of serotonin on augmentation. Further experiments were designed to characterize the magnitude and reversibility of the effects of 5-HT.

3.2 Protocol

The recovery of activity-dependent synaptic enhancement in the presence of serotonin was compared to that in the absence of serotonin in twelve preparations. Attempts were made to reverse the effects of serotonin by continuously rinsing the preparation with normal NVH in six preparations.

The standard protocol to examine the recovery of augmentation (stimulation at 30 secs at 15 Hz followed by 1 Hz for 90 secs) was applied repetitively at 3 minute intervals. The rest period between stimulus trains was introduced to reduce the possible buildup of PTP which might interfere with the measurement of augmentation. The preparation was exposed to salines in the following sequence: normal [Ca^{2+}] NVH for 1 hour, 1 μM serotonin in NVH for 2 hours, and then normal [Ca^{2+}] NVH for 2 hours to wash out 5-HT (see Figure 8). The details of dissection and signal acquisition have already been described in Materials and Methods (see Chapter II).

3.3 Results and data analysis

3.3.1 Control data

It was found that the decay time constant of augmentation was consistently longer in the presence of serotonin than that without serotonin (see Figure 9a, b). This phenomenon was highly

Figure 8. **Experimental protocol to examine the recovery time course of synaptic enhancement in the presence and in the absence of serotonin.**

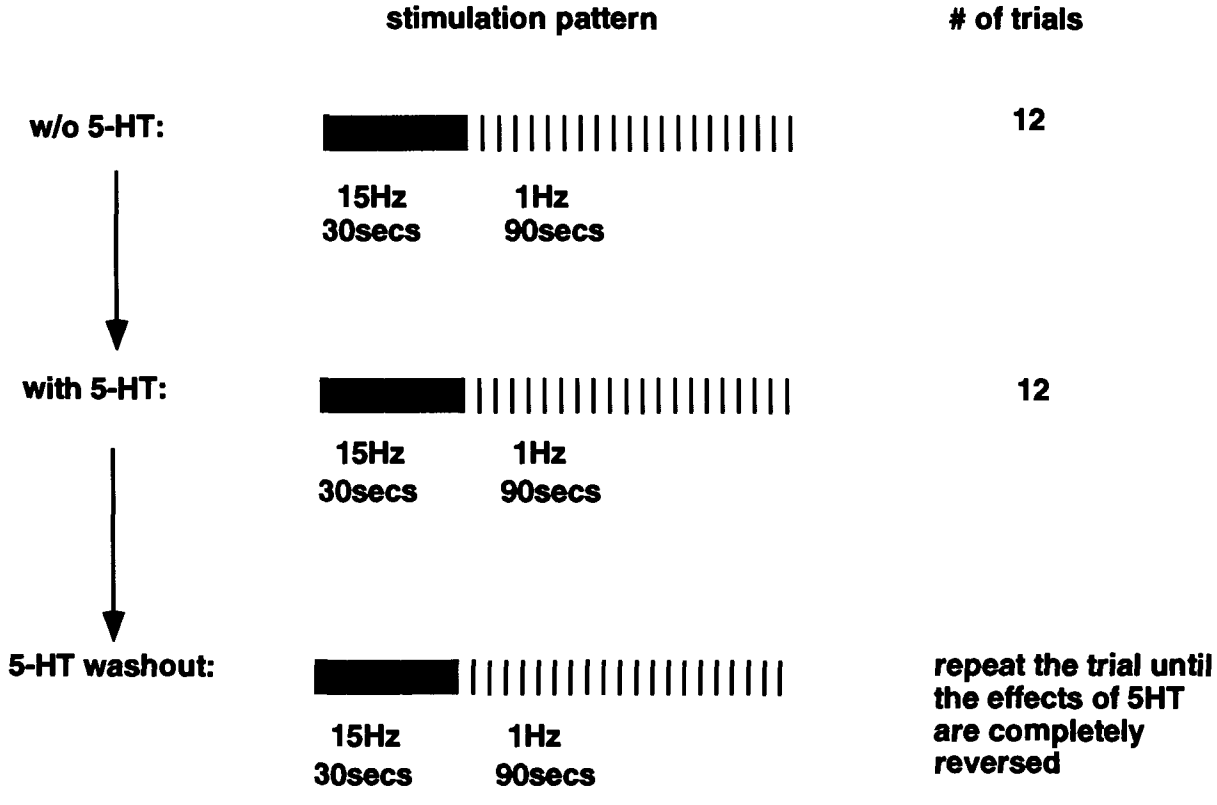
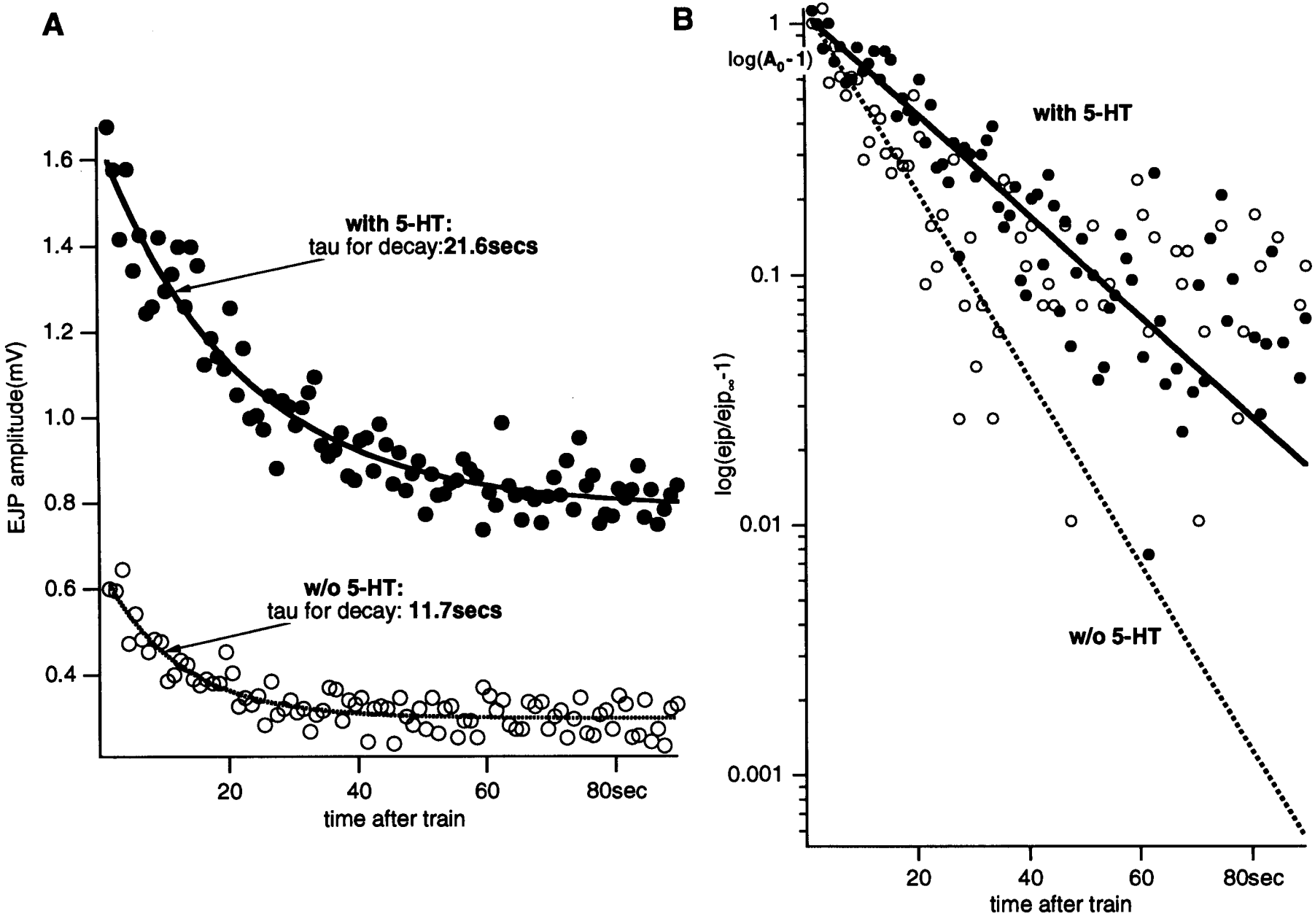

stimulation pattern
# of trials
w/o 5-HT:
12
15Hz
30secs
1Hz
90secs
with 5-HT:
12
15Hz
30secs
1Hz
90secs
5-HT washout:
repeat the trial until the effects of 5HT are completely reversed
15Hz
30secs
1Hz
90secs

Figure 9. Serotonin prolongs the recovery of synaptic enhancement.

The effect of serotonin on the recovery of synaptic enhancement on one claw after a 30 sec 15 Hz stimulus train. (A) *Open circles* are the amplitude of EJPs in the absence of serotonin averaged from 12 repeated trials. *Solid circles* are the amplitude of EJPs in the presence of serotonin averaged from 12 repeated trials. The data were collected on one preparation within 3 hours with temperature maintained at approximately 15°C. *The dotted line* is an exponential fit to the data without 5-HT with an estimated time constant of 11.7 seconds. *The solid line* is an exponential fit to the data during the application of 5-HT with an estimated time constant of 21.6 seconds. Compared to the recovery of synaptic enhancement without 5-HT, the decay of synaptic enhancement (mainly augmentation) is significantly prolonged by the application of 5-HT. (B) The logarithmic plot of (A) after the normalization of EJPs. The normalization procedure was implemented by dividing the amplitude of testing EJPs by the EJP control amplitude (in this case, under 1 Hz stimulation). Two control amplitudes (without 5-HT and with 5-HT) were approximated by the calculated values at $t = \infty$ according to their respective exponential fits. The magnitude of augmentation (A) during the test trial was thus estimated by the ratio of testing EJP amplitude to EJP control amplitude. The slope of the lines is equal to the reciprocal of the time constant for the recovery. It is also noted that A_0 (the initial magnitude of augmentation) after serotonin application remains the same as before the application of 5-HT.


A
B
EJP amplitude(mV)
1.6
1.4
1.2
1.0
0.8
0.6
0.4
with 5-HT:
tau for decay:21.6secs
w/o 5-HT:
tau for decay: 11.7secs
20
40
60
80sec
time after train
log(A0-1)
1
0.1
0.01
0.001
log(ejp/ejp∞-1)
with 5-HT
w/o 5-HT
20
40
60
80sec
time after train

reproducible (see Table 1).

Table 1. Comparison of the recovery time constant of synaptic enhancement with and without serotonin

Experiment #	τ w/o 5HT(sec)	τ with 5HT(sec)	τ with 5HT/τ w/o 5HT
1	9.8	36.7	3.8
2	12.6	19.7	1.6
3	12.0	44.8	3.8
4	11.4	29.6	2.6
5	11.7	21.6	1.8
6	11.1	27.3	2.5
7	14.9	66.3	4.5
8	17.3	35.2	2.0
9	5.4	25.9	4.8
10	13.6	34.9	2.6
11	3.7	23.2	6.4
12	13.0	35.0	2.7
Mean±SEM	**11.4±1.1**	**33.3±3.7**	**3.2±0.4**

A bar chart (Figure 10) was used to summarize the data.

A paired-data t-test was used to determine the significance of the difference between the two sets of time constants. With the calculated t =5.46 ($P(|t| \geq 5.46) < 0.001$), the time constant of recovery from EJP enhancement was significantly changed during the application of serotonin.

Further statistical test was carried out to determine the extent of difference between the two sets of time constants. Using the t distribution, the 95% confidence interval was computed according to $|\pm t_{0.01\ (2),\ n-1} * \text{SEM}|$ (=0.9). Therefore, $P[\ 3.2-0.9 \leq \mu_d \leq 3.22+0.9\ ] = 95\%$. This result means that the presence of serotonin will likely (95% confidence) increase the time constant of recovery from EJP enhancement by at least 230% (= 320%-90%).

Figure 10. A bar chart to summarize the difference in time constant of recovery from synaptic enhancement (n = 12).

The application of serotonin appreciably increased the time constant from 11.4±1.1 seconds (mean±SEM, n=12) to 33.3±3.7 seconds (mean±SEM, n=12). A paired data t-test shows a significant difference between the two sets of time constants ($P(|t| \geq 5.46) < 0.001$, n=12).

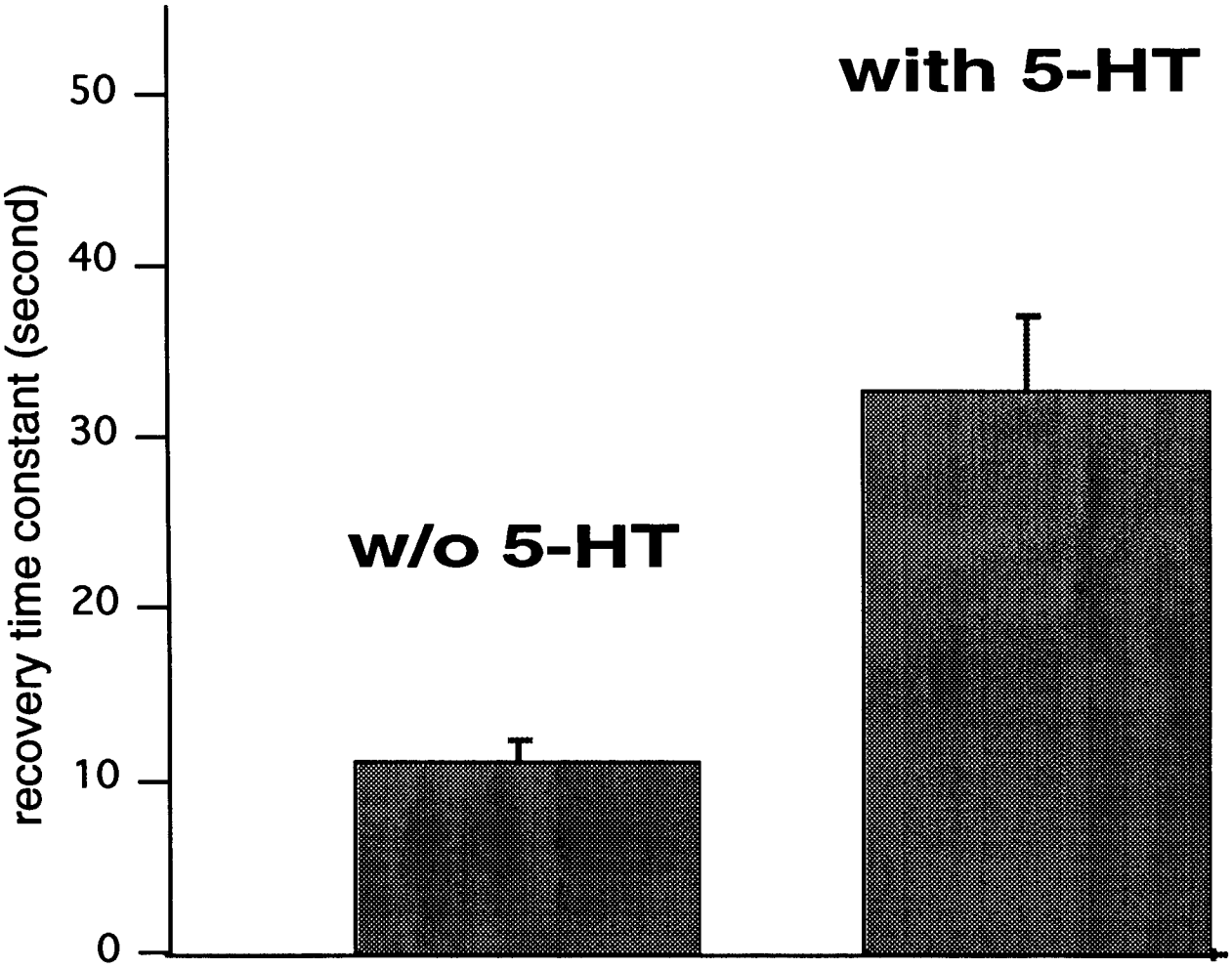

recovery time constant (second)
0
10
20
30
40
50
w/o 5-HT
with 5-HT

Table 2. Comparison of the initial magnitude of synaptic enhancement (A_0) with and without serotonin

Experiment #	A_0 w/o 5HT	A_0 with 5HT
1	2.3	2.0
2	2.3	2.2
3	2.1	1.9
4	2.2	2.2
5	2.2	2.7
6	1.9	1.6
7	1.4	1.7
8	1.9	1.8
9	2.2	1.7
10	1.7	1.7
11	2.6	1.7
12	2.9	2.3
Mean±SEM	**2.1±0.1**	**2.0±0.1**

Figure 11 is a graphical summary of these data.

A Paired data t-test on the initial amplitude of augmentation shows that there is no statistically significant change in A_0 with the application of 5-HT ($t = 1.51$, $0.1<P<0.2$, $n=12$).

3.3.2 Reversibility of the effect of serotonin on recovery τ

In six experiments, the time course of the decay of synaptic enhancement was examined during the washout of 5-HT. It was found that the time constant of decay gradually recovered towards the pre-serotonin level during the washing (see Figure 12 as a typical result). Complete recovery usually required about 140 minutes.

These washout experiments indicate that the serotonin-induced lengthening of the recovery is reversible. This evidence argues against the possibility that the fatigue or "rundown" of the preparation was responsible for the slower recovery of augmentation.

3.3.3 Correlation between serotonergic enhancement and prolongation of ADSE recovery

Since both serotonergic enhancement of the EJP amplitude (Dixon and Atwood 1985) and serotonin-induced prolongation of recovery from synaptic enhancement are reversible upon

Figure 11. 5-HT does not significantly reduce the initial magnitude of augmentation (n=12).

A bar chart comparing the initial magnitude of synaptic enhancement A_0 with and without 5-HT. A_0 without 5-HT is 2.1±0.1 (mean±SEM, n=12). A_0 with 5-HT is 2.0±0.1 (mean±SEM, n=12). The mean values are represented by the bars and SEMs are represented by the error bars. A paired data t-test shows no significant difference between A_0 without 5-HT and A_0 with 5-HT ($0.1<P<0.2$, n=12).

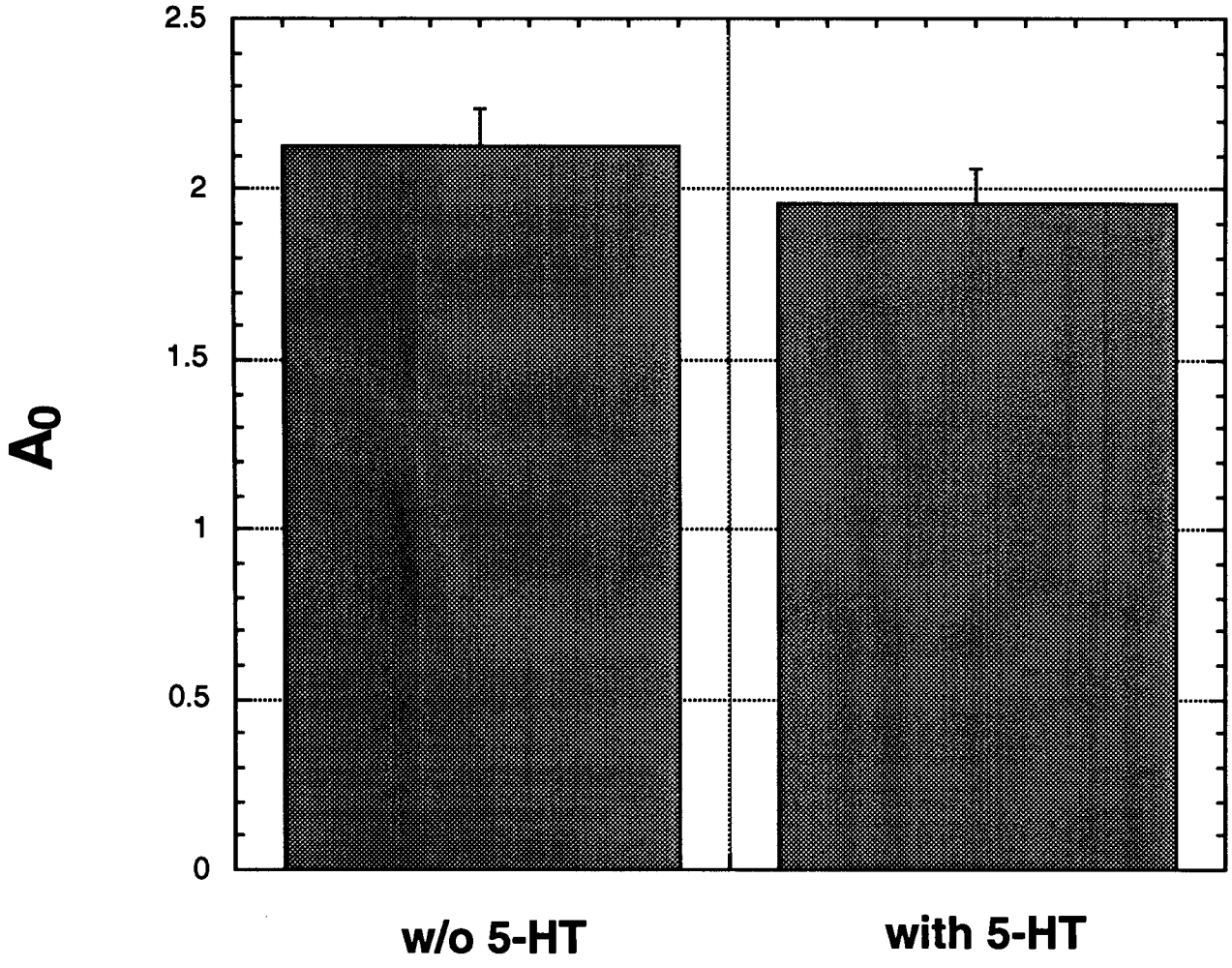
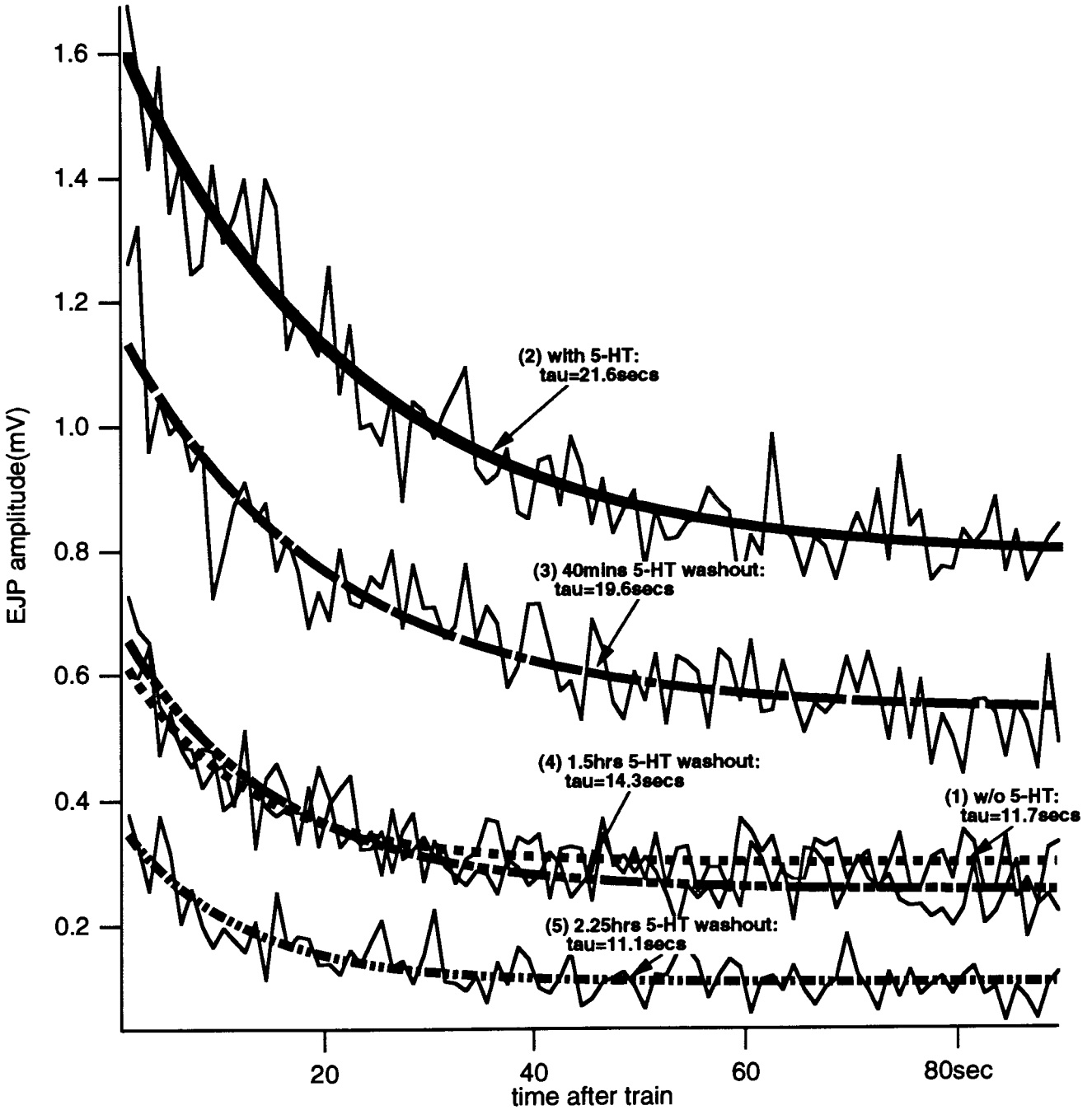

2.5
2
1.5
1
0.5
0
A_0
w/o 5-HT
with 5-HT

Figure 12. The prolongation of the recovery of augmentation reverses during 5-HT washout.

The data demonstrate that the amplitudes of EJPs as well as the time constants of recovery from synaptic enhancement gradually return to pre-serotonin levels as the washing of serotonin proceeds. Each trace presented in the figure is the average of 12 trials done in a block with about 20 minutes between each block of trials during washout. The numbers in the brackets indicate the sequence in which each trace was collected. The amplitude of EJPs at the completion of washout (2.25 hours) appears to be smaller than that of pre-serotonin ones. This is probably due to a decreased input impedance of intracellular amplifier resulting from muscle movement during long periods of recording (6 hours), and should not affect the measurement of the time course of recovery.

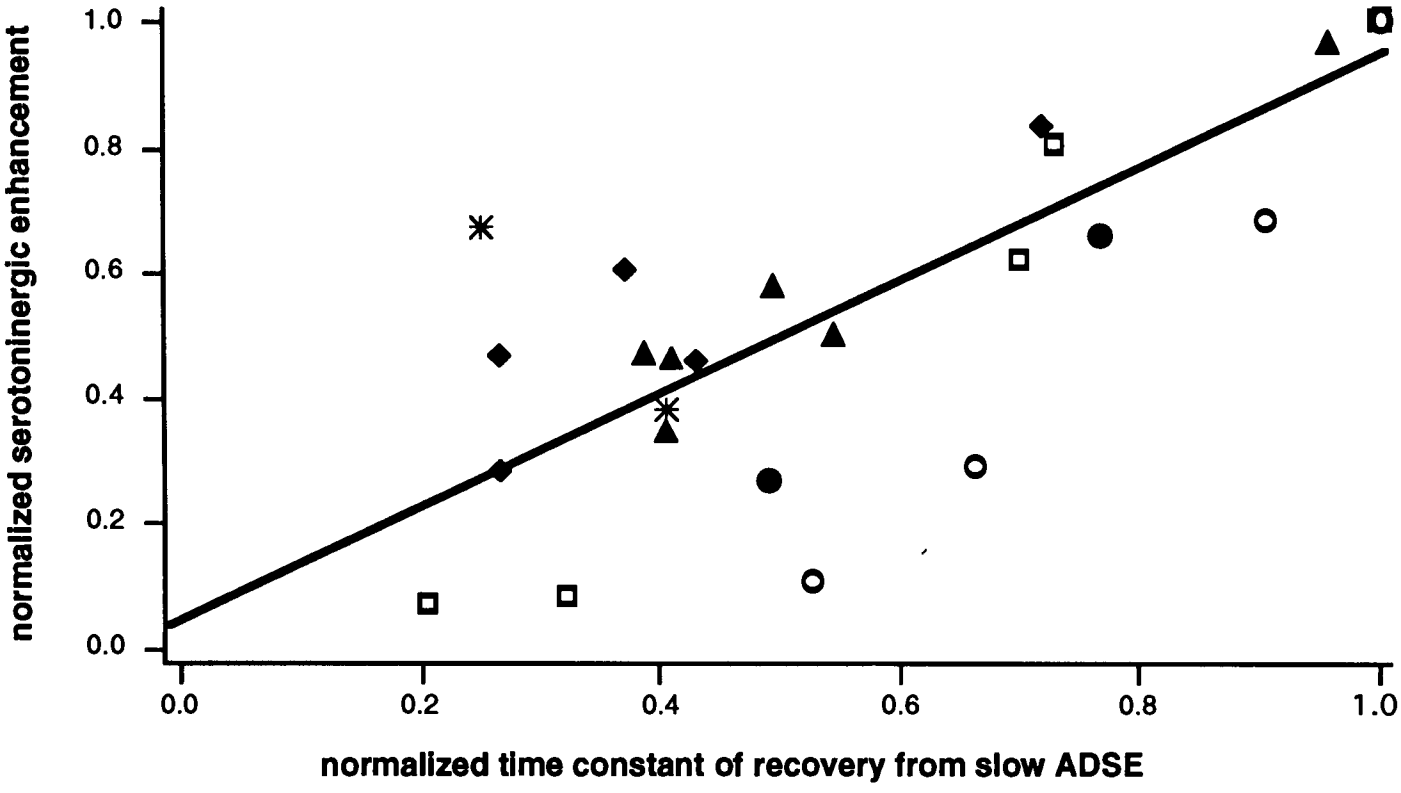

1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
EJP amplitude(mV)
(2) with 5-HT:
tau=21.6secs
(3) 40mins 5-HT washout:
tau=19.6secs
(4) 1.5hrs 5-HT washout:
tau=14.3secs
(1) w/o 5-HT:
tau=11.7secs
(5) 2.25hrs 5-HT washout:
tau=11.1secs
20
40
60
80sec
time after train

washing with normal NVH solution, further analysis was undertaken to examine whether they recovered at the same rate.

A close correspondence between serotonergic EJP enhancement and serotonin-induced prolongation of recovery time constant was observed (n=22, including the data from three experiments in which washing was terminated before complete recovery). Figure 13 summarizes this correlation, showing slope = 0.91, intercept = 0.04, r = 0.84 (linear correlation coefficient). Further statistics on r were carried out to determine if there is a positive correlation between these two processes. With the calculated $t = 6.92 > t_{0.05(1),\, n-2}$ (= 1.73), where n is the number of data pairs, a positive correlation between the two processes was statistically identified. The strong correlation between the reversal rate of serotonergic enhancement and serotonin-induced prolongation of recovery time constant suggests that these two processes could be interrelated by a common intermediate or final biochemical product.

Figure 13. Correlation between serotonergic EJP enhancement and serotonin-induced prolongation of recovery from activity-dependent synaptic enhancement.

Time constants of recovery from augmentation were estimated using an exponential fitting function beginning 1.5 secs following the offset of tetanic stimulation. The general form of those functions is $K_0 + K_1 * e^{(-t / \text{time constant})}$. Since K_0 is a good estimation of the amplitude of EJPs at the control level ($t = \infty$) (refer to Chapter II), the magnitude of serotonergic enhancement is represented by the ratio of K_0 in the presence of 5-HT to K_0 in the absence of 5-HT. Different symbols represent the data from different experiments. To summarize the data from six washout experiments, both serotonergic enhancement and the time constant of recovery from augmentation are normalized by their corresponding data in the presence of 5-HT before the washout commences. Slope = 0.91, intercept = 0.04, r = 0.84 (linear correlation coefficient).


normalized serotoninergic enhancement
1.0
0.8
0.6
0.4
0.2
0.0
0.0
0.2
0.4
0.6
0.8
1.0
normalized time constant of recovery from slow ADSE

Chapter IV
Mechanisms Unrelated to Calcium Dynamics

4.1 Saturation and depression

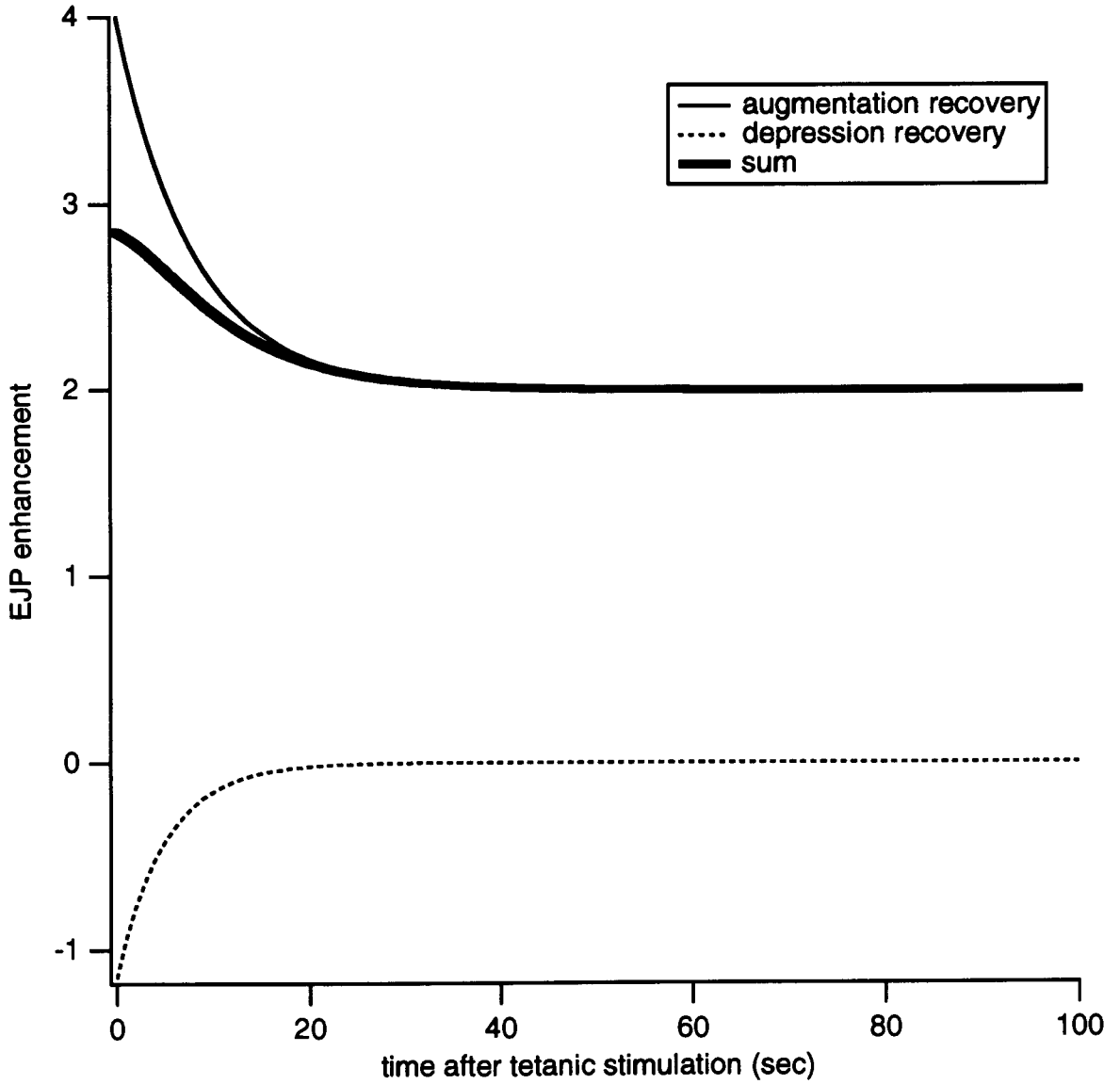
4.1.1 Introduction

There are many possible explanations for the slower recovery of synaptic enhancement observed with serotonin. It is well known that serotonin by itself enhances transmitter release by 2-5 fold. When serotonergic enhancement combines with activity-dependent synaptic enhancement, it is possible that the transmitter release machinery is saturating. In this instance, release might not be capable of further increase due to a variety of reasons such as a limited number of release sites or a maximal rate of vesicle mobilization in the presynaptic terminal. Following the offset of the stimulus train in this case, transmitter release would accordingly decay from a level which is lower than it might have reached if release did not saturate, leading to the appearance of a slower decay of augmentation.

Tetanic stimulation can also result in synaptic depression, which in fact exists at many synapses. In these preparations, repetitive stimulation first dramatically increases the amplitude of postsynaptic potentials as a result of the buildup of short-term facilitation, augmentation, and PTP, and then decreases the PSP amplitude below its previous level. This subsequent drop in PSP amplitude reflects synaptic depression and is thought to result from a decrease in the number of quantal packets of transmitter released by each impulse (Magleby 1987). Despite the fact that the crayfish opener muscle preparation normally displays very little synaptic depression and saturation of release (Chapter II), these possibilities can not necessarily be excluded in the presence of 3-5 fold serotonergic enhancement. If the cell gradually recovers from synaptic depression after the 15 Hz train, the decay of augmentation combined with the recovery from depression could result in a process characterized by an exponential curve with a slower time constant (see Figure 14).

Figure 14. A model demonstrating that the sum of augmentation decay and hypothetical depression recovery could result in a process characterized by a single exponential curve with a longer time constant than that of recovery from augmentation.

Thin solid line represents the decay of augmentation with a time constant of 8 seconds. *Dashed line* represents the exponential recovery from synaptic depression with a typical time constant of 5.0 seconds (Magleby 1987). *Thick solid line* is the sum of the recovery from augmentation and the recovery from depression, which could be roughly fitted by an exponential curve with a slower time constant of 11.4 seconds (42% slower than that of augmentation).


4
3
2
1
0
-1
EJP enhancement
augmentation recovery
depression recovery
sum
0
20
40
60
80
100
time after tetanic stimulation (sec)

However, this simple model predicts that, even if depression occurred in these experiments, depression alone could not completely account for the three-fold increase in time constant of recovery from synaptic enhancement seen with serotonin. Nonetheless, the possibility that depression might be partly responsible (about 40%) was worth further investigation.

4.1.2 Experimental protocol

To test these possibilities, further experiments were undertaken based on the following rationale. These experiments were to examine whether serotonin-induced prolongation of recovery of augmentation persisted under conditions where transmitter release was artificially reduced to a level at which saturation and/or depression were unlikely to occur (or at least be greatly reduced). If the prolongation of the time constant of recovery disappeared or was significantly reduced, saturation of release and/or synaptic depression might be significant factors contributing to the effect.

Previous study indicates that decreasing extracellular calcium concentration drastically reduces the number of quantal packets of transmitter released by a nerve impulse, presumably by reducing calcium entry (Dodge and Rahamimoff 1967). Thus, decreasing the amount of transmitter release in the presence of serotonin can be implemented by reducing extracellular calcium concentration. A detailed experimental protocol is illustrated in Figure 15.

4.1.3 Results and data analysis

Six complete experiments were performed. Figure 16 shows a typical result. Table 3 presents all the raw data, and a bar chart (Figure 17a) is used to summarize these data.

Figure 15. Experimental protocol to examine the effect of reducing extracellular cellular calcium concentration on the prolongation of recovery from synaptic enhancement in the presence of serotonin.

A standard protocol was adopted consisting of a 15 Hz 30 second conditioning train followed by 1 Hz testing impulses to map out the decay of augmentation. In 50% of normal $[Ca^{2+}]$ NVH (without 5-HT) where the EJP amplitude is relatively small, the number of trials averaged was increased to 16-25 so that a reasonable S/N ratio could be achieved.

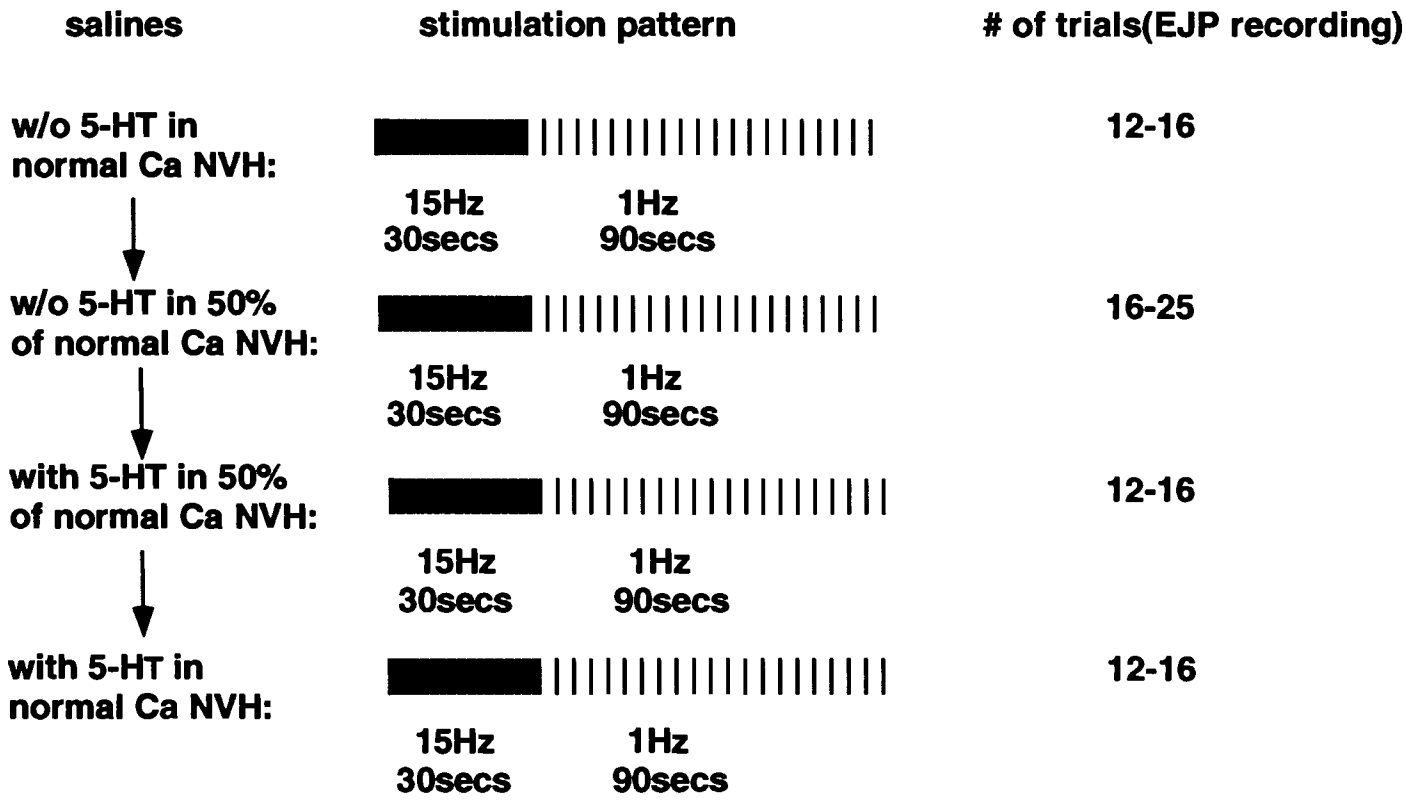

salines
stimulation pattern
# of trials(EJP recording)
w/o 5-HT in normal Ca NVH:
15Hz 30secs
1Hz 90secs
12-16
w/o 5-HT in 50% of normal Ca NVH:
15Hz 30secs
1Hz 90secs
16-25
with 5-HT in 50% of normal Ca NVH:
15Hz 30secs
1Hz 90secs
12-16
with 5-HT in normal Ca NVH:
15Hz 30secs
1Hz 90secs
12-16

Figure 16. Reducing extracellular calcium concentration by 50% does not affect the magnitude of serotonin-induced prolongation of ADSE.

Recovery of synaptic enhancement beginning 1.521 seconds after tetanic stimulation in a series of salines with two different calcium concentrations (50% and 100% of normal) was recorded in one claw. The numbers in brackets indicate the sequence in which each set of data (an average of 12-25 trials each set) was collected. The magnitude of the prolongation of recovery from synaptic enhancement in the presence of serotonin did not diminish with the decreased extracellular calcium concentration.

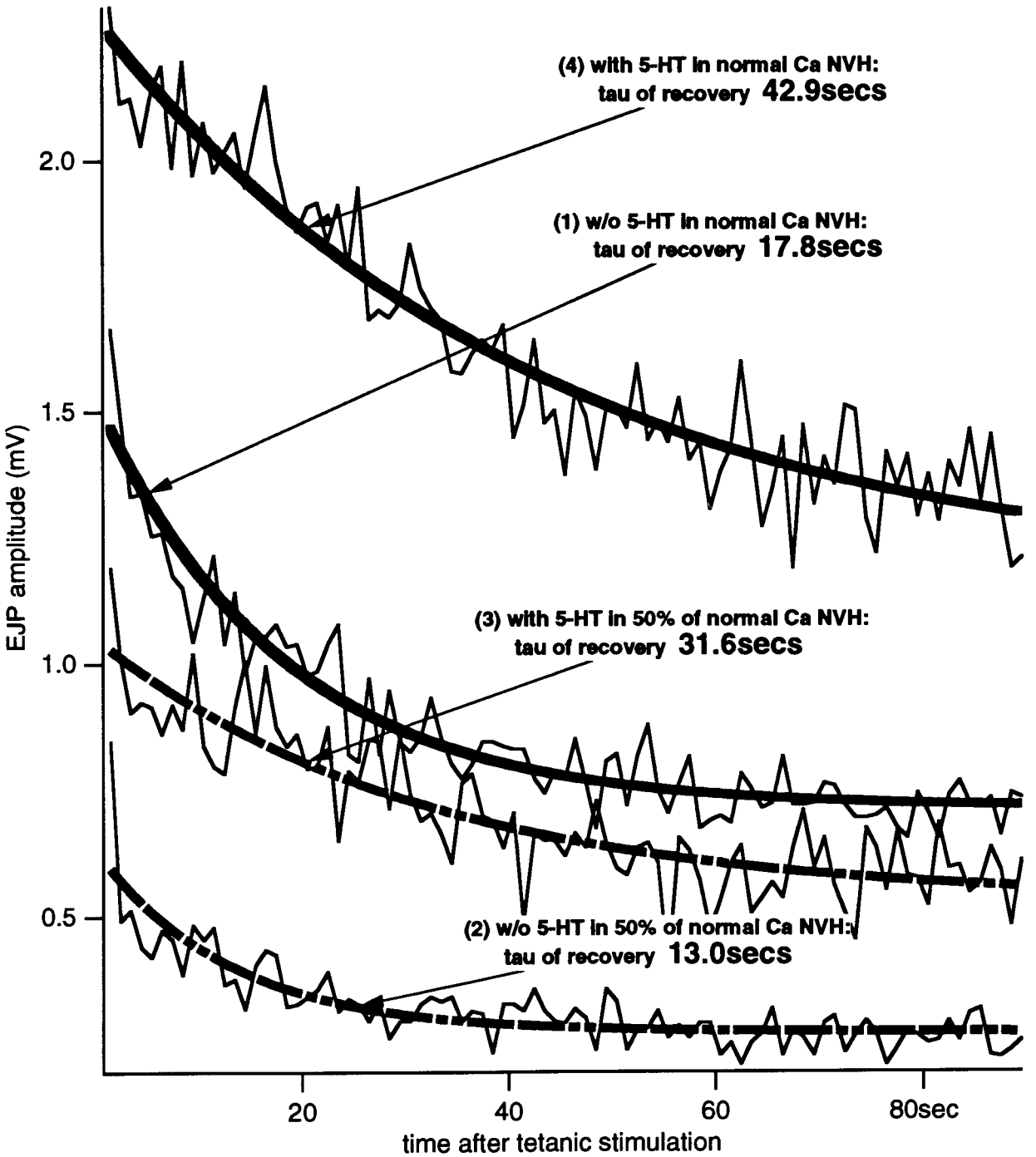
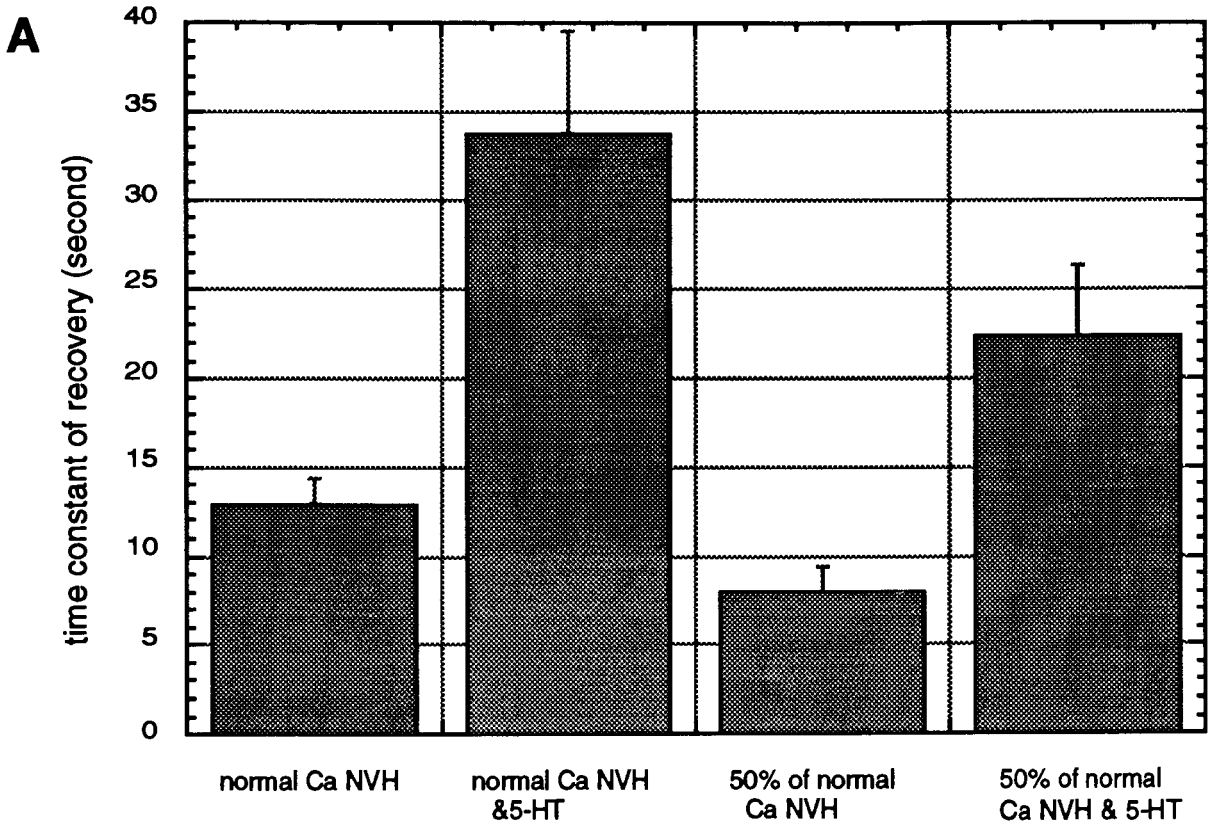

(4) with 5-HT in normal Ca NVH:
tau of recovery 42.9secs
(1) w/o 5-HT in normal Ca NVH:
tau of recovery 17.8secs
(3) with 5-HT in 50% of normal Ca NVH:
tau of recovery 31.6secs
(2) w/o 5-HT in 50% of normal Ca NVH:
tau of recovery 13.0secs
EJP amplitude (mV)
2.0
1.5
1.0
0.5
20
40
60
80sec
time after tetanic stimulation

Figure 17. The effect of 5-HT on the time constant of recovery from augmentation in 50% of normal [Ca^{2+}] NVH and normal [Ca^{2+}] NVH.

(A) A bar chart summarizing different time constants of recovery in different salines (n=6). In normal [Ca^{2+}] NVH, the presence of 5-HT changes the time constant of recovery from synaptic enhancement after tetanus from 12.9±1.5 seconds (mean±SEM) to 33.7±5.9 seconds (mean±SEM); In 50% of normal [Ca^{2+}] NVH, the time constant of recovery was increased from 8.0±1.4 seconds (mean±SEM) to 22.4±4.0 seconds (mean±SEM). (B) The extent of serotonin-induced prolongation of the time constant, as represented by the ratio of τ with 5-HT to τ without 5-HT, in normal [Ca^{2+}] NVH (2.8±0.6, mean±SEM, n=6) is compared with that in 50% of normal [Ca^{2+}] NVH (3.0±0.4, mean±SEM, n=6). A paired data t-test shows no significant difference between these two ratios ($P>0.5$, n=6).


A
time constant of recovery (second)
40
35
30
25
20
15
10
5
0
normal Ca NVH
normal Ca NVH &5-HT
50% of normal Ca NVH
50% of normal Ca NVH & 5-HT

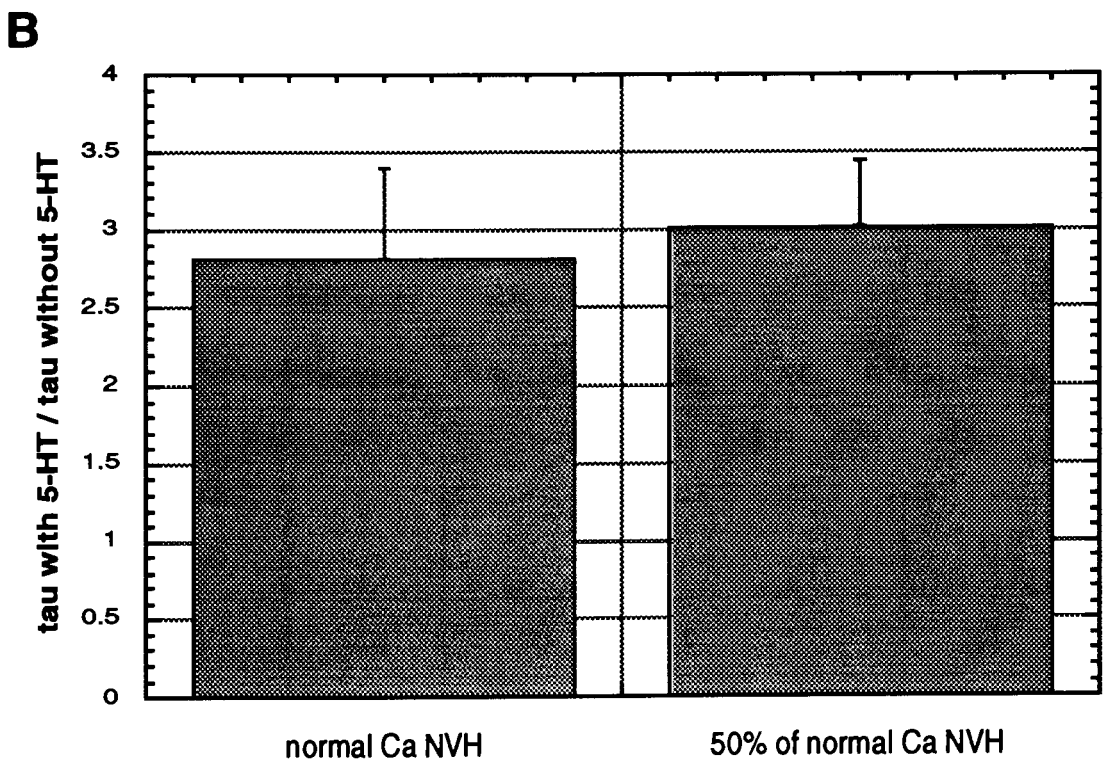

B
tau with 5-HT / tau without 5-HT
4
3.5
3
2.5
2
1.5
1
0.5
0
normal Ca NVH
50% of normal Ca NVH

Table 3. Comparison of recovery time constants in different NVH salines

# of experiment	τ in normal $[Ca^{2+}]$ NVH (sec)	τ in 50% of normal $[Ca^{2+}]$ NVH (sec)	τ in 50% of normal $[Ca^{2+}]$ NVH & 5-HT (sec)	τ in normal $[Ca^{2+}]$ NVH & 5-HT (sec)
1	17.6	12.2	33.3	60.4
2	14.0	8.2	33.6	33.0
3	12.3	8.6	12.3	17.2
4	6.2	4.5	14.4	33.6
5	13.0	3.6	15.1	28.9
6	14.0	11.1	25.7	29.1
Mean±SEM	**12.9±1.5**	**8.0±1.4**	**22.4±4.0**	**33.7±5.9**

The following analysis was implemented in order to evaluate the extent of serotonin-induced prolongation of recovery in normal $[Ca^{2+}]$ NVH as opposed to that in reduced $[Ca^{2+}]$ NVH (50% of normal). The ratios of time constants (τ with 5-HT / τ without 5-HT) in normal $[Ca^{2+}]$ NVH and 50% of normal $[Ca^{2+}]$ NVH were calculated for each experiment (as shown in Table 4). Figure 17b summarizes those ratios graphically.

Table 4. Ratios of recovery time constants in normal $[Ca^{2+}]$ and 50% of normal $[Ca^{2+}]$ NVH

τ with 5HT / τ w/o 5HT	(1)	(2)	(3)	(4)	(5)	(6)	Mean± SEM
normal $[Ca^{2+}]$ NVH	3.4	2.4	1.4	5.4	2.2	2.1	**2.8±0.6**
50% of normal $[Ca^{2+}]$ NVH	2.7	4.1	1.4	3.2	4.2	2.3	**3.0±0.4**

A paired-sample t-test was applied to determine if the difference between two sets of ratios

(normal and 50% of normal [Ca^{2+}] salines) was statistically significant. With the calculated t =0.30 (P>0.5, n=6), reducing extracellular calcium concentration, which should have greatly relieved the possible saturation of transmitter release and/or synaptic depression, did not affect the prolongation of recovery of ADSE.

It is also noted that 50% of normal [Ca^{2+}] NVH saline seems to reduce the time constant of augmentation to some extent (refer to Figure 17a and Table 3). Using a two tailed paired sample t-test, a significant difference is detected between the time constant in normal [Ca^{2+}] saline and the time constant in 50% of normal [Ca^{2+}] saline ($t = 4.35$, $0.005 < P(|t| \geq 4.350) < 0.01$). A one tailed paired sample t-test further confirms that the time constant with 50% of normal [Ca^{2+}] saline, is reduced by at least 2.5 seconds ($t = 2.10$, $0.025 < P(|t| \geq 2.097) < 0.05$).

4.1.4 Discussion

It is thus concluded that the saturation of transmitter release and/or synaptic depression are unlikely to play a significant part in the observed prolongation of recovery from augmentation by serotonin.

These results also indicate that, when the concentration of extracellular Ca^{2+} was decreased by 50%, the time constant of recovery from augmentation was reduced as well. Previous study has shown that reducing extracellular calcium concentration reduces calcium entry, thereby affecting the concentration of intracellular calcium ions that accumulate in presynaptic terminals during a tetanus train (Dodge and Rahamimoff 1967). Based on previous observations under normal conditions without 5-HT (Delaney and Tank 1994), this reduction is likely caused by a faster recovery of residual calcium, perhaps due to a reduced gradient against which Ca^{2+} pumps must work.

4.2 Changes in action potentials

4.2.1 Introduction

As reviewed in chapter I, in *Aplysia,* serotonin's facilitatory effect seems to be exerted through the broadening of the action potential as a result of a decreased K^+ efflux. Yet, no broadening of the action potential was observed at crustacean nmjs (Dixon and Atwood, 1985). For the experimental paradigm adopted in this study differs from that used previously (Dixon and Atwood, 1985) in that tetanic stimulation is applied in conjunction with serotonin, it is necessary to ensure that there is no additional serotonin-evoked change of presynaptic action potentials involved in the prolongation of augmentation recovery.

It has been suggested that augmentation correlates with the recovery of a persistent hyperpolarization which builds up during trains of action potentials (Bittner and Baxter 1991, also see Figure 6). Since the recovery of augmentation is prolonged by serotonin, it is possible that the rate of decay of this slow hyperpolarization is slowed by serotonin as well. This experiment was also designed to elucidate any possible role for the recovery of hyperpolarization.

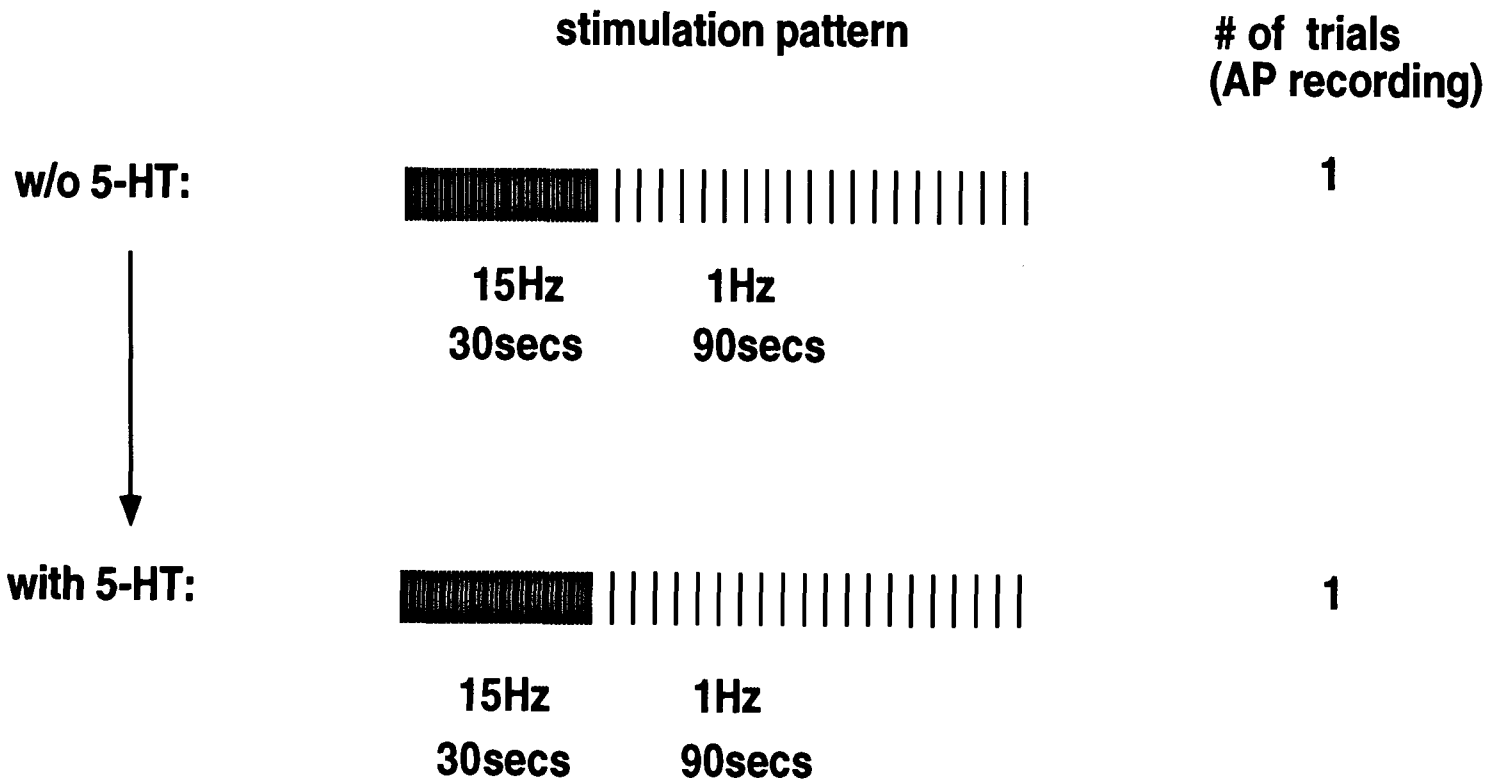
4.2.2 Protocol

The experimental paradigm is illustrated in Figure 18. An intracellular recording electrode with high input impedance (30 MΩ when filled with 3 M KCl) was used to penetrate the excitor axon and record action potentials. An operational amplifier amplified the signal by 100 times before the signal was acquired by MacAdio A/D board. The signal was also DC coupled so that the slow drift of membrane potential (including hyperpolarizations) could be detected.

4.2.3 Results

By graphically superimposing action potentials on each other during the tetanic stimulation, the action potentials in the presence of serotonin were carefully compared with those in the absence

Figure 18. **Experimental protocol to test for a 5-HT-associated change in action potentials during the standard augmentation trials (15 Hz 30 secs followed by 1 Hz 90 secs).**


stimulation pattern
# of trials
(AP recording)
w/o 5-HT:
1
15Hz
30secs
1Hz
90secs
with 5-HT:
1
15Hz
30secs
1Hz
90secs

of serotonin. The comparison shows that neither the amplitude nor the shape of the individual action potentials is changed by serotonin (see Figure 19).

The presence of serotonin did not affect the rate of recovery of the slow hyperpolarizations following 15 Hz stimulation either (for a typical result, see Figure 20). Table 5 presents the time constant for the recovery of membrane potential following tetanus in nine experiments. Figure 21 is a graphical summary of the data.

Table 5. Time constant of recovery of presynaptic membrane potential after 15 Hz 30 second train

Exper-iment #	(1)	(2)	(3)	(4)	(5)	(6)	(7)	(8)	(9)	Mean± SEM
w/o 5-HT (sec)	7.3	14.5	3.1	15.7	6.8	10.6	14.5	10.9	15.9	11.0± 1.5
with 5-HT (sec)	9.2	11.0	3.3	17.3	6.7	10.9	14.6	11.8	12.5	10.8± 1.4

A paired sample t-test shows no significant difference between the rate of repolarization with 5-HT and that without 5-HT ($|t| = 0.34$, $0.7<P<0.8$, $n=9$).

However, the amplitude of hyperpolarization was increased with the application of serotonin (see Figure 20). This was probably due to a slight depolarization of axonal membrane potential (2-5 mV) in the presence of 5-HT (also see Dixon and Atwood, 1985), which pulled the membrane potential farther away from the K^+ equilibrium potential. Consequently, a larger hyperpolarization resulted.

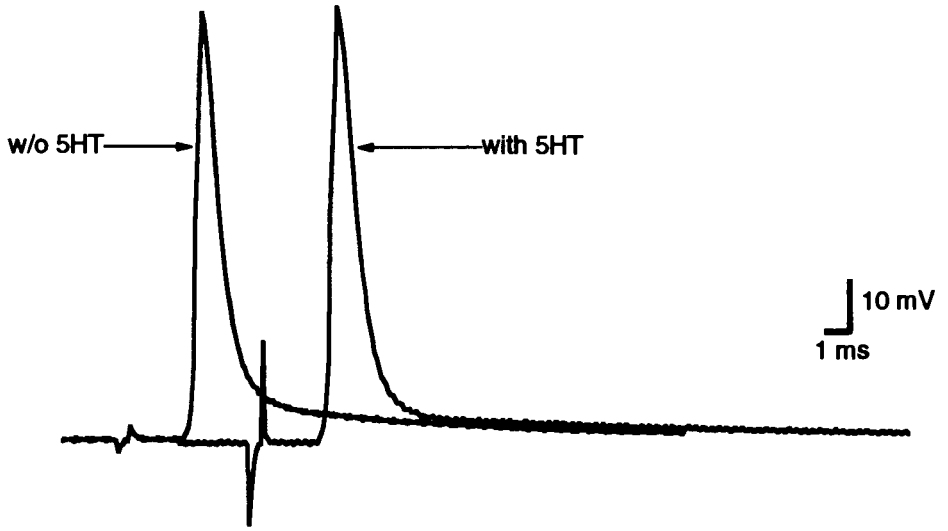
4.2.4 Conclusion

The depolarization or hyperpolarization of membrane potential can affect the amount of transmitter that is being released (Atwood and Wojtowicz 1986). The correlation between the recovery time constant of augmentation and recovery time constant of hyperpolarization (both are

Figure 19. A comparison of the widths and heights of individual action potentials during an augmentation trial.

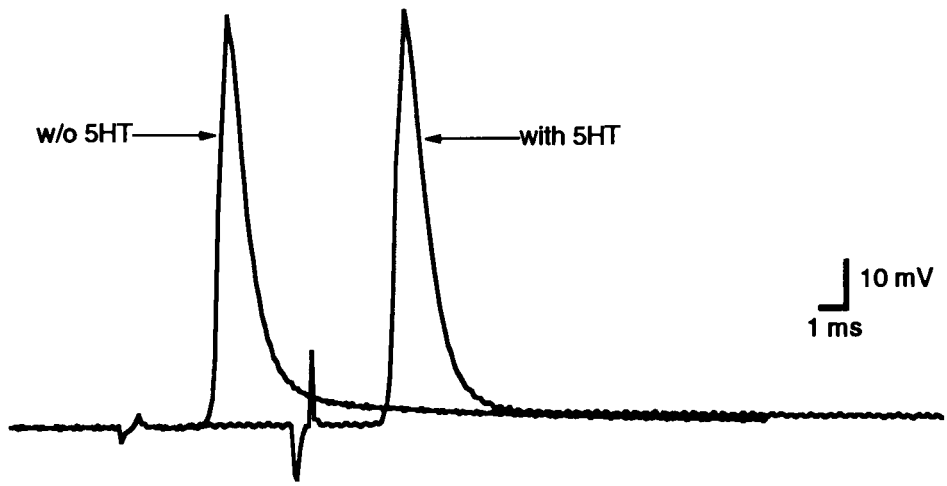
The action potentials with 5-HT and without 5-HT (A) at 2 seconds after the termination of 15 Hz stimulus train; (B) at 20 seconds after the termination of 15 Hz stimulus train; (C) at 40 seconds after the termination of 15 Hz stimulus train are graphically aligned in order to compare their heights and widths. There is no significant 5-HT-associated change in the shape and the amplitude of action potentials during the application of serotonin.

A



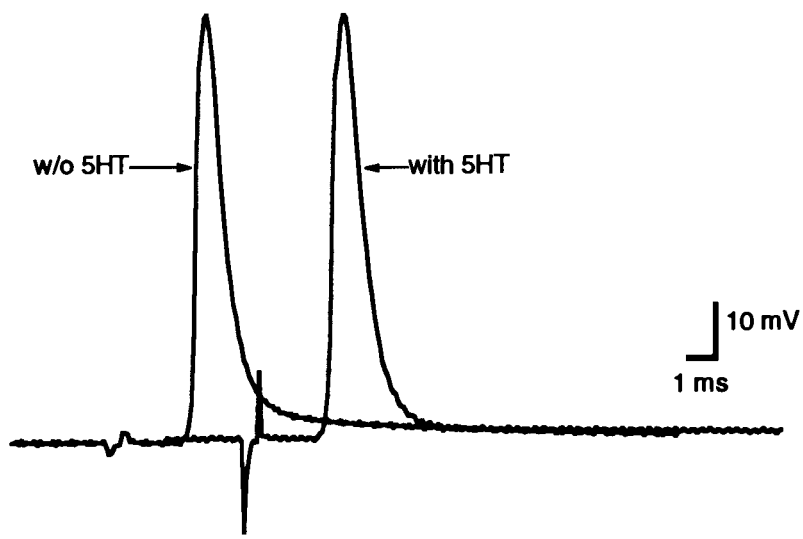
AP at 2 seconds after the termination of 15 Hz stimulus train

B



AP at 20 seconds after the termination of 15 Hz stimulus train

C



AP at 40 seconds after the termination of 15 Hz stimulus train

Figure 20. A comparison of the rate at which presynaptic membrane potential returns to pre-tetanus levels following a high frequency stimulation (15 Hz, n=1).

The membrane potential of excitor axon was closely monitored during and after 15 Hz tetanic stimulation. The presence of serotonin does not slow down the repolarization rate of membrane potential following tetanus (τ=14.53 seconds without 5-HT, τ =14.64 seconds with 5-HT).

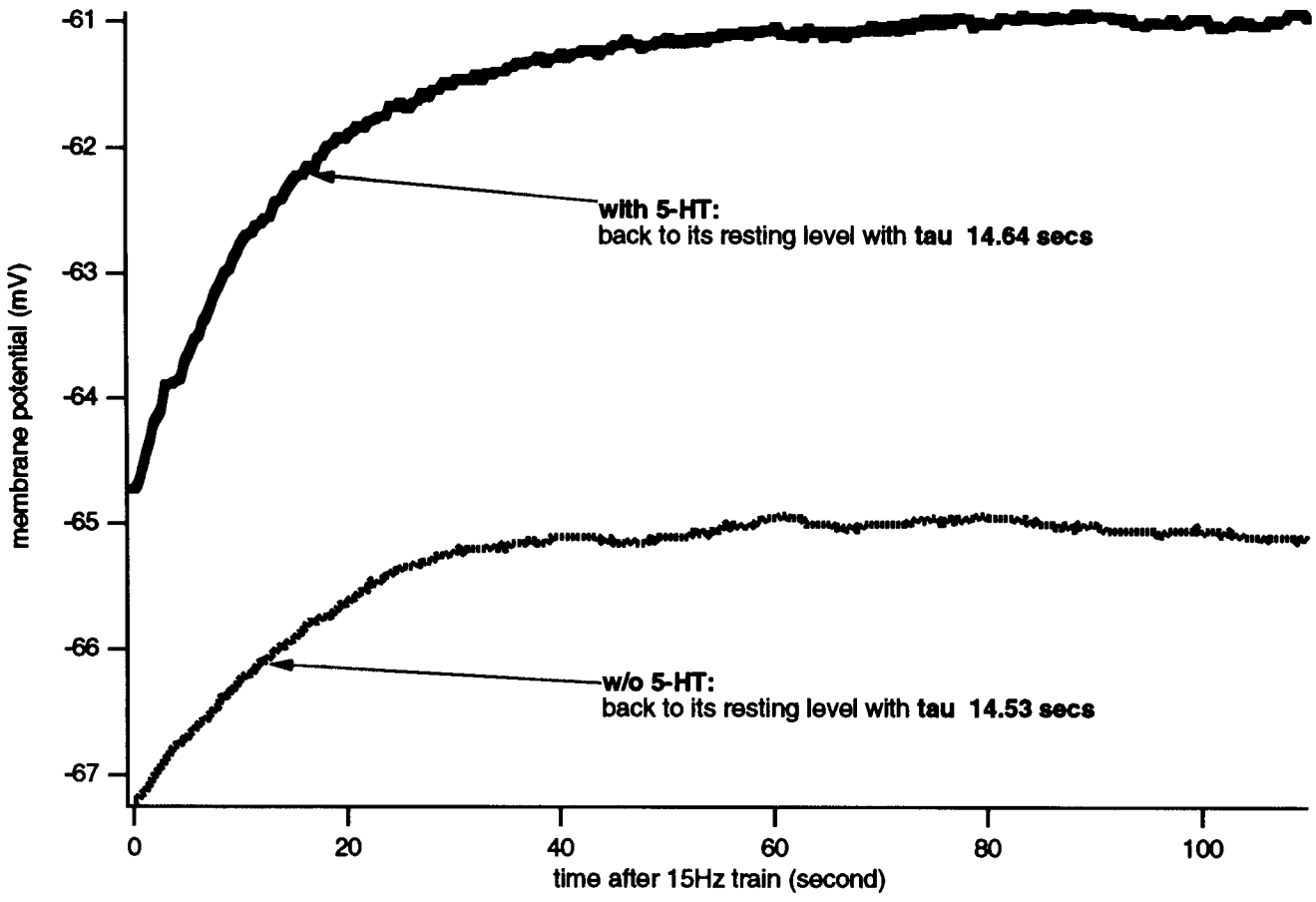
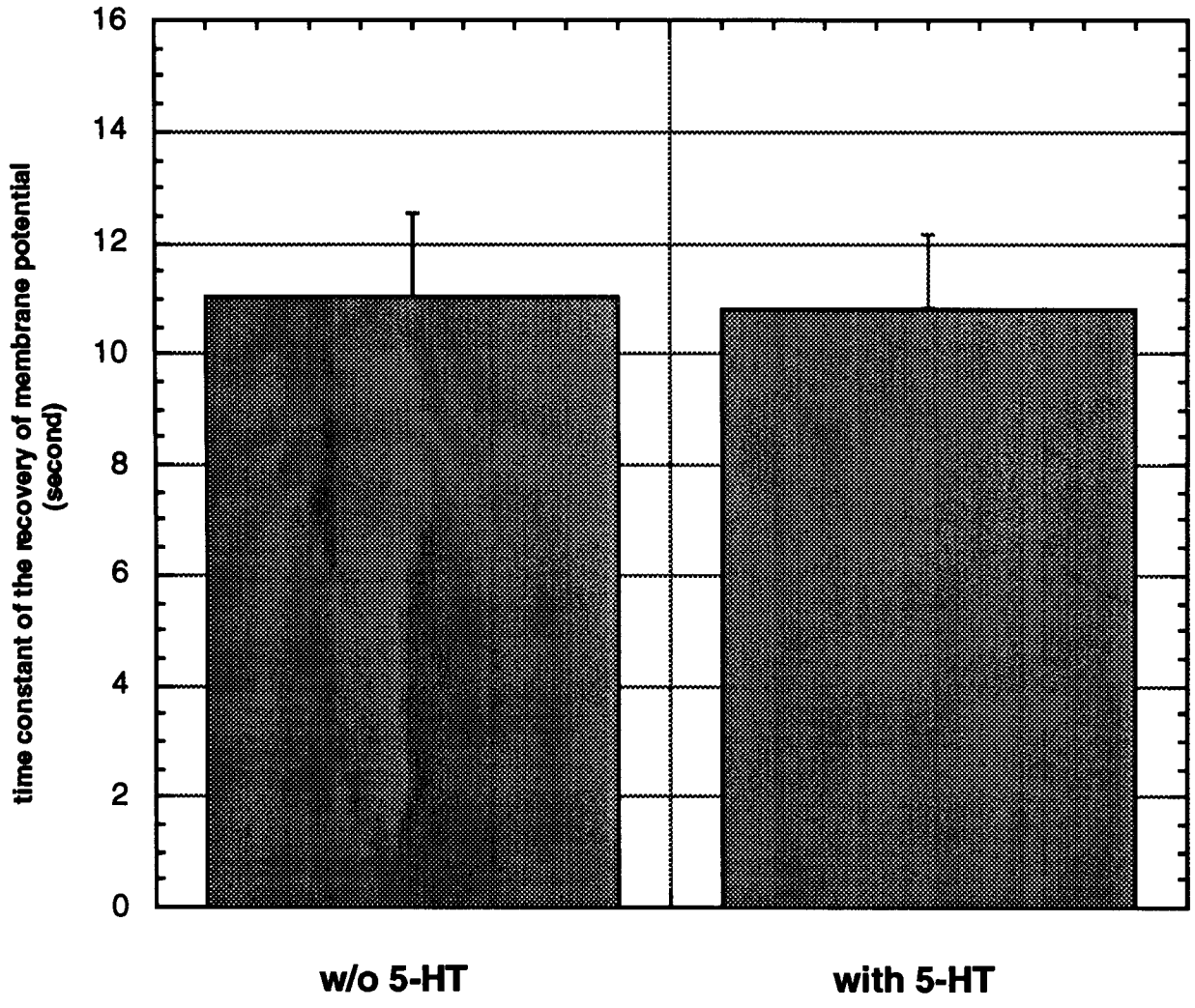

membrane potential (mV)
-61
-62
-63
-64
-65
-66
-67
0
20
40
60
80
100
time after 15Hz train (second)
with 5-HT:
back to its resting level with tau 14.64 secs
w/o 5-HT:
back to its resting level with tau 14.53 secs

Figure 21. The presence of serotonin does not slow the repolarization rate of presynaptic membrane potential (n=9).

A bar chart summarizing the rate of repolarization in nine experiments. Before the application of 5-HT, the time constant of recovery of membrane potential is 11.03±1.516 seconds (mean±SEM, n=9). After the application of 5-HT, the corresponding time constant is 10.82±1.373 seconds (mean±SEM, n=9). A paired data t-test shows no significant difference between the two rates ($0.7<P<0.8$, n=9).


16
14
12
10
8
6
4
2
0
time constant of the recovery of membrane potential (second)
w/o 5-HT
with 5-HT

around 10 secs under normal conditions at 15.5°C) led to the speculation that the hyperpolarization built up during repetitive stimulation directly or indirectly produces augmentation (Bittner and Baxter 1991). However, based on the action potential recordings in these experiments, the rate of repolarization did not change with the application of 5-HT. The recovery of hyperpolarization following tetanic stimuli, therefore, could not account for the prolongation of ADSE by serotonin.

Using the standard paradigm for testing augmentation in the presence of serotonin, no additional 5-HT-associated broadening of action potentials was observed after tetanic stimulation. Therefore, 5-HT-associated changes in the amplitude and shape of action potentials are not responsible for the prolonged recovery of augmentation.

Chapter V

The Role of Calcium Dynamics in Serotonin-induced Prolongation of ADSE

5.1 Fura-2 calcium imaging

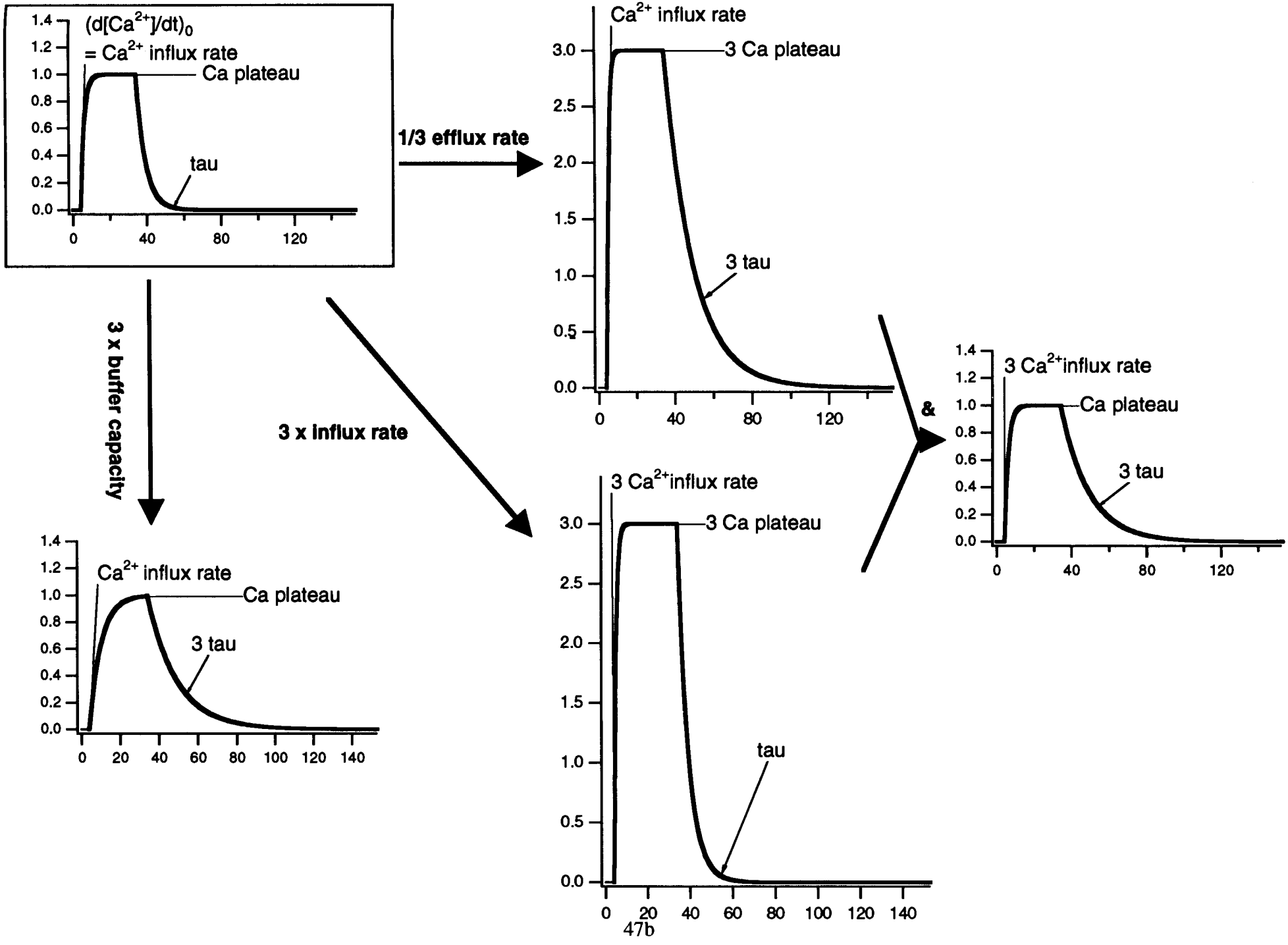
5.1.1 Introduction

The change of intracellular residual calcium concentration (residual calcium dynamics) is thought to determine the expression of augmentation. Therefore, the slower decay of augmentation after a high frequency train might simply result from a slower recovery of calcium in the presence of serotonin.

Three factors determine the dynamics of residual calcium (Figure 22): the rate of calcium influx, the rate of calcium efflux and the intracellular calcium buffer capacity (defined by the ratio of buffer concentration to the dissociation constant of the buffer). Intracellular Ca^{2+} will accumulate whenever the rate of calcium influx is greater than the rate of Ca^{2+} efflux. While calcium efflux rate appears to be a function of the intracellular free Ca^{2+} level (Tank et al. 1995), the amount of Ca^{2+} influx with each action potential appears to be constant. Thus, the net rate of calcium influx is a linear function of stimulation frequency up to 20 Hz (Tank et al. 1995). In our experiments, the calcium influx rate is constant throughout the 15 Hz conditioning train. During a sustained stimulus train the efflux rate will increase with the accumulation of intracellular free Ca^{2+} and eventually catch up to the Ca^{2+} influx rate. A plateau Ca^{2+} level will be reached at this point. After tetanus, however, $[Ca^{2+}]$ recovers due to the extrusion of the calcium ions by surface pumps as calcium influx is terminated with the offset of the stimulation. Since the change of intracellular calcium concentration determines the expression of augmentation, the steady-state level of augmentation is expected to correspond to the plateau level of free $[Ca^{2+}]$ in presynaptic terminals (Delaney and Tank 1994), and the slower decay of augmentation in the presence of 5-HT could

Figure 22. A schematic illustration of three ways to alter intracellular residual calcium dynamics during and after tetanic stimulation.

Either decreasing calcium efflux rate or increasing intracellular calcium buffer capacity could result in a slower recovery of residual calcium. Prolonging the recovery of residual calcium by 3 fold while maintaining the same level of calcium plateau could be achieved by (1) tripling the intracellular calcium buffer capacity or (2) increasing Ca^{2+} influx rate 3 fold while simultaneously decreasing Ca^{2+} efflux rate by 3 fold. Increasing buffer capacity does not affect the steady-state level of $[Ca^{2+}]_i$, but slowing both the buildup and decay of $[Ca^{2+}]_i$. The second way slows the recovery of $[Ca^{2+}]_i$ without changing the rate of buildup and the steady-state level of $[Ca^{2+}]_i$.


(d[Ca2+]/dt)0
= Ca2+ influx rate
Ca plateau
tau
1/3 efflux rate
Ca2+ influx rate
3 Ca plateau
3 tau
3 x buffer capacity
3 x influx rate
&
3 Ca2+influx rate
Ca plateau
3 tau
Ca2+ influx rate
Ca plateau
3 tau
3 Ca2+influx rate
3 Ca plateau
tau

reflect a slower recovery of intracellular residual calcium.

A slower recovery of residual calcium could be achieved by two ways, either a decreased calcium efflux rate or an elevated intracellular calcium buffer capacity. If the effects of 5-HT on the slow phase of activity-dependent synaptic enhancement result from increased intracellular Ca^{2+} buffering, achieved by changing either the amount or the affinity of endogenous Ca^{2+} buffer, 5-HT should only change the rate at which Ca^{2+} builds up and decays, not the plateau Ca^{2+} level. In other words, the presence of serotonin would produce a prolonged recovery of residual calcium while the plateau level of $[Ca^{2+}]$ would stay the same. Since augmentation is dictated by the dynamics of free Ca^{2+} removal in terminals, the recovery of augmentation should be prolonged and the steady-state level of augmentation at the end of the sustained stimulation would be the same as that in the absence of 5-HT.

If the extrusion rate is decreased, calcium recovery after the tetanus would be prolonged. However, during the sustained stimulation, presynaptic residual calcium would build up more quickly and reach a higher steady-state than that in the absence of serotonin. Accordingly, augmentation should decay from a higher level with a longer time constant following the offset of the stimulus train. This does not match our data, where no consistent increase or decrease of steady state level of augmentation (represented by A_0) was observed with the application of serotonin (refer to Figure 9b, Table 2 and Figure 11). A mechanism involving the change of Ca^{2+} pump rate would predict a corresponding inverse change in Ca^{2+} influx rate, which was not suggested by a previous study (Delaney et al. 1991). In order to reach the same steady-state level, the decreased rate of influx must equally balance the decreased rate of efflux.

5.1.2 Experimental protocol

This series of experiments attempted to address the core question: does serotonin prolong the recovery of augmentation by affecting the calcium dynamics? And if so, which factor causes the change of calcium dynamics? To address these questions, microfluorimetric fura-2 imaging (Delaney et al. 1989) was used to carefully examine the Ca^{2+} buildup and recovery in response to

15 Hz stimulation in the presence and absence of serotonin.

i) Imaging

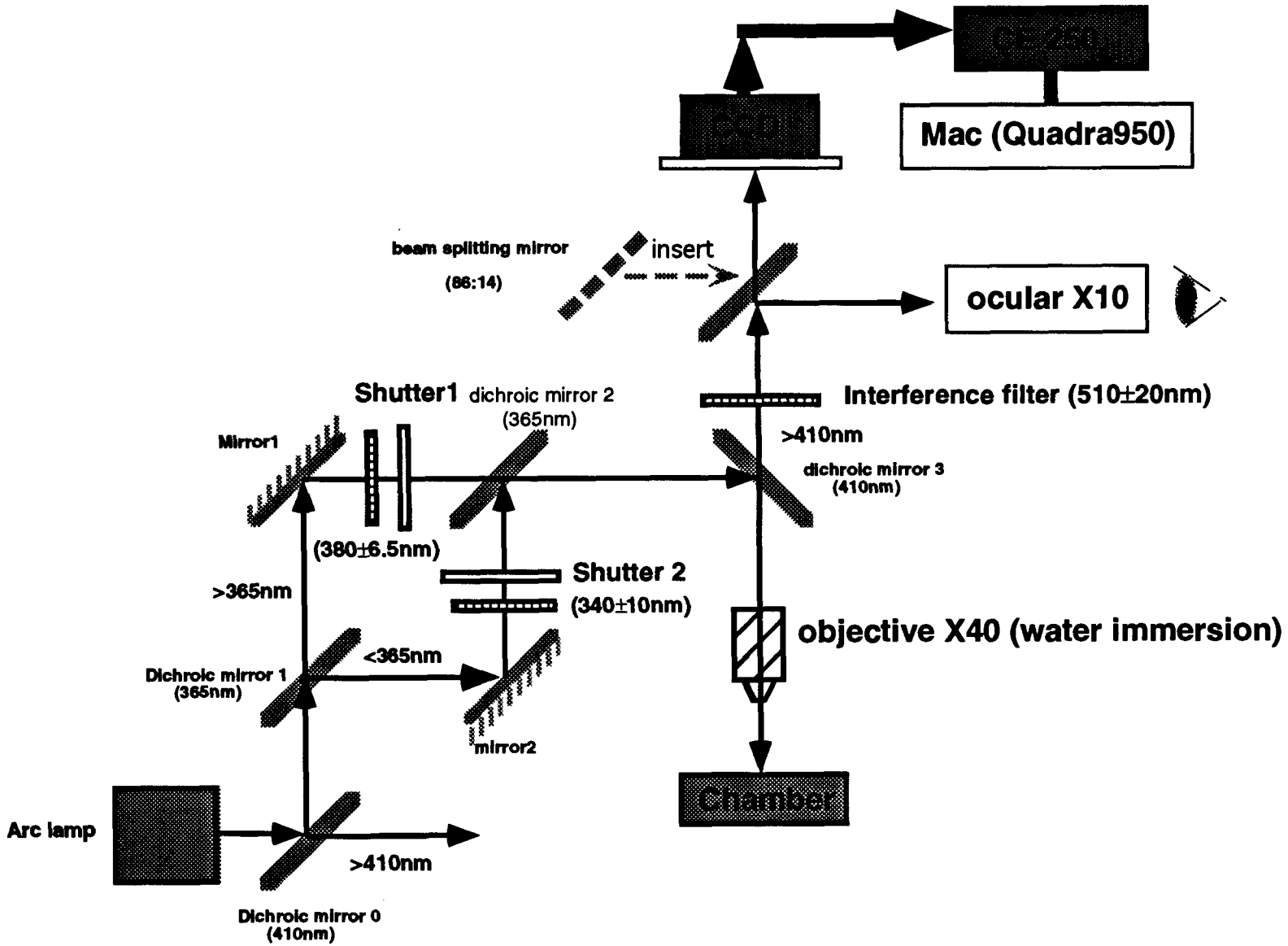
Fura-2 binds calcium ions with an estimated apparent dissociation constant of 865 nM at crayfish neuromuscular junctions (Delaney et al. 1989). Ca^{2+} binding to fura-2 subsequently shifts the peak of its excitation spectrum from 340 nm to 380 nm, while the peak of emission spectrum remains at 510 nm. The ratio of emission intensities obtained from 340/380 nm excitation pairs can be used to indicate $[Ca^{2+}]_i$ independent of pathlength or fura content in cytoplasm.

Fura-2 was iontophoretically injected into the preterminal axon of the excitor neuron by continuous hyperpolarizing current (8-10 nA for 20-30 mins) through a glass microelectrode filled with 22 mM Fura-2 (pentapotassium salt, Molecular probes) dissolved in 200 mM KCl (50-80MΩ)(see Figure 5). To allow fura-2 to diffuse evenly into distal terminals, we waited one hour before starting to collect images.

Fluorescence spectroscopy, fluorescence microscopy, electronic imaging, and digital image processing are the basic elements that need to be incorporated into a functional fluorescence ratio imaging system (Bright et al. 1989). In our system, a 12-bit cooled CCD camera (Photometrics CH250, Tuscon, AZ) acquired the background fluorescence from the surface of muscle fibres adjacent to the imaged terminals. This background was later subtracted from the total fluorescence. 2x2 binning of pixels and 400 msec exposures were typically used with the CCD. A series of dichroic mirrors and two electronically controlled shutters allowed the rapid alternation of excitation wavelengths (340 nm, 380 nm) (see Figure 23). Emission fluorescence was filtered through an interference filter of 510±20 nm. Pairs of ratioed images obtained with alternating 340 nm and 380 nm excitation were used to calculate $[Ca^{2+}]_i$ according to Equation 5 of Grynkiewicz et al. (1985). The calcium concentration was monitored by repetitive measurements of the same terminal during and after the 15 Hz stimulus train. Whenever possible, intracellular recording of EJP amplitudes from a postsynaptic muscle fibre was obtained immediately thereafter. Image acquisition and analysis were implemented by custom software on a Macintosh computer (Quadra

Figure 23. A schematic diagram illustrating our fluorescence ratio imaging system.

A fluorescence ratio imaging microscope system requires the integration of the following basic elements: a light source, an excitation light control device, a wavelength exchanger, microscope, a low-light level camera, an image processor, with computer coordinated control over all aspects of the system. In our system, exposure time is precisely controlled by two electronic shutters. A beam splitting mirror can be inserted to split some emission light (14%) into the ocular lenses of the microscope in case the terminal needs to be simultaneously monitored during the imaging.


Mac (Quadra950)
beam splitting mirror
(86:14)
insert
ocular X10
Shutter1
dichroic mirror 2
(365nm)
Interference filter (510±20nm)
>410nm
dichroic mirror 3
(410nm)
Mirror1
(380±6.5nm)
Shutter 2
(340±10nm)
>365nm
objective X40 (water immersion)
Dichroic mirror 1
(365nm)
<365nm
mirror2
Chamber
Arc lamp
>410nm
Dichroic mirror 0
(410nm)

950).

The following two procedures were adopted to minimize the error in [Ca^{2+}] measurement rendered by the muscle movement between acquisition of the image pairs. First, a beam splitting mirror (86:14) was added along the light path so that 14% of fluorescence from the illuminated sample was shunted to the ocular lenses of the microscope. In this way, muscle movement could be closely monitored and the terminal could be refocused if it moved out of focus with the muscle contraction during the imaging. Second, to overcome the intrinsic noise of the CCD and compensate for the 14% loss in the intensity of fluorescence acquired by the CCD camera, sets of ratioed images collected from 2 repeated trials were averaged. An adequate S/N ratio could be obtained without significant photobleaching.

Ideally, sampling frequency should be set as high as possible in order to follow the quick buildup of intracellular [Ca^{2+}] during a high frequency stimulus train. However, the sampling rate is limited by the exposure time and the mechanical limit for the alteration of excitation wavelengths. The possibility of using 200 msec exposure time instead of the standard 400 msec was considered. But images taken under 200 msec exposure could have a S/N ratio that is too low to measure calcium concentration accurately even if averaging sets of ratioed images collected from repeated trials could ameliorate part of the problem. A better way to speed up the sampling rate meanwhile maintaining the 400 msec exposure time is to operate the CCD camera in the frame transfer mode.

It is also worth mentioning that, in principle, it is possible to measure Ca^{2+} influx rate if the initial buildup of calcium concentration could be closely followed. The initial slope of the rising Ca^{2+} concentration reflects solely the Ca^{2+} influx rate because calcium extrusion rate and leak-in rate are still equal as the train commences. However, on many occasions, accurate measurements of calcium concentration at the onset of 15 Hz stimulus train were not possible due to the movement of the terminals during an initial contraction of the muscle. The quantitative measurement of initial slope was thus not implemented.

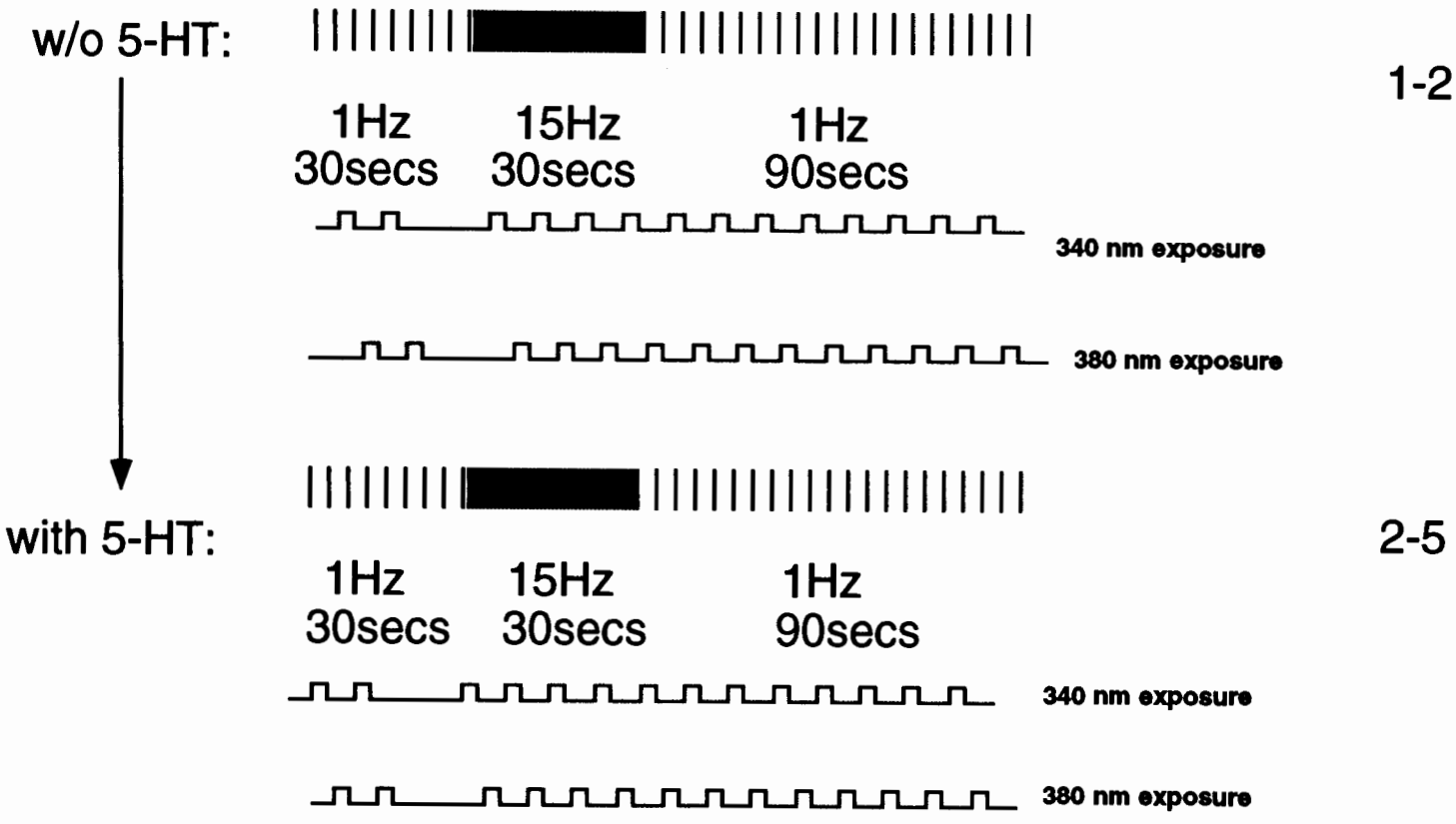
ii) Paradigm

The experimental paradigm illustrated in Figure 24 was used to determine whether the

Figure 24. Experimental protocol to examine the buildup and recovery of intracellular calcium concentration before and after the application of serotonin.

stimulation pattern

# of trials([Ca]$_i$ imaging)



decay of intracellular calcium concentration following a train is slowed by 5-HT.

Several images were taken during the 1 Hz stimulation before the onset of 15 Hz tetanus to obtain the control $[Ca^{2+}]_i$. The preparation was sequentially exposed to 340 nm and 380 nm excitation wavelength during 15 Hz conditioning train and 1 Hz testing impulses thereafter. The exposure time was set to 400 msecs. The emission light was collected by a CCD camera which was subsequently used to form consecutive images indicative of $[Ca^{2+}]_i$ change.

5.1.3 Results and data analysis

Almost all the measurements indicate that $[Ca^{2+}]_i$ recovery is not prolonged by serotonin. Figure 25 shows a typical result and Figure 26 summarizes the data from seven claws (total 41 terminals).

Statistical analysis using a paired-data t-test indicates no significant difference ($P >> 0.5$, $n=41$) between the time constant of $[Ca^{2+}]_i$ recovery in the presence of serotonin (13.6 ± 0.9, mean±SEM) and that in the absence of serotonin (14.5 ± 0.8, mean±SEM).

We further computed the 95% confidence limits of the slope and intercept of linear regression line (Figure 26) to be 0.89 ± 0.23 and 1.51 ± 1.70, respectively. Since 1 and 0 fall within the confidence intervals of slope and intercept respectively, this linear regression line is not statistically different from a line with slope = 1 and intercept = 0. From this test, it could be concluded that the recovery of $[Ca^{2+}]_i$ with 5-HT strongly correlates with the recovery of $[Ca^{2+}]_i$ in the absence of 5-HT.

5.1.4 Discussion

Since the $[Ca^{2+}]_i$ recovery with 5-HT follows the time course similar to that without 5-HT, the extrusion rate of Ca^{2+} pump apparently remains the same after the perfusion of serotonin. Although the quantitative measurement of the rate of calcium influx was not implemented, the fact that $[Ca^{2+}]_i$ seems to reach the same plateau level (refer to Figure 24) led us to conclude that the rate of Ca^{2+} influx through voltage-gated calcium channels had not been changed either. By the

Figure 25. 5-HT application does not alter $[Ca^{2+}]_i$ buildup during tetanic stimulation or the $[Ca^{2+}]_i$ recovery following the tetanic stimulation.

The time constant of $[Ca^{2+}]_i$ recovery τ = 5.6 seconds without 5-HT and 5.6 seconds with 5-HT, while the time course of recovery from slow ADSE is prolonged over 3 fold by the presence of 5-HT (τ = 20.2 seconds with 5-HT). The recovery time constant of slow ADSE in the absence of serotonin is in the range of 5-11 seconds (previous experimental data), which normally coincides with that of $[Ca^{2+}]_i$ decay (Delaney and Tank 1994). The magnitude of ADSE (A) during the test trial is estimated by the ratio of testing EJP amplitude to EJP control amplitude.

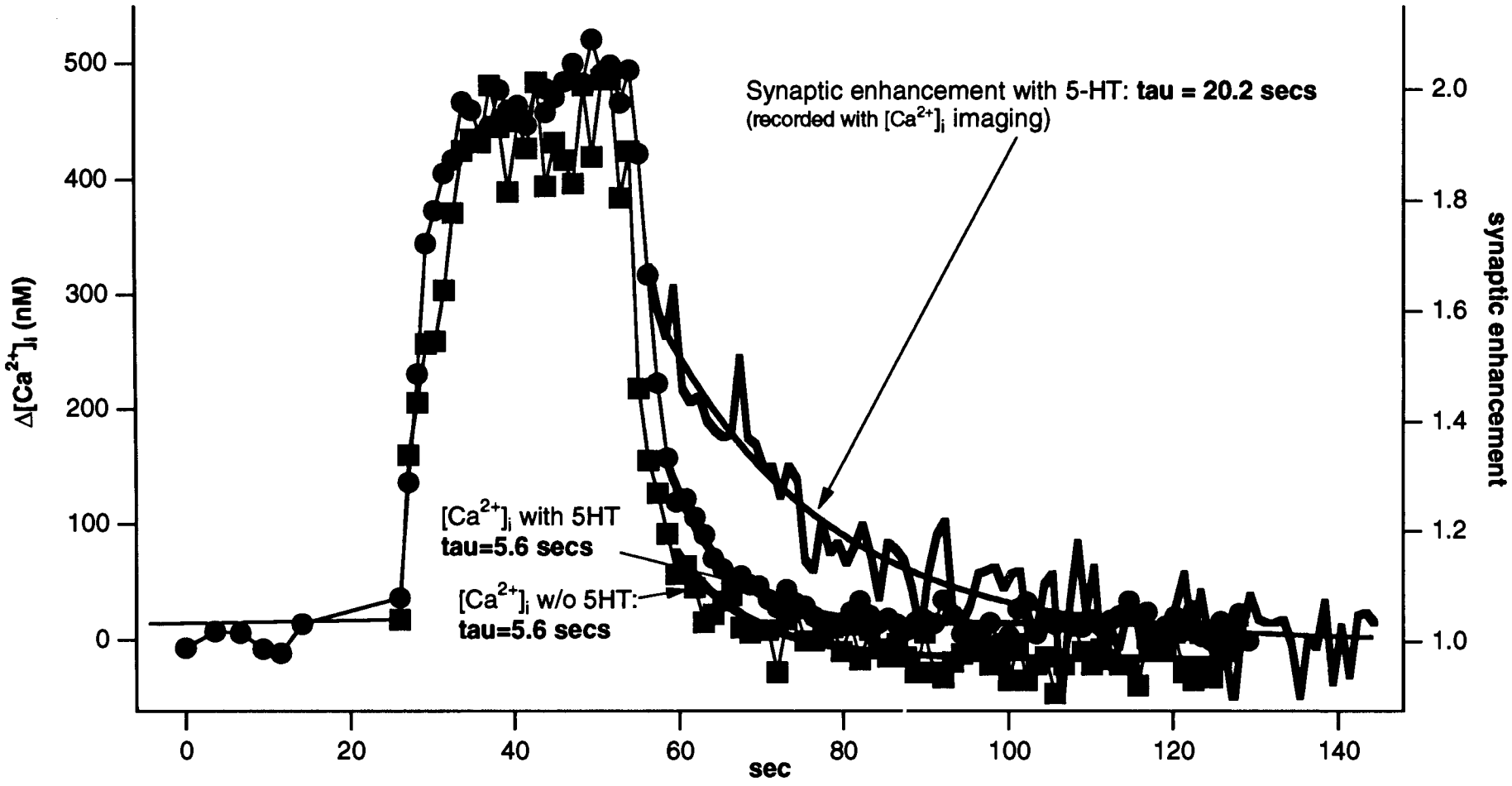
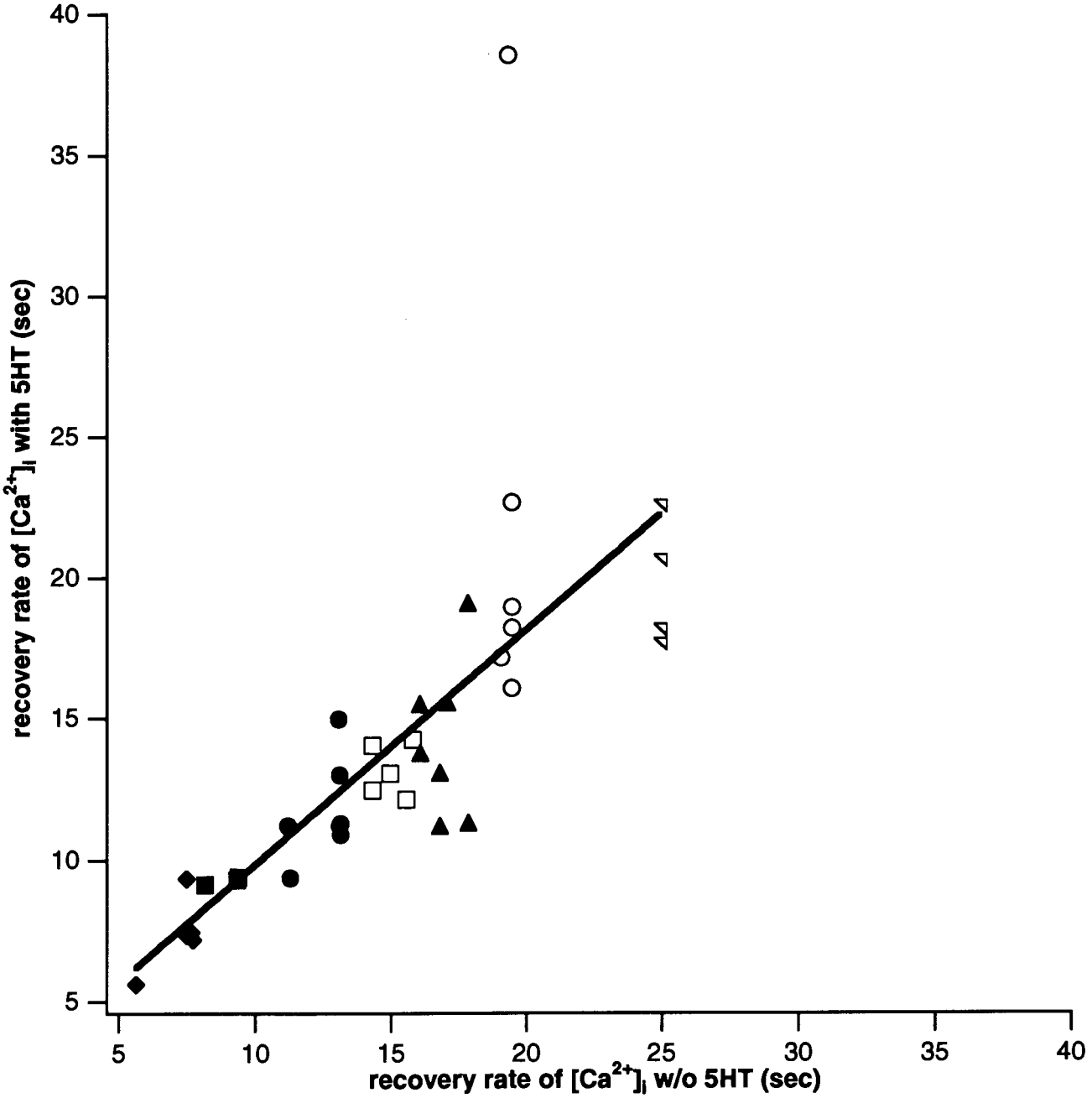

Synaptic enhancement with 5-HT: tau = 20.2 secs
(recorded with [Ca2+]i imaging)
[Ca2+]i with 5HT
tau=5.6 secs
[Ca2+]i w/o 5HT:
tau=5.6 secs
Δ[Ca2+]i (nM)
synaptic enhancement
sec
500
400
300
200
100
0
2.0
1.8
1.6
1.4
1.2
1.0
0
20
40
60
80
100
120
140

Figure 26. A plot delineating the relationship between the time constant of $[Ca^{2+}]_i$ recovery with 5-HT and without 5-HT (n=41).

Different sets of symbols stand for the data obtained in different experiments under various conditions, i.e., different temperatures and different fura-2 content. The number of terminals measured in each experiment ranges from 3-7 (total 41 terminals). A paired-data t-test indicates no significant difference ($P >> 0.5$, n=41) between the time constant of $[Ca^{2+}]_i$ recovery in the presence of serotonin (13.6±0.9, mean±SEM) and that in the absence of serotonin (14.5±0.8, mean±SEM). *Solid line* is a linear fit to the data with slope = 0.83, intercept = 1.51 seconds, r = 0.76 (linear correlation coefficient).


40
35
30
25
20
15
10
5
recovery rate of [Ca2+]i with 5HT (sec)
5
10
15
20
25
30
35
40
recovery rate of [Ca2+]i w/o 5HT (sec)

rationale discussed in introduction (5.1.1), serotonin-induced intracellular calcium buffering is also excluded. All the evidence points to a conclusion that, with 5-HT, there is a slower kinetic process that predominates the recovery of synaptic enhancement other than the change of $[Ca^{2+}]_i$.

Two working hypotheses are proposed. From the data it seems that the presence of serotonin may affect the intracellular biochemical reactions in such a way that the change of calcium concentration no longer determines the recovery of augmentation. It could be further assumed that the slower recovery from EJP enhancement observed in previous experiments no longer reflects the recovery rate of augmentation. In *Aplysia*, accumulated intracellular Ca^{2+} brought in by a train of action potentials exerts a synergistic effect on the serotonin-activated cAMP pathway (Abrams et al. 1991). Following the tetanus, the enhanced cAMP pathway will reverse to the pre-tetanic state with a much slower time constant. Since the approach that is taken to investigate these biochemical processes is to measure the final amount of transmitter released, the effect of the recovery from enhanced cAMP on transmitter release, may masquerade the recovery from residual Ca^{2+}-driven augmentation which occurred under normal conditions. Therefore, the slower recovery from EJP enhancement after the train may represent the recovery rate of enhanced cAMP production, instead of the recovery of traditionally defined augmentation.

It is also possible that residual Ca^{2+} still plays a role in the process of recovery of synaptic enhancement, although the change of $[Ca^{2+}]_i$ no longer dictates the recovery of augmentation. The series of reactions which are responsible for augmentation before the application of 5-HT still account for this synaptic enhancement after the application of 5-HT. The only thing that differs is that serotonin might significantly alter the rate of one reaction in the series, so that this reaction predominates over the change in $[Ca^{2+}]_i$ and becomes the new rate-limiting step. If this is the case, the change of residual calcium concentration is still involved in the process of recovery from synaptic enhancement. The change of residual calcium dynamics, thus, should be reflected during the recovery, although not as dominantly as that in the absence of serotonin. The detailed mechanisms at the biochemical level, however, need to be clarified by further investigation.

5.2 Increasing Ca^{2+} buffer capacity

5.2.1 Introduction

Introduction of exogenous Ca^{2+} buffer into presynaptic terminals slows the buildup and recovery of $[Ca^{2+}]_i$ (Delaney and Tank 1994). EDTA was chosen for this study since it is a kinetically slow buffer and does not significantly block synaptic transmission, unlike a fast buffer such as BAPTA and EGTA which reduce the amount of transmitter released at moderate concentrations (Regehr et al. 1994). If changing calcium dynamics exerts an effect on the prolongation of augmentation, it would provide evidence for the possible role of residual calcium in the phenomenon. Conversely, if the first hypothesis is true, the recovery of synaptic enhancement is not a residual Ca^{2+}-driven process, then changing calcium dynamics would have little impact on the recovery of this enhancement.

5.2.2 Protocol

EDTA (200 mM EDTA, 150 mM KCl) was iontophoresed into the excitor axon via a glass microelectrode by continuous hyperpolarizing current (5-10 nA for 60 mins or more, depending upon the desired EDTA concentration in the terminal). Typically, the buffer was injected at one or two points in the axon and allowed to diffuse 1-2 hr to ensure a uniform distribution of buffer in the terminals. An even distribution of buffer was critical since the experiments were conducted over an interval of 2-3 hours and if the concentration of buffer were to change during this period, it would alter the time constant of recovery independent of any effects of 5-HT.

The experimental procedure is illustrated in Figure 27.

5.2.3 Results

Table 6 presents the data obtained from six EDTA experiments.

Figure 27. **Experimental protocol to examine the effect of exogenous buffer EDTA on the serotonin-induced prolongation of ADSE.**

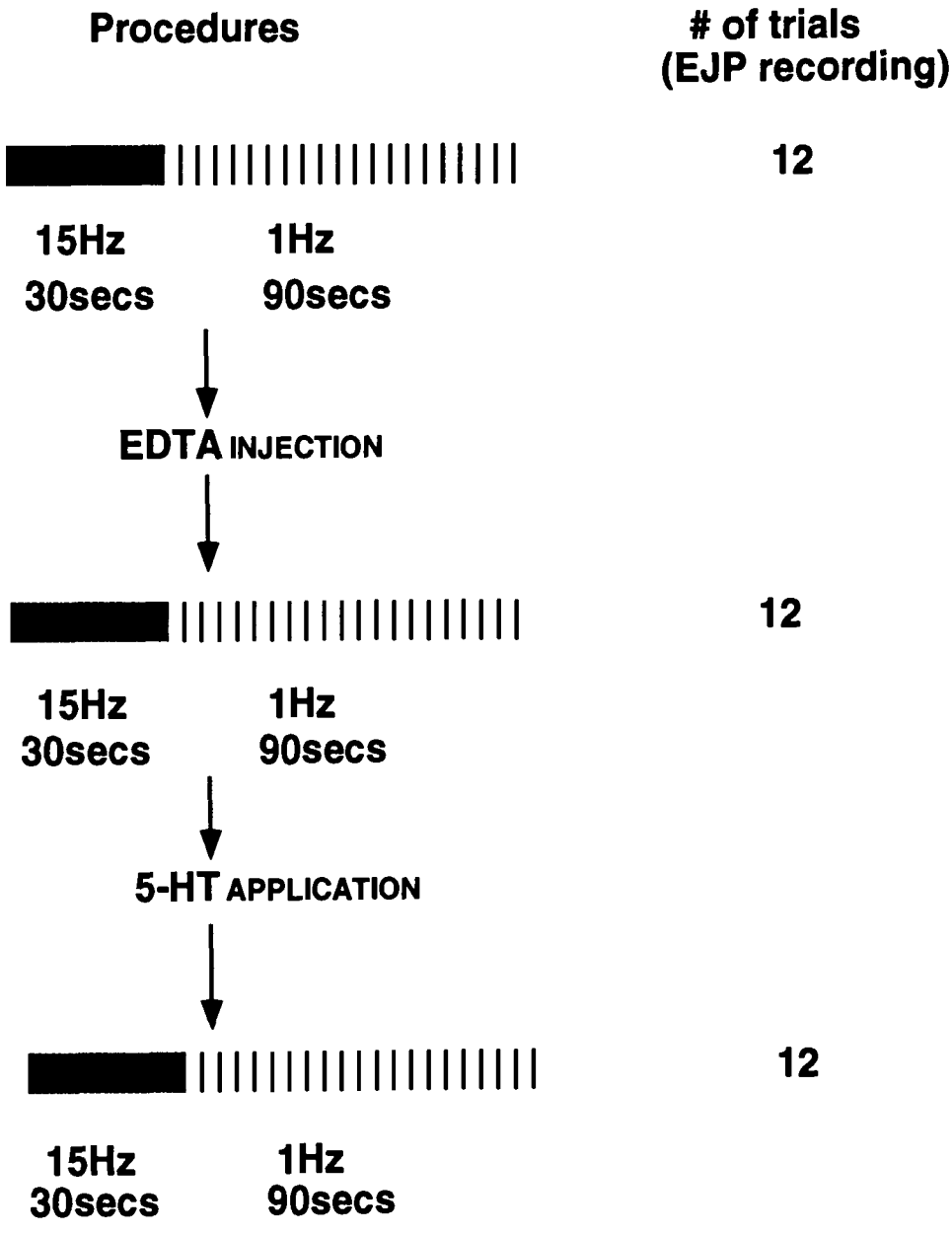

Procedures
# of trials
(EJP recording)
12
15Hz
30secs
1Hz
90secs
EDTA INJECTION
12
15Hz
30secs
1Hz
90secs
5-HT APPLICATION
12
15Hz
30secs
1Hz
90secs

Table 6. Time constants of recovery from synaptic enhancement with exogenous buffer injection and serotonin application

# of experiment	normal τ (sec) w/o EDTA	τ (sec) with EDTA	τ (sec) with EDTA&5-HT
1	6.9	11.5	31.1
2	8.3	25.3	32.3
3	5.1	18.5	25.0
4	10.6	38.6	33.3
5	5.0	22.0	29.0
6	8.1	34.6	32.6
Mean±SEM	**7.3±0.9**	**25.1±4.1**	**30.5±1.3**

Compared with the prolongation caused by serotonin in the absence of EDTA (refer to the previous data in chapter III), the amount of prolongation in the presence of EDTA seems to be greater (see Figure 28). A two sample t-test was conducted to confirm that the amount of increase in time constant caused by 5-HT with EDTA (n=6) is greater than that without EDTA (n=12). With t = 1.79, the presence of EDTA does facilitate the serotonin-induced prolongation of recovery from synaptic enhancement ($0.025 < P(|t| \geq 1.79) < 0.05$).

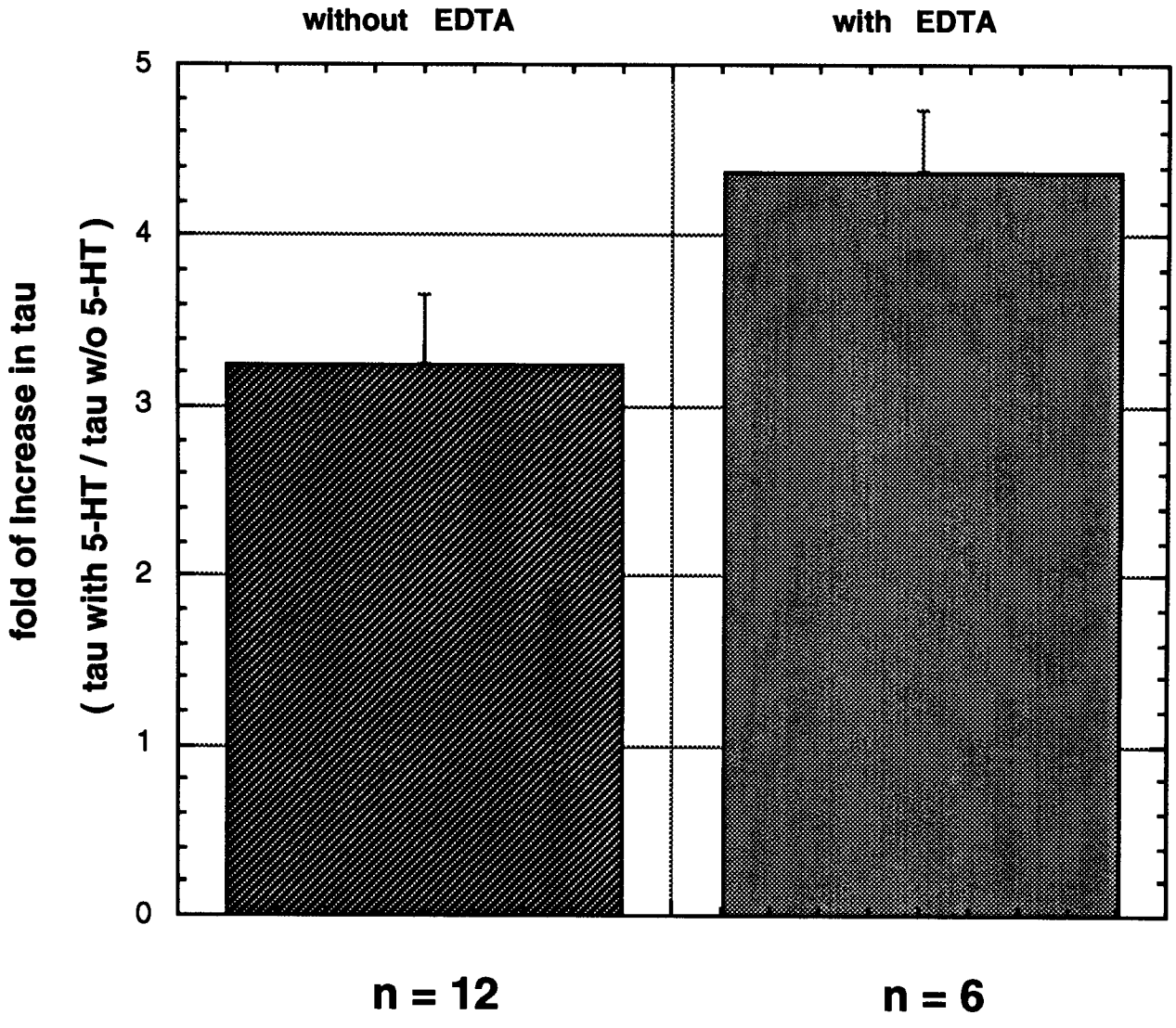
Further two-sample t tests indicate that there is a significant difference in the control time constant of recovery between these two groups of animals ($0.02 < P(|t| \geq 2.426) < 0.05$, df=16). In addition, the time constant in the presence of both 5-HT and EDTA (n=6) is not significantly different from the time constant with the addition of only 5-HT (n=12, $0.6 < P < 0.7$, t = 0.52, df=16). Therefore, it appears that the group of animals in which EDTA was injected had a slightly shorter time constant to begin with in normal NVH so that the fold of increase in the time constant of recovery after the application of 5-HT seems greater.

5.2.4 Discussion

A definite conclusion can not yet be drawn as to whether the prolongation of ADSE is

Figure 28. A bar chart showing that the addition of EDTA seems to facilitate the serotonin-induced prolongation of ADSE.

The data were obtained from two groups of crayfishes (n=12 without EDTA injection; n=6 with EDTA injection). Without injected EDTA, the increase in time constant following the application of serotonin (as estimated by the ratio of the time constant with 5-HT to the time constant without 5-HT) is 3.2±0.4 fold (mean±SEM, n=12, refer to previous data in Table 1 and Figure 10). While in the presence of EDTA, the factor of increase in time constant is 4.4±0.4 (mean±SEM, n=6). There is a significant difference between these two ratios ($0.025 < P(|t| \geq 1.79) < 0.05$, df = 16).


without EDTA
with EDTA
fold of Increase in tau
(tau with 5-HT / tau w/o 5-HT)
5
4
3
2
1
0
n = 12
n = 6

dependent upon the recovery of residual calcium. However, the fact that the magnitude of prolongation seems to be increased by the presence of EDTA appears to be consistent with the hypothesis that residual calcium is still involved in the recovery of ADSE, although not as the dominant rate-limiting step.

In a simplistic model, the following first-order reaction can be assumed to describe the process of synaptic enhancement by residual calcium. As an initial assumption, it is taken that Ca^{2+} binds to a substrate molecule (S) to produce an activated product that increases the probability of transmitter release in a first order kinetic reaction:

$$Ca^{2+} + S \Leftrightarrow CaS^* \qquad (1)$$

The forward and reverse rate constant of this reaction k^+ and k^- have units of $sec^{-1}nM^{-1}$ and sec^{-1} respectively. The dissociation constant of the reaction is K_d ($= k^- / k^+$). If the K_d of this calcium-binding reaction is greatly reduced by 5-HT, the dissociation rate of Ca^{2+} from CaS* can become the new rate limiting step in the recovery of [CaS*] rather than the rate at which Ca^{2+} is extruded by Ca^{2+} pumps. Since the amount of synaptic enhancement is assumed to be proportional to the level of CaS* in this model, the recovery of ehancement, conceptually, must be associated with the rate of CaS* breakdown. Thus, any reduction of k^- or increment of k^+ which decreases the dissociation rate of CaS*, will in effect prolong the recovery of synaptic enhancement.

A question still remains: does this model predict no change of initial amplitude of synaptic enhancement in the presence of serotonin?

The magnitude of synaptic enhancement (in this case, augmentation A_0) caused by the repetitive stimulation is defined as the fraction of increase in a test EJP amplitude after a tetanus over a control EJP amplitude. Therefore, A_0 is represented by the ratio of [CaS*] at the end of prolonged stimulation ($[CaS^*]_0$) to [CaS*] at the resting level ($[CaS^*]_{rest}$). Since the reaction is at equilibrium state at rest and at the end of a prolonged tetanus, $[CaS^*]_0$ and $[CaS^*]_{rest}$ can be expressed by

$$[CaS^*] = (k^+/ k^-) [Ca^{2+}] [S] \quad (2)$$

Apparently, (k^+/ k^-) can be canceled out in the division ($A_0 = \{(k^+/ k^-) [Ca^{2+}]_0 [S]_0\} / \{(k^+/ k^-) [Ca^{2+}]_{rest} [S]_{rest}\}$, such that

$$A_0 = \{[Ca^{2+}]_0 [S]_0\} / \{[Ca^{2+}]_{rest} [S]_{rest}\} \quad (3)$$

with $[S] = [S]_{total} - [CaS^*] = [S]_{total} - (k^+/ k^-) [Ca^{2+}] [S]$, we obtain

$$[S] = [S]_{total} / \{ 1+ (k^+/ k^-) [Ca^{2+}]\}. \quad (4)$$

By substituting [S] in (3), $A_0 = \{[Ca^{2+}]_0 / [Ca^{2+}]_{rest} \} \{ K_d+[Ca^{2+}]_{rest} \} / \{ K_d+[Ca^{2+}]_0\}$

If $K_d >> [Ca^{2+}]$, A_0 reduces to $[Ca^{2+}]_0 / [Ca^{2+}]_{rest}$ (5)

Therefore, the magnitude of augmentation at the end of a sustained stimulus is not dependent upon the value of k^+ and k^- as long as $\mathbf{K_d >> [Ca^{2+}]_i}$. If serotonin acts on k^+ or k^- and K_d remains >> $[Ca^{2+}]_i$, there will not be a significant change in the magnitude of augmentation during the application of serotonin. It seems that, provided that K_d remains far greater than $[Ca^{2+}]_i$, this model might be able to explain why A_0 remains largely unchanged after the application of serotonin (Figure 9b).

Interestingly, this model also predicts that an increment of dissociation constant (k^+/ k^-) should enhance the baseline transmitter release by the same fraction as the prolongation of recovery of synaptic enhancement. This enhanced transmitter release at rest is presumed to be serotonergic enhancement of the individual EJPs. Therefore, the two seemingly different effects of serotonin should decay at the same rate along with the washout of serotonin, which matches the experimental

observation (Figure 12). Nonetheless, this conceptual model needs more elaboration. Especially, whether or not the reduction of K_d within the range of $K_d >> [Ca^{2+}]_i$ could bring about 3-5 fold increase in time constant of recovery needs to be addressed by further kinetic analysis (see next section 5.3).

5.3 A kinetic model

Numerical simulation is carried out to address the following question: does serotonin prolong the recovery of augmentation by reducing the dissociation constant of the calcium-binding reaction? Qualitative aspects of the model under equilibrium conditions (t = 0 and ∞) have been previously discussed (5.2.4). In this section, the nonequilibrium process is examined.

The time evolution of the reaction (1) can be described by the following differential equation:

$$d[CaS^*]/dt = k^+ [Ca^{2+}] [S] - k^- [CaS^*] \qquad (6)$$

By substituting [S] with (3), we obtain

$$d[CaS^*]/dt = k^+ [Ca^{2+}] [S]_{total} - [CaS^*] (k^+ [Ca^{2+}] + k^-) \qquad (7)$$

Under the condition that $[Ca^{2+}]$ follows an exponential decay with a time constant τ (experimental data), i. e.,

$$[Ca^{2+}] = [Ca^{2+}]_{rest} + ([Ca^{2+}]_0 - [Ca^{2+}]_{rest}) e^{(t/\tau)} \qquad (8)$$

This linear differential equation has an analytical solution:

$$[CaS^*] (t) = e^{(-a*t - b*\tau* e^{(-t/\tau)} + b*\tau)} * [\int_0^t (c + d*e^{(-t/\tau)} * e^{(a*t + b*\tau* e^{(-t/\tau)} - b*\tau)}) dt] \qquad (9)$$

where $a = [Ca^{2+}]_{rest}(k^+[S]_{total}-1)$; $b = k^+[S]_{total}([Ca^{2+}]_0-[Ca^{2+}]_{rest})$; $c = k^+[Ca^{2+}]_{rest}$; $d = k^+([Ca^{2+}]_0-[Ca^{2+}]_{rest})$.

Numerical simulation of the kinetic model was performed on a UNIX platform. The integration term of the solution was calculated by the Romberg integration method with a relative precision of 0.001. Simulation results were then transferred from UNIX to a Macintosh computer and imported to Igor to produce graphical decay curves of [CaS*] versus time.

This kinetic simulation shows that, when K_d ($=k^-/k^+$) $>>[Ca^{2+}]$, the time course of [CaS*] basically follows the decay of $[Ca^{2+}]$ (e.g., K_d = 6 μM, see Figure 29).

This numerical model also shows that decreasing k^- by 6000-fold produces a 3-fold prolongation of the recovery of [CaS*]. However, this change brings K_d into a range where K_d is comparable to $[Ca^{2+}]_i$, which, according to the previous discussion in 5.2.4., A_0 can no longer be maintained. Figure 30 demonstrates that A_0 is greatly reduced under conditions that produce a 3 fold increase in time constant. Moreover, with K_d in the nM range, this substrate has absorbed essentially all the residual Ca^{2+} in the cytoplasm. As a consequence, there are not enough free calcium ions for fura-2 to bind and produce a sufficient fluorescent signal. This does not match the experimental observations.

Based on this model, it is concluded that reducing the dissociation constant of the Ca^{2+}-binding reaction is not likely to prolong the augmentation 3-4 fold without changing its initial amplitude.

Figure 29. A simulated recovery of [CaS*] follows the same time course as that of $[Ca^{2+}]_i$ (K_d = 6 μM).

The simulated recovery of [CaS*] is represented by a solid line corresponding to the left axis scale. The recovery of $[Ca^{2+}]_i$, corresponding to the right axis scale, is represented by the dotted line (experimental data).

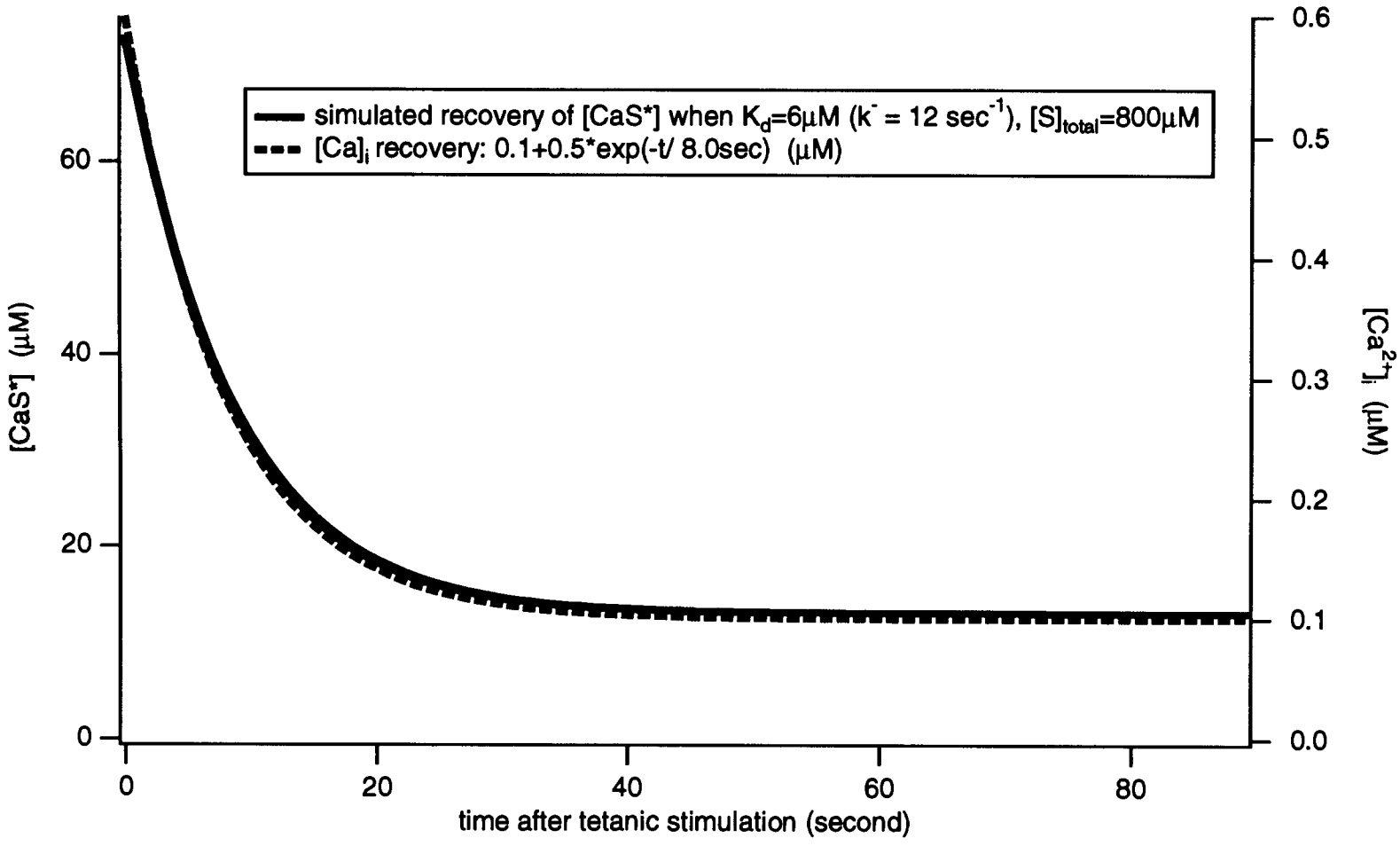
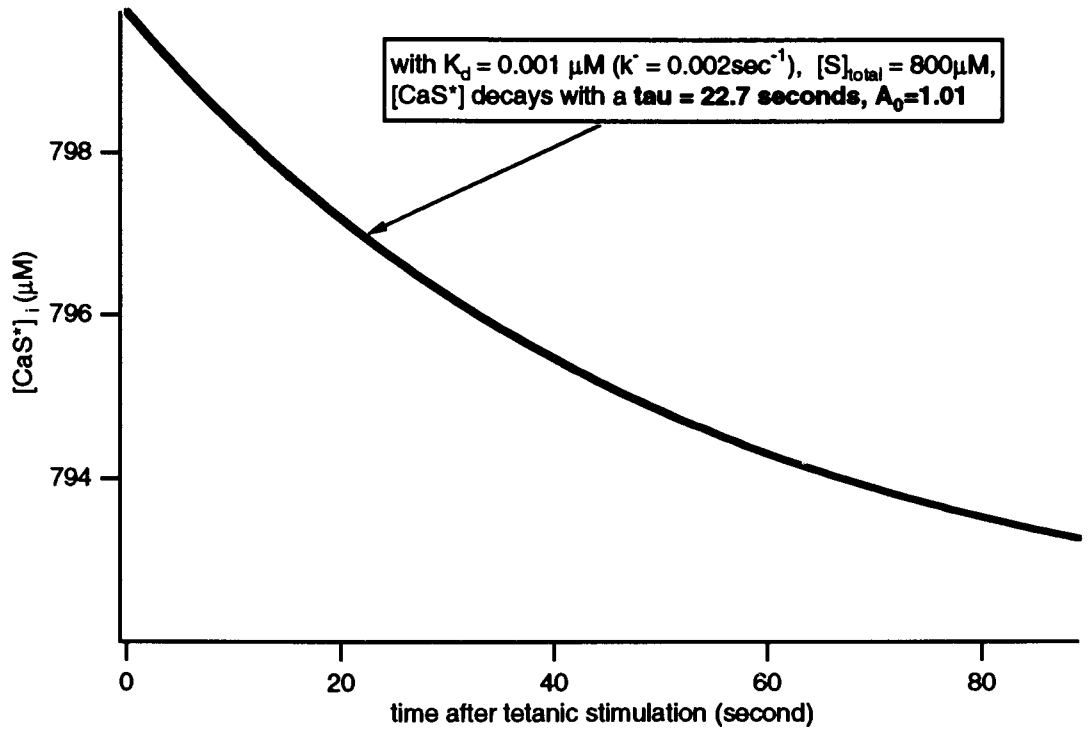

simulated recovery of [CaS*] when K_d=6μM (k^- = 12 sec^{-1}), $[S]_{total}$=800μM
$[Ca]_i$ recovery: 0.1+0.5*exp(-t/ 8.0sec) (μM)
[CaS*] (μM)
$[Ca^{2+}]_i$ (μM)
0
20
40
60
80
0.0
0.1
0.2
0.3
0.4
0.5
0.6
time after tetanic stimulation (second)

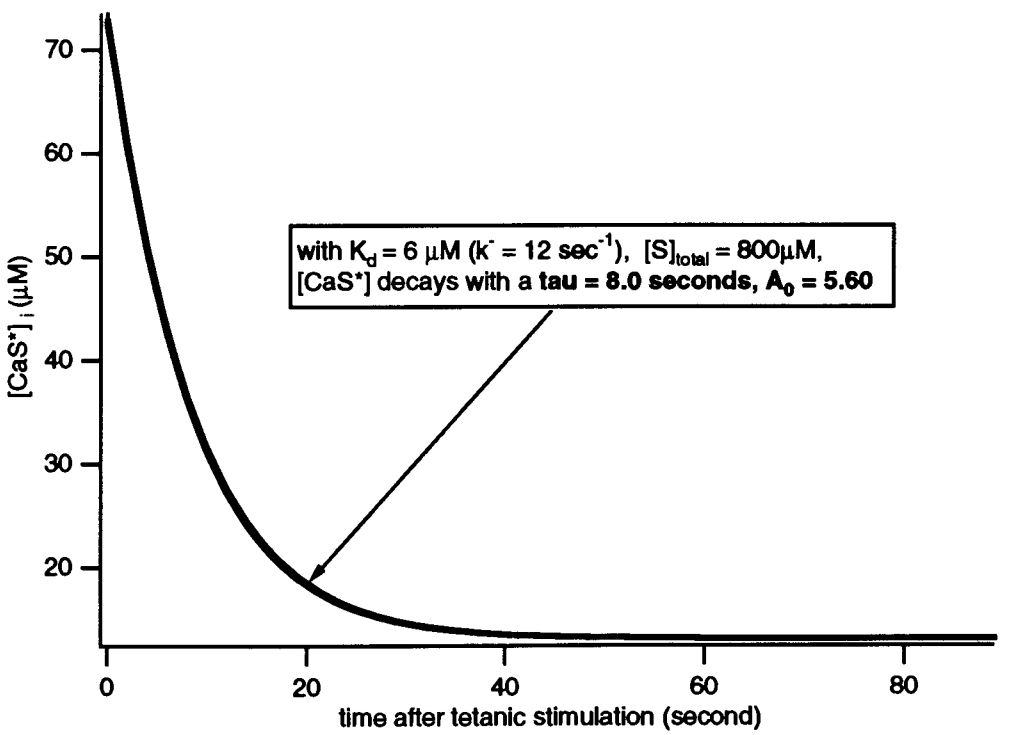
Figure 30. Reducing K_d to 0.001μM results in a recovery of [CaS*] with an approximately threefold longer time constant (22.7 seconds) as compared to that with K_d = 6μM (8.0 seconds).

In this model, the magnitude of augmentation is assumed to be directly proportional to the amount of CaS*. Thus, in addition to the prolonged recovery of enhancement, the magnitude of the enhancement, as measured by $[CaS^*]_0 / [CaS^*]_\infty$, is appreciably decreased (from 5.60 to 1.01) by the reduction in K_d.

A


with K_d = 0.001 μM (k^- = 0.002sec^{-1}), $[S]_{total}$ = 800μM,
[CaS*] decays with a **tau = 22.7 seconds, A_0=1.01**
798
796
794
$[CaS^*]_i$ (μM)
0
20
40
60
80
time after tetanic stimulation (second)

B


with K_d = 6 μM (k^- = 12 sec^{-1}), $[S]_{total}$ = 800μM,
[CaS*] decays with a **tau = 8.0 seconds, A_0 = 5.60**
70
60
50
40
30
20
$[CaS^*]_i$ (μM)
0
20
40
60
80
time after tetanic stimulation (second)

Chapter VI

General Conclusions and Discussions for Future Directions

6.1 General conclusions

(1) In this study, a previously unknown neuromodulatory effect of serotonin has been discovered at crayfish opener neuromuscular junction. Serotonin (1 μM) prolongs the recovery of the slow phase of activity-dependent synaptic enhancement (normally called augmentation) by three fold without changing the initial magnitude of augmentation measured immediately after the conditioning train.

(2) Several possible cellular mechanisms that could be responsible for this serotonin-induced prolongation of ADSE were examined. It is found that:

a) Residual calcium dynamics are not changed by 5-HT, therefore, these can not account for the prolonged recovery of slow ADSE in the presence of serotonin.

b) Washout of serotonin-induced prolongation of slow ADSE is strongly correlated with washout of serotonergic enhancement of individual EJPs. This suggests that the same biochemical cascades that are involved in serotonergic enhancement (e.g., phosphatidyl inositol and adenylate cyclase cascades) are likely to be responsible for the prolonged ADSE as well.

c) Fatigue of the preparation, saturation of transmitter release, synaptic depression, and changes in presynaptic action potentials are not primarily responsible for the prolongation of recovery from ADSE.

6.2 Functional significance of the prolongation of synaptic enhancement

6.2.1 Functional significance of activity-dependent short term synaptic plasticity

Activity-dependent changes in synaptic efficacy are important for neural functions.

Neurons in the nervous system typically operate through bursts of impulses rather than through single isolated spikes. Use-dependent synaptic plasticity, which is a likely consequence of these bursts, is thus widely distributed at various levels of neural circuits. Since synapses are constantly modified to reflect their history of use as though the synapse "remembers" its previous activity, this type of synaptic behavior is sometimes called "synaptic memory". In addition to long-term stimulation-induced changes such as long term potentiation (LTP), synaptic modifications on a relatively shorter time scale (including augmentation) could provide the nervous system with a wider dynamic range and more flexibility in storing various kinds of information. Short-term activity-dependent synaptic plasticity has been widely observed in the mammalian CNS (e.g., Regehr et al. 1994; Zengel et al. 1980), and thoroughly characterized at invertebrate synapses and vertebrate neuromuscular junctions (for review see Atwood and Wojtowicz 1986; Magleby 1987). Although the physiological functions of short-term activity-dependent synaptic plasticity are not clear in many instances, it is believed that the nervous system is designed to utilize these synaptic memories. Short term activity-dependent synaptic plasticity might serve as the biophysical basis of some forms of short term memory and psychophysical phenomena such as priming (Regehr et al. 1994; Tank et al. 1995). The molecular substrate of explicit memory has been traditionally related to LTP in the CA1 region of hippocampus. However, recent studies indicate that the presence of short term activity-dependent synaptic enhancement (PTP in particular) and the elevation of $[Ca^{2+}]_i$ over a critical threshold may be required for inducing and stabilizing LTP (Malenka 1993). In other words, short term activity-dependent synaptic plasticity might be involved either directly or indirectly to facilitate the formation of long-term memory.

6.2.2 Functional significance of neuromodulation of short term activity-dependent synaptic plasticity

It is well established that nerve cells act to integrate information in the nervous system. In any neural circuit, the information carried by nerve signals is conveyed from one synapse to another. The output of the circuit not only depends upon the connectivity of the neurons, but also

relies heavily on the input-output relationship of individual neurons that constitute the circuit. Previously, it was reasoned that short term synaptic plasticity such as augmentation (as a type of input-output function of a single neuron) might play an essential role in determining the output of the neural circuit based upon previous neural activity. Any factor that alters the input-output relationship of individual neurons could, in turn, affect the output of neural circuits. Consider serotonin-modified augmentation discovered in this study as an example: normally when serotonin is not present, the synapse would have a short memory of previous stimulation and would actively integrate temporal information over a 20-sec period. If subsequent nerve impulses follow within 20 seconds, the neuron will release a greater amount of transmitter for each impulse. When the synapse receives neuromodulation from serotonin, however, the synapse has a much longer memory (60-80 seconds) and is able to integrate temporal information carried by nerve impulses over a couple of minutes, information that would otherwise be neglected or unintegrated. Undoubtedly, such neuromodulation of activity-dependent plasticity would have a significant impact on the output of the circuits, and eventually on the computations performed by large scale neural networks and nervous systems.

i) At the circuit level

The discovery that serotonin could prolong a form of activity-dependent synaptic plasticity presents a new opportunity for elucidating the cellular mechanisms by which serotonin modulates the output of neural circuits. For instance, it has been found, in stomatogastric nervous system of the crab, that the release of serotonin from GPR cells (gastropyloric receptor) has pronounced effects on the pyloric motor pattern (Katz and Harris-Warrick, 1990a). These effects include a prolonged increase of the pyloric cycle frequency. Prior to the input of serotonin, the pattern is slow and irregular. Upon receiving 5-HT, the cycle frequency is increased to 0.6 Hz and prolonged over a minute so that it remains as high as 0.4 Hz at the time of second stimulus train. Consequently, the cycle frequency reaches its peak within 5 cycles, where it normally would have taken many more cycles to reach the peak if serotonin were not present. In this case, serotonin might be involved in altering the motor pattern by prolonging the recovery of activity-dependent

synaptic enhancement of individual neurons.

The finding that serotonin can modify the time course of ADSE could also lead to a better understanding of the synchronization of different circuits that are otherwise separated from each other. An essential requirement for the synchronization of circuits is the overlapping of the time-frames of their activities. For example, if the firing of two pattern generators never occurs at the same moment, synchronization of these two patterns would be virtually impossible. However, if serotonin acts to lengthen the period of activity in either generator so that they overlap in time, the probability of synchronization between the two would be greatly enhanced. In fact, ample evidence (Katz and Harris-Warrick 1990b) suggests that neurohormones induce the fusion of different CPGs into a single conjoint network that produces a combined motor pattern distinguished from the previous ones. In some other cases, the modulatory input can recruit neurons from one functional CPG into another.

The general concept introduced here, although still at a speculative stage, may apply not only to CPGs, but also to various neural networks. No matter how large the network is, it must be made up of small circuits like CPGs interconnected with each other, and ultimately, any circuit is constituted by individual neurons. In addition, many tasks performed by small circuits, such as synchronization and prolonged excitation, are also performed by large scale networks. Thus, resolving how these tasks are carried out by nervous systems would eventually rest on the plasticity of individual synapses, which are the building blocks of any complex neural circuits. Neuromodulators such as serotonin could play important instructive roles in determining the moment-to-moment output of a neural circuit simply by altering the synaptic strengths and intrinsic cellular properties of the circuit neurons in a fashion which changes their temporal integration properties.

ii) From a behavioural perspective

5-HT has been implicated in a huge diversity of behavioral and physiological processes including learning (reviewed by Byrne 1987) and neuronal development (Lauder 1990). This is not surprising considering the ubiquitous distribution of 5-HT-containing axon terminals

throughout CNS. More impressively, there is a striking similarity of 5-HT-modified behavior in vertebrates and invertebrates, despite the enormous differences in their gross body morphology and general organization of their nervous systems (brain rather than nervous ganglion). 5-HT seems to enhance motor output and suppress sensory input in both vertebrates and invertebrates (Jacobs and Fornol 1993). Implicit learning and memory, as opposed by its explicit counterpart which involves a conscious awareness of what is learned and recalled, do not involve the hippocampus, but involve the specific sensory and motor systems used during the learning process (Jessel and Kandel 1993). As a result, implicit forms of learning can be studied in simple invertebrate nervous systems and the mechanisms underlying the physiological effects of serotonin in crustacean could potentially be extended to vertebrate systems. So far, there are still many intriguing possibilities regarding the cellular events that underlie serotonin-modified behavioural phenomena. The finding that serotonin prolongs the time course of synaptic enhancement might have recovered a missing link towards the understanding of these phenomena. In this regard, the knowledge of the effect of serotonin on the time course of synaptic enhancement could bear tremendous behavioral implications.

6.3 Discussion for future directions

6.3.1 Ideal systems to demonstrate the behavioral impact of the change of the time course of synaptic enhancement

Neural networks are highly adaptable to ever changing conditions. The output of CPG used for generating simple rhythmic movements was initially believed to be strictly determined by its pattern of connectivity. It is now thought to be surprisingly flexible according to a number of experimental reports (e.g., Dickinson et al 1990; Katz and Harris-Warrick, 1990b). The output of the circuits could be orchestrated by neuromodulatory input from the CNS, either directly through neural pathways or indirectly through the action of neurohormones.

Neuromodulation of circuits has been demonstrated in many systems. In *Aplysia*, several

identified interneurons modulate the motor networks underlying feeding and learning behavior by releasing serotonin (Weiss et al. 1981). In *Tritonia*, one of the component neurons in the CPG for generating the escape swimming pattern releases serotonin. This intrinsic neuromodulation may be important for certain types of learning by lowering firing threshold and onset latency (Katz et al. 1994). More interestingly, in the moth *Manduca sexta*, 5-HT has been reported to extend the time window during which pheromone-induced behavior can be elicited and general motor activity can be enhanced (Kloppenburg and Hildebrand 1995). 5-HT also reduces the conditioned olfactory responses in honeybees (Menzel et al. 1988). The stomatogastric nervous system of decapod crustaceans is one of the best characterized systems for the study of the neuromodulation of motor patterns because of its well characterized circuitry with identifiable neurons. The actions of modulatory input include rewiring circuit diagram (Nagy and Moulins 1987), coordinating multiple CPGs (Katz and Harris-Warrick, 1990b), and creating conjoint CPGs (Dickinson et al. 1990), not simply initiating and maintaining motor patterns.

To specifically demonstrate how the lengthening of the recovery from synaptic enhancement could affect the output of circuits, a model system in which short-term activity-dependent synaptic plasticity determines the output of the circuit is required. In the meantime, serotonin, *in vivo*, should have access to this circuit. Only in this kind of model system would it be possible to clearly demonstrate how the application of serotonin alters the activity pattern of the circuit by acting on activity-dependent plasticity of individual neurons, and to ensure this modulation could actually take place *in vivo*.

6.3.2 Further exploration of biochemical reactions underlying the prolongation

The first step to investigate the detailed biochemical reactions responsible for the prolongation of augmentation caused by serotonin would be to confirm the involvement of PKC and cAMP pathways. This could be done by utilizing either a blocker or an activator of these two pathways. The fact that these blockers and activators can work at different levels of these two pathways could be very advantageous for us in examining the biochemical reactions in greater

detail. For example, 1μM forskolin activates adenylate cyclase while presynaptic injection of SQ22,536 inhibits the activity of adenylate cyclase. Exposure to micromolar IBMX which inhibits phosphodiesterase increases the cAMP concentration, while the Walsh inhibitor (PKI) could directly activate PKA after injected into the presynaptic terminals (Dixon and Atwood 1989b). There are also many drugs which either stimulate or inhibit the PKC pathways. The application of a phorbol ester (TPA) could act as a direct activator of protein kinase C. Meanwhile, PKC concentration could be increased by injecting IP_3 into presynaptic axon. RA233, a potent phospholipase C inhibitor, can be used to block PKC pathway (Dixon and Atwood 1989a). If the blocker of a certain biochemical pathway eliminates the serotonin-induced prolongation of slow ADSE, or the activator of a certain biochemical pathway replicates the serotonin-induced prolongation, then this pathway is likely to be involved in the action of serotonin. Likewise, the inhibitor or the activator of a specific protein could help determine the role of this protein in the serotonin-induced prolongation of activity-dependent synaptic enhancement.

One way to monitor the dynamics of cAMP activity in single cells is to employ a fluorescent indicator (FlCRhR) whose fluorescence intensity is dependent upon the concentration of cAMP (Adams et al. 1991). By using similar imaging techniques that examine the change of intracellular calcium concentration, the activity level of PKA pathways during the application of serotonin could be observed closely.

A likely calcium-binding protein in cytoplasm is calmodulin. Ca^{2+} and calmodulin, once they form a complex, are able to activate a variety of target proteins such as protein kinase C, adenylate cyclase and CaM Kinases. Therefore, tetanic stimulation that produces Ca^{2+}-driven augmentation, via calmodulin, could potentially interact with serotonin-activated PKA and PKC pathways (see Figure 4). CaM Kinase II has been suggested to phosphorylate synapsin I in the induction of augmentation (Delaney and Tank 1994). There is a CaM Kinase II inhibitor (KN-62) which could be used to examine the role of CaM Kinase II in the serotonin-induced prolongation of augmentation (Kamiya and Zucker 1994). Using calmidazolium (a calmodulin inhibitor) although it may lack the specificity, the involvement of calmodulin could be tested (Kamiya and Zucker

1994). By combining these blockers and activators, the biochemical mechanisms underlying serotonin-induced prolongation of activity-dependent synaptic enhancement could be determined.

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Appendix

List of Abbreviations

A	augmentation
ADSE	activity-dependent synaptic enhancement
AP	action potential
CNS	central nervous system
CPG	central pattern generator
K_d	dissociation constant
EDTA	ethylenediaminetetraacetic acid
EJP	excitatory junctional potential
EPSP	excitatory postsynaptic potential
k^+	forward rate constant
GABA	gamma-aminobutyric acid
GPR	gastropyloric receptor
5-HT	5-hydroxytryptamine or serotonin
IP_3	inositol 1,4,5-trisphosphate
LTP	long term potentiation
nmj	neuromuscular junction
NVH	normal van Harrevald's saline
PSP	postsynaptic potential
PTP	posttetanic potentiation
PLC	phospholipase C
PKC	protein kinase C
PKA	protein kinase A
k^-	reverse rate constant
F	short-term facilitation or paired-pulse facilitation