University of Alberta

Roles of 5-HT in the Regulation of Neurite Outgrowth in an Identified Serotonergic Neuron C1 from *Helisoma trivolvis*

by

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A thesis submitted to the Faculty of Graduate Studies and Research in partial fulfillment of the requirements for the degree of Doctor of Philosophy in Specialization of Physiology and Cell Biology

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Edmonton, Alberta

Fall, 2006

Your file Votre référence
ISBN: 978-0-494-22982-8
Our file Notre référence
ISBN: 978-0-494-22982-8

ABSTRACT

A considerable amount of evidence has accumulated to support the hypothesis that 5-HT (5-hydroxytryptamine) is an important regulatory neurotransmitter in both developing molluscan nervous systems and mammalian brains. Neuronal pathfinding and neurite extension are crucial in forming initial neural networks of developing nervous systems. Thus, our knowledge of the putative 5-HT roles in regulating regenerative neurite outgrowth from molluscan culture studies may allow us to better understand the development of nervous systems.

5-HT is known to be a diffusible factor that influences neurite outgrowth. Neurite outgrowth consists of four essential phases; initiation, elongation, guidance and cessation. The present study tested two hypothesized regulatory roles of 5-HT in neurite outgrowth using serotonergic neuron C1 cultures from *Helisoma trivolvis*; (1) a tonic inhibitory role on the onset of neurite elongation and (2) differential modulatory roles on neurite elongation.

5-HT inhibited the onset of neurite elongation in a dose-dependent manner. This inhibition was prevented by prior treatment of neuron C1 with *pCPA*, a tryptophan hydroxylase inhibitor. High concentrations of 5-HT (50 and 100 μM) reduced the probability of neurite elongation, causing neuronal necrotic death. *Helisoma* brains were found to release 5-HT into culture media, called standard brain-conditioned medium (s-CM). The s-CM was then found to inhibit the onset of neurite elongation, suggesting that 5-HT may have a

tonic inhibitory effect on the phase transition from neurite initiation to elongation.

Application of 5-HT resulted in differential effects on neurite outgrowth at different elongation rates. In slow-growing neurites, lower concentrations of 5-HT facilitated elongation, whereas higher concentrations of 5-HT inhibited neurite elongation or caused growth cone collapse. In fast-growing neurites, 5-HT inhibited neurite elongation or caused growth cones to collapse, in a dose-dependent manner. Neurites with collapsed growth cones usually returned to elongation after the removal of 5-HT. These differential effects of 5-HT on elongation could be due to the changes of intracellular Ca^{++} levels in individual neurites including their growth cones.

Taken together, 5-HT is suggested to be an extrinsic regulator in the phase transition of neurite outgrowth that acts in a differential regulatory fashion for the onset of elongation and control of elongation rate, during neuronal regeneration.

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5,7-DHT, 5,7-dihydroxytryptamine

5-HIA, 5-hydroxyindoleacetaldehyde

5-HT, 5-hydroxytryptamine

5-HTP, 5-hydroxytryptophan

8-OH-DPAT, 8-hydroxy-2-di-n-propylamino-tetralin

AC, adenylate cyclase

Ach, acetylcholine

ADF, actin-depolymerizing factor

ADNF, activity-dependent neurotrophic factor

ANOVA, analysis of variance

ApCdc42, *Aplysia* cell division cycle 42 (GTP binding protein, 25kDa)

ApHM, *Aplysia* hemolymph

Arp2/3, actin related protein 2/3 complex

ART, artemin

ATP, adenosine triphosphate

B19, *Helisoma* buccal ganglionic neuron 19

B4, *Helisoma* buccal ganglionic neuron 4

B5, *Helisoma* buccal ganglionic neuron 5

BDNF, brain-derived neurotrophic factor

BSA, bovine serum albumin

C1, *Helisoma* cerebral ganglionic neuron 1

cAMP, cyclic adenosine monophosphate

CAMs, cell adhesion molecules

CBC, cerebrobuccal connectives

CCD, charge-coupled device

CD44, cell surface glycoprotein

Cdc42, cell division cycle 42 (GTP binding protein, 25kDa)

cDNA, chromosomal deoxyribonucleic acid

C-domain, central domain

CGC, cerebral giant cell

CM, conditioned medium

CNS, central nervous system

CNTF, ciliary neurotrophic factor

CT-1, cardiotrophin-1

DA, dopamine

dic, days in culture

DIC, differential interference contrast

DM, defined culture medium

DMSO, dimethyl sulfoxide

ECL, enhanced chemo-luminescence

ECM, extracellular matrix

EGF, epidermal growth factor

ELISA, enzyme-linked immunosorbent assay

ENC1, embryonic neuron C1

EPSP, excitatory postsynaptic potential

F-actin, filamentous actin

FGF, fibroblast growth factor

FITC, fluorescein isothiocyanate

FMRFamide, Phe-Met-Arg-Phe-NH_2

Fura-2 AM, fura-2 acetoxymethyl ester

GABA, γ-aminobutyric acid

G-actin, globular actin

GAP-43, growth-associated protein 43

GDNF, glial cell-lined derived neurotrophic factor

GDP, guanosine diphosphate

GnRH, gonadotropin-releasing hormone

GTP, guanosine triphosphate

HB-GAM, heparin-binding growth-associated molecules

HEPES, 4-2-hydroxyethyl-1-piperazineethanesulfonic acid

HPLC, high-performance liquid chromatography

HPLC-ED, HPLC with electrochemical detector

HSP70, heat shock protein 70

IBMX, isobutyl methylxanthine

IGF, insulin-like growth factor

IL, interleukin

IM, intramuscular

KA, kainatic acid

KLH, keyhole limpet hemocyanin

L1-CAM, cell adhesion molecule L1

LGCs, neuroendocrine light green cells

LIF, leukemia inhibitory factor

LIM-Ks, LIM domain (named from the Lin-11, Isl-1 and Mec-3 genes) kinases

MAG, myelin-associated glycoprotein

MAO, monoamine oxidase

MAPKs, mitogen-activated protein kinases

m-CM, modified conditioned medium

MEK, mitogen-activated protein kinase kinase

mGnRH, mammalian gonadotropin-releasing hormone

MIP, molluscan insulin-related neuropeptide

MLN, median lip nerve

mRNA, messenger ribonucleic acid

NB medium, neural basal medium

NCAM, neuronal cell adhesion molecule

NCAM, neuronal cell adhesion molecule

NGF, nerve growth factor

NO, nitric oxide

NT, neurotrophin

NTN, neurtrin

OSM, oncostatin-M

P1, *Helisoma* pedal ganglionic neuron 1

P5, *Helisoma* pedal ganglionic neuron 5

PAGE, polyacrylamide gel-electrophoresis

PBS, phosphate-buffered saline

PC-12, pheochromocytoma

*p*CPA, *p*-chlorophenylalanine

PDGF, platelet-derived growth factor

P-domain, peripheral domain

*p*EPA, *para*-ethynylphenylalanine

PI3, phosphatidylinositol 3

PKC, protein kinase C

PLC, phospholipase C

PSP, persephin

RHAMM, receptor for hyaluronan (HA)-mediated motility

RNAi, RNA interference

S100B, S100 calcium binding protein, beta (neural)

s-CM, standard conditioned medium

SCP_b, small cardioactive peptide B

SDS, sodium dodecyl sulfate

SEM, standard error of mean

SOS, sodium octyl sulfate

TBS, tris-buffered saline

TNF, tumor necrosis factor

TRITC, tetramethyl rhodamine isothiocyanate

Trk, tropomyosin-related receptor kinase

TTBS, TBS plus Tween-20

TTX, tetrodotoxin

VAPs, velvet antler polypeptides

WASP, Wiskott-Aldrich syndrome protein

INTRODUCTION

1. General Introduction

Our brain is an amazingly complex neuronal network where an estimated 100 billion highly specialized neurons communicate with each other by electrical and chemical signals. These sites of communication are called synapses. At electrical synapses, current generated by an impulse in the presynaptic nerve terminal spreads into the next cell through low-resistance channels. The more common chemical synapse consists of a fluid-filled gap between the presynaptic and postsynaptic membranes. Thus, at chemical synapses, direct current flow to the next cell is prevented, and instead, the presynaptic nerve terminal releases neurotransmitters that activate their receptors on the postsynaptic membrane. Several neurochemicals may be classified as neurotransmitters including amino acids (glutamate, glycine and γ-aminobutyric acid), neuropeptides (enkephalin, cholecystokinin, substance P, corticotropin, endorphin, and somatostatin), and biogenic amines (dopamine, norepinephrine, epinephrine, tyramine, octopamine and 5-hydroxytryptamine).

Biogenic monoamines, including catecholamines and indoleamines, have attracted much attention due to a variety of roles in the nervous system. 5-hydroxytryptamine (5-HT, also called serotonin) was the first identified monoamine in the central nervous system (CNS) (Gaddum, 1953; Twarog and Page, 1953). Since that time, enormous strides have been made towards understanding its roles in behavior, learning and memory, development,

synaptic transmission, and neuroplasticity. Interestingly, 5-HT is one of the earliest developing neurotransmitter systems found in vertebrates as well as invertebrates, and has been suggested to have putative roles in regulating neuronal development (Lauder and Bloom, 1974; Lauder et al., 1982; Goldberg and Kater, 1989; Lauder, 1990; Marios and Croll, 1992; Kempf et al., 1997). Several studies have even shown that serotonergic neurons exert critical modulatory influences in the developing CNS, in both vertebrates and invertebrates (Lauder and Krebs, 1978; Lauder et al., 1981; Diefenbach et al., 1998).

Studies on physiological mechanisms in neurons are often hampered due to the small size of most neurons and their inaccessibility *in vivo*. On account of the large size and identifiability of specific neurons in the invertebrate CNS, a number of complicated physiological mechanisms have been elucidated in many molluscan models such as *Aplysia, Helisoma, Lymnaea and Helix*. These molluscs provide excellent experimental systems to address questions relevant to neuronal regeneration and development. A neuron-specific *in vitro* experimental comparison between different types of neurons can be performed using the identifiable single neuron culture system from those molluscs. For example, hypothetical regulatory roles of neurotransmitters in the development of nervous systems have been elucidated with previous studies of molluscan single neuron culture systems, where 5-HT was shown to modulate the neurite outgrowth, synapse formation, and physiological activity of specific target cells (Haydon et al., 1984 & 1987;

Price and Goldberg, 1993; Koert et al., 2001). A considerable amount of evidence has accumulated supporting the hypothesis that 5-HT is an important regulatory neurotransmitter in both developing molluscan nervous systems (Goldberg and Kater, 1989; Goldberg et al., 1991; Goldberg, 1995; Diefenbach et al., 1995; Diefenbach et al., 1998) and mammalian brains (Sikich et al., 1990; Riad et al., 1994; Yan et al., 1997; Hery et al., 1999). Since neuronal pathfinding and neurite extending processes are important in forming initial neural networks of developing nervous systems, our knowledge of the 5-HT regulation of neurite outgrowth from molluscan culture studies may provide a greater ability to understand the development of nervous systems.

An important goal in neurobiology is to understand the mechanisms that regulate neuronal development and regeneration. Neuronal development is a process whereby embryonic neuronal precursor cells differentiate into neurons, acquiring their intrinsic features in response to some extrinsic factors found in the local environment. Neuronal development is comparable to neuronal regeneration, which has precise and neuron-specific mechanisms that respond to extrinsic factors to reform original neuronal interactions and physiological functions (Anderson et al., 1980; Mason and Muller, 1982; Murphy et al., 1985; Murrain et al., 1990; Mason and Muller, 1996). The primary effects of these extrinsic factors are to initiate and/or regulate neurite outgrowth, which are key steps in neuronal regeneration after injury as well as

during neuronal development. This suggests that neuronal regeneration may involve recapitulation of some aspects of neuronal development.

5-HT is an extrinsic factor involved in the regulation of both developmental and regenerative neurite outgrowth. However, in most studies on developing and regenerating neurons, different neuronal subtypes have been used, and these cells may exhibit different regulatory mechanisms in neurite outgrowth. Thus, the roles of 5-HT in regulating both developmental and regenerative neurite outgrowth should, at the very least, be tested on the same type of neuron. For example, 5-HT regulatory mechanisms for the developmental neurite outgrowth of an identified developing embryonic serotonergic neuron (Diefenbach et al., 1995) may be compared with those of the regenerative neurite outgrowth of an identified adult serotonergic neuron. Therefore, complimentary studies on regenerative neurite outgrowth of adult neurons are required. Conversely, potential mechanisms for the 5-HT-influenced cytoarchitectural formation in adult neurons could be compared with embryonic neurite outgrowth to understand developmental 5-HT regulatory processes.

Many studies in vertebrates and invertebrates, have demonstrated specific roles of neurotransmitters in regulating the neurite outgrowth of different types of neurons. The initial studies were performed in experiments on identified molluscan neurons that were isolated from adult ganglia, and cultured under conditions that promoted the regeneration of new neurites. These studies suggested that 5-HT served a neuron-specific inhibitory role in

the regulation of neurite outgrowth (Haydon et al., 1987; Murrain et al., 1990). The neuron-specific regulatory role of 5-HT in neurite outgrowth must be related to the expression of serotonergic receptors in individual neurons. Most of the 5-HT inhibitory effects were induced through 5-HT hetero-receptors found in non-serotonergic neurons. In addition, some previous studies suggested an interesting possibility that serotonergic neurons may inhibit their neurite elongation through autoregulatory receptors during the development and/or the regeneration of nervous systems (Haydon et al., 1984; Whitaker-Azmitia and Azmitia, 1986; Haydon et al., 1987; Diefenbach et al., 1995). However, these previous studies have actually been focused on responses through 5-HT homo-receptors found in other serotonergic neurons, and nothing is yet known about how self-released 5-HT directly or indirectly modulates the neurite outgrowth of the same serotonergic neuron through autoreceptors. Thus, it is of interest to examine how 5-HT affects regulatory serotonergic neurons to control their own neurite outgrowth through 5-HT autoreceptors (Figure 1) (for concepts of autoreceptors, hetero- & homo-receptors, see Galzin et al., 1985; Moret, 1985; Westerink et al., 1990; Hen, 1992; Cooper et al., 1996; Stamford et al., 2000).

The studies presented herein examined direct effects of 5-HT on the neurite outgrowth of neuron C1, an identified serotonergic neuron from the cerebral ganglion of *Helisoma trivolvis*, a fresh water pond snail. Cellular morphological changes in the soma, neurites and growth cones of neuron C1 after 5-HT treatments were characterized. 5-HT inhibited the transition from

the neurite initiation phase of outgrowth to the elongation phase of outgrowth, and regulated the rate of neurite extension.

In *Helisoma*, a pair of cerebral serotonergic neuron C1s project to the buccal ganglia, and modulate feeding behavior through the release of 5-HT (Granzow and Kater, 1977; Murphy, 2001). These neurons are morphologically and functionally similar to collateral hypothalamic projections of serotonergic neurons in mammals (Parent, 1981). Thus, knowledge of 5-HT effects on regenerative neurite outgrowth obtained from the present studies can be helpful to understand the functional recovery of feeding behaviors accompanying the reconstruction of hypothalamic neuronal projections. Moreover, the present studies introduced a neuronal culture model which demonstrates a unique biphasic inhibitory / facilitatory response to 5-HT that depends upon dose and the outgrowth state of the neuron. This regulatory model will help us to understand how 5-HT released from some serotonergic neurons *in vivo* regulates projecting neurites of other serotonergic neurons or their own elongating neurites.

2. Background Information

I will now review the current information on neurite outgrowth in invertebrate, mainly molluscan, and vertebrate neuronal systems. The following sections provide a fundamental basis at the cellular and molecular levels to interpret results in the present thesis.

2.1. 5-HT metabolism in gastropods and vertebrates; Critical differences in inactivation

Serotonin was initially discovered about 50 years ago in the CNS and in the myenteric plexus of the gut of vertebrates (Gaddum, 1953; Feldberg and Toh, 1953; Erspamer, 1963; Page, 1976). High concentrations of 5-HT were subsequently found in the enterochromaffin cell system of the gastrointestinal tract and in blood platelets of vertebrates (Zucker et al., 1954; Barter and Pearse, 1955; Solcia and Sampietro, 1967). The presence of 5-HT has also been demonstrated in several invertebrates. For example, 5-HT was first identified in molluscan nervous system (Welsh and Moorhead, 1959; Welsh, 1968). Buznikov and colleagues (1964) demonstrated the presence of 5-HT in sea urchin embryos from early cleavage division stages through gastrulation. Moreover, 5-HT has been shown to be present in many identifiable neurons of *Helisoma trivolvis* in both embryos and adults (Goldberg and Kater, 1989; Diefenbach et al., 1998).

5-HT is an indole-monoamine, and many features of its synthesis, storage, release and inactivation are similar to the processes occurring in

tissues which synthesize other monoamines. 5-HT in both vertebrates and invertebrates is synthesized from the aromatic amino acid L-tryptophan, which is actively taken up into 5-HT neurons by means of a carrier mechanism for large neutral amino acids. L-Tryptophan is hydroxylated to 5-hydroxytryptophan (5-HTP) by the enzyme tryptophan hydroxylase (Sjoerdsma et al., 1955; Kruk and Pycock, 1991; Cooper et al., 1996). Tryptophan hydroxylase is only found in the cytoplasm of 5-HT-containing neurons and is the rate-limiting enzyme in the synthesis of 5-HT (Jequier et al., 1969). *p*-Chlorophenylalanine (*p*CPA) is known as an irreversible competitive inhibitor of tryptophan hydroxylase (Gál and Whitacre, 1982). The tryptophan hydroxylase requires molecular oxygen and a pteridine cofactor for its activity. 5-HTP is decarboxylated in the cytoplasm to 5-HT by the non-specific enzyme aromatic L-amino acid decarboxylase. 5-HT storage mechanisms have many features in common with catecholamine storage processes. From a previous study on the subcellular localization of 5-HT in an identified serotonin-containing molluscan neuron, 5-HT was believed to be bound within small-granulated vesicles, and in particles which resemble lysosomes (Cottrell and Osborne, 1970). Furthermore, previous studies suggest that 5-HT in vesicles exists in a granular complex with acidic secretory proteins (chromogranin or secretogranin), divalent metal ions (Ca^{++} or Mg^{++}) and adenosine triphosphate (ATP) (Reiffen and Gratzl, 1986; Bargsten and Grube, 1992; Carnell and Moore, 1994). Stored neuronal 5-HT is released into the synaptic cleft by the process of

exocytosis in response to action potentials and drugs. This release is dependent upon an influx of calcium into the neuron (Kruk and Pycock, 1991; Cooper et al., 1996).

Serotonin released into the synaptic cleft is transported back into the presynaptic neuron through a high-affinity, energy-dependent, active transport mechanism (Gerschenfeld et al., 1978; Parent, 1981; Kruk and Pycock, 1991). It is then acted on by monoamine oxidase (MAO) and converted into 5-hydroxyindoleacetaldehyde (5-HIA). However, there is a critical difference in the 5-HT inactivation mechanisms between vertebrates and invertebrates. Although low levels of monoamine oxidase were detected in the pond snail, *Lymnaea stagnalis* (Hiripi, 1970) and the mussel, *Anodonta cygnea* (Hiripi and Slanki, 1971), some studies suggest that in general, gastropods do not contain high levels of MAO and inactivate 5-HT via γ-glutamyl conjugation rather than by oxidative deamination (Sloley and Goldberg, 1991). In *Helisoma* (Sloley and Goldberg, 1991), *Aplysia* (McCaman et al., 1985), and *Helix* (Sloley et al., 1990), 5-HT is converted to γ-glutamyl 5-HT through the activity of γ-glutamylamine synthetase (Battelle et al., 1988). This monoamine-inactivation was also observed in other invertebrates such as the cockroach (Sloley and Downer, 1984) and earthworm (Sloley, 1994). In addition to the γ-glutamyl conjugation system, released 5-HT can be reused through reuptake mechanisms, as demonstrated in some giant serotonergic neurons in the CNS of *Aplysia* (Gerschenfeld et al., 1978), *Mytilus* (Burrell and Stefano, 1981) and the land

snail *Helix pomatia* (Osborne et al., 1975). Clomipramine, a tricyclic antidepressant, is known to block the reuptake of 5-HT in the molluscan nervous system (Osborne et al., 1975). Figure 2 and 3 illustrate the pathways of synthesis and inactivation/breakdown of 5-HT with multiple enzyme systems and their cofactors.

2.2. Serotonergic systems in *Helisoma* brain and neuron C1

Molluscan nervous systems are considered to be excellent experimental model systems to address questions relevant to neuronal development and regeneration. The nervous systems consist of a circumoesophageal ring (brain), pedal cords, and visceral loops (Laverack and Dando, 1979). Roles of gastropod serotonergic systems in modulating feeding behaviors have long been examined in the neurobiological literature.

1) Organization and structure of the central ganglionic ring

The brain of gastropod molluscs consists of ganglia arranged in a ring around the esophagus. The ganglia are attached via connectives and the ring structure is called the circumoesophageal ganglionic ring. The central ganglionic ring is enveloped in a bi-layered connective tissue sheath consisting of muscle cells, fibroblasts and globular cells containing glycogen. The sheath is penetrated by capillaries from arterial branches (Pentreath and Cottrell, 1970), which supply the brain with oxygen and nutrients. Each ganglion consists of a cortical layer of neuronal somata, whose axons

project through the neuropile (Bulloch and Horridge, 1965). Typically the neurons of gastropods are either unipolar, with one axon emanating from the soma, or pseudo-unipolar, with one axon that bifurcates (Kandel, 1991). In contrast to vertebrates, no dendrites directly sprout from the soma, but axons near the soma extend dendrite-like neurites. These dendritic neurites can be visualized by injecting the dye Lucifer Yellow into the soma (Murphy et al., 1985).

The brain of *Helisoma* consists of five paired ganglia (cerebral, buccal, pedal, pleural and parietal) and one unpaired ganglion (visceral; Figure 4). The different ganglia appear at specific times during CNS development, with the cerebral ganglia developing first, followed by the pedal, buccal, pleural, parietal and then the visceral ganglia appearing in an undetermined sequence (Goldberg and Kater, 1989; Goldberg, 1995). The left cerebral ganglion has an anterior extending part, which is immunoreactive for gonadotropin - releasing hormone (GnRH) (Young et al., 1999), and nitric oxide synthase (unpublished data). Each ganglion in the brain contains several hundred neurons including many neurosecretory cells (Dorsett and Roberts, 1980; Goldberg and Cavers, 1993). The easily identifiable neuronal somata are located in the outer layer of the ganglia. The inner neuropile area is composed of an extensive network of neurites, axonal tracts and glial cells.

2) Serotonergic expression of the central ganglionic neurons

The presence of neurotransmitters is a distinctive feature of neurons. Even though the accumulation of neurotransmitters into certain endocrine and glial cells may occur, the biochemical detection of a neurotransmitter within a cell is still a strong indicator of neuronal identity. Initially, it was suggested that 5-HT was found in a particular type of ganglionic neuron in gastropods (Stefani and Gerschenfeld, 1969). Subsequently, it has been demonstrated that two giant cerebral neurons from land and fresh water snails contain detectable quantities of 5-HT (Cottrell, 1970, 1971, 1974 & 1976; Cottrell and Osborne, 1970). Parallel studies using sensitive enzymatic micromethods showed that homologous giant cerebral neurons in *Aplysia californica* contained 4 – 6 pmoles of 5-HT in their somata (Weinreich et al., 1973; Brownstein et al., 1974). In *Limax* and *Helix*, giant serotonergic neurons contain about 1 μg of 5-HT (for review, see Parent, 1981). Using high-performance liquid chromatography (HPLC), a recent study on *Lymnaea* showed that the cerebral giant cells (CGC) are also serotonergic (Koert et al., 2001).

5-HT phenotypic expression after immunostaining is ganglion-specific depending on developmental stages. Serotonergic neurons increase in number postembryonically, with different ganglia showing increases at different developmental times (Goldberg and Kater, 1989). A previous study comparing the 5-HT immunoreactive expression between wild-type and laboratory-reared albino strain *Helisoma* (Diefenbach and Goldberg, 1990),

showed that rearing conditions might precociously alter the developmental time course of gastropod nervous systems. In addition, 5-HT cell clusters from the cerebral and pedal ganglia of *Helisoma* revealed distinct developmental patterns for individual clusters (Diefenbach and Goldberg, 1990). At least five serotonergic neuronal clusters have been observed in each cerebral ganglion (Goldberg and Kater, 1989). The cerebral serotonergic neuron C1 and the pedal serotonergic neuron P5 show prominent 5-HT immunoreactivity at postembryonic stages. 5-HT-like immunoreactivity of neuron C1 has also been shown in isolated cell culture (Murphy et al., 1985). 5-HT immunoreactive neurons are also found in the central ganglionic ring from other molluscs such as a pulmonate, *Lymnaea stagnalis* (Croll and Chiasson, 1989), a prosobranch, *Littorina littorea* (Croll and Lo, 1986) and an opisthobranch, *Pleurobranchia* (Bulloch and Horridge, 1965). The distribution of individual serotonergic neurons in *Lymnaea* is similar to that in the *Helisoma* CNS (Kemenes et al., 1989). Thus, the distribution of *Lymnaea* 5-HT neurons may be used as a reference to identify 5-HT containing neurons in *Helisoma*. The only major difference in the distribution of 5-HT-immunoreactivity is that neuron P5 in the right pedal ganglion of *Helisoma* stains positively for 5-HT while its homologue in *Lymnaea*, the serotonergic neuron LPeD1, is actually located in the left pedal ganglia of *Lymnaea* (Kemenes et al., 1989; Croll and Chiasson, 1989; Goldberg and Kater, 1989). Accordingly, the left pedal ganglionic neuron in

Helisoma has been identified as a dopamine-immunoreactive neuron P1, homologous to the right pedal ganglionic neuron RPeD1 in *Lymnaea*.

3) The identified cerebral serotonergic neuron C1 of *Helisoma trivolvis*; a neuron involved in the control of feeding activity

Because of their easy identifiability, special attention has been paid to a pair of large serotonergic interneurons in the cerebral ganglia from a wide range of molluscs including *Aplysia, Helisoma, Helix, Limax, Lymnaea, Planorbis, Pleurobranchaea and Tritonia* (Dorsett et al., 1973; Pentreath et al., 1973; Dorsett, 1974; Berry and Pentreath, 1976; Gillette and Davis, 1977; Granzow and Kater, 1977; Gelperin et al., 1978; Weiss et al., 1978; Pentreath and Berry, 1978; Bulloch and Dorsett, 1979; Dorsett and Willows, 1979; Granzow and Rowell, 1981). Those neurons share many homologous features (Weiss and Kupfermann, 1976). Thus, the metacerebral giant cells of some snails are thought to be homologous to *Helisoma* serotonergic cerebral cells (neuron C1) (Granzow and Kater, 1977; Granzow and Rowell, 1981).

The identifiable, large serotonin-containing neuron C1s in *Helisoma* are a pair of bilaterally symmetrical cells in the anterior region of the cerebral ganglia, which project axons into the buccal ganglia via the cerebrobuccal connectives (CBC) (Granzow and Kater, 1977; Granzow and Rowell, 1981). The axon of neuron C1 branches profusely in the buccal ganglia, making synapses with numerous neurons including neuron B19 that control the

muscles involved in feeding (Gadotti et al., 1986). Feeding behaviors in *Helisoma* are suggested to arise from a triphasic motor pattern including protraction, retraction and hyper-retraction (Quinlan and Murphy, 1996; Murphy, 2001). Serotonergic neuron C1 and the FMRFamide-containing (Phe-Met-Arg-Phe-NH_2) neuron Pl 1 are also known to be two descending modulatory neurons for feeding activity (Granzow and Kater, 1977; Murphy, 1990; Murphy, 2001). An electrophysiological study showed that cerebral neuron C1 produces excitatory input to the feeding motor program contained in the buccal ganglia (Granzow and Kater, 1977). Furthermore, action potentials within neuron C1 evoked excitatory postsynaptic potentials (EPSP) in buccal ganglionic neuron B19 that were blocked by the 5-HT partial antagonist methysergide (Gadotti, 1985), suggesting that serotonergic neuron C1s are involved in feeding. During the depletion of 5-HT using 5,7-dihydroxytryptamine (5,7-DHT), the EPSP elicited by the neuron C1 onto the buccal motor neuron B19 was significantly decreased (Gadotti et al., 1986). However, the feeding activity is not always initiated and driven by serotonergic neuron C1. Dopamine, GABA and small cardioactive peptide B (SCP_b) have also been shown to start and modulate the feeding program, generating a central pattern of neuronal activity that can be depressed by FMRFamide (Trimble and Barker, 1984; Trimble et al., 1984; Weiss et al., 1984; Lukowiak and Murphy, 1987; Murphy, 1990 & 2001).

2.3. Advantages of using molluscan neuronal models and a single cell culture system for the study of neurite outgrowth

There are diverse experimental model systems that have enhanced our understanding of nervous system development. Various invertebrates provide large, identifiable and easily accessible neurons as well as relatively simple nervous systems that are well suited for neurobiological study. The large size of molluscan neurons is experimentally important because it is directly associated with enhanced synthesis and transport of presynaptic materials, and the extent of postsynaptic innervation (Gillette, 1991). In addition, the knowledge obtained from simple invertebrate neurobiological studies often leads to an understanding of the much more complicated neural mechanisms of higher vertebrate systems (Goldberg, 1995 & 1998). In fact, our current understanding of various physiological mechanisms underlying cell excitability, synaptic transmission, learning and memory and neuroplasticity is largely based on studies with identified neurons from adult molluscs (Jacklet et al., 2004; Green et al., 1996; Bailey et al., 1994; Frolkis et al., 1984; Gillette, 1983; Takeuchi et al., 1977 & 1976). The roles and actions of neurotransmitters during neural development is one area of developmental neurobiology where studies on model invertebrate systems have had a particularly significant impact. For example, some physiological roles of 5-HT have been thoroughly studied, demonstrating the utility of molluscan neurons as excellent models for nervous system function. The pulmonate freshwater pond snail *Helisoma trivolvis*, for example, provides

many large neurons such as buccal ganglion neurons B4, B5 and B19, the cerebral ganglionic neuron C1, the pedal ganglionic neuron P1 and P5, and the embryonic neuron C1 (ENC1) that are easily identifiable and experimentally accessible (Granzow and Kater, 1977; Granzow and Rowell, 1981; Murphy et al., 1985; Gadotti et al., 1986; Haydon et al., 1987; Bulloch and Ridgway, 1989; Goldberg, 1998; Diefenbach et al., 1998). In contrast to higher vertebrate experimental systems, most of these identifiable neurons have provided us with new opportunities to study the nervous system at the cellular level.

The large identified neurons from adult *Helisoma* have been recognized as one of the most useful model systems for studying neurite outgrowth and growth cone function. These neurons, like other adult molluscan identified neurons, are easily accessible for various *in vitro* cell culture analyses due to their minimal phenotypic variability and large somata (For review, see Goldberg, 1998). Buccal ganglionic neurons B4, B5 and B19 have often been used in cell cultures for the examination of their regenerative neurite outgrowth (Haydon et al., 1987; Mattson and Kater, 1987; Cohan et al., 1987; Mattson et al., 1988; McCobb et al., 1988; Berdan and Bulloch, 1990; Polak et al., 1991; Kater and Mills, 1991; Berdan and Ridgway, 1992; Williams and Cohan, 1994; Davenport et al., 1996; Torreano and Cohan, 1997; Goldberg, 1998; Rehder and Cheng, 1998). Moreover, buccal ganglionic neurons seem to be attractive for certain types of studies such as those that test whether neurons are responsive to 5-HT (Price and Goldberg,

1993). Pedal ganglionic neurons P1 and P5 have also been used for examining electrophysiological properties and growth cone motility during neurite outgrowth (Haydon et al., 1987; McCobb and Kater, 1988; Guthrie et al., 1989). However, the serotonergic cerebral ganglionic neuron C1 has never been studied in cell culture (Murphy et al., 1985) for regenerative neurite outgrowth despite its many advantages for regenerative studies. In addition, it is one of only two identifiable serotonergic neurons in *Helisoma* that to date, can be isolated and cultured in a single cell environment. Personal efforts to culture neuron C1 suggested that there might have been a tonic inhibitory factor in culture conditions, which specifically inhibited neurite outgrowth of neuron C1. These properties made neuron C1 very attractive for determining the autoregulatory effects of 5-HT on neurite outgrowth and elongation.

2.4. What is neurite outgrowth?

Developing neurons extend neurites along the guided pathways, and the precision of these projections has a profound impact on neural circuit assembly. Early developmental errors in neuronal pathfinding can be associated with the absence of guidance cues or outgrowth-promoting factors. Similarly, the neurite extension of regenerating adult neurons that are recovering from neuronal injury can be influenced with various extrinsic growth-related factors. Thus, both developing embryonic and regenerating

adult neurons rely on neuronal growth-promoting factors to establish a proper neuronal network.

Neurite outgrowth is a morphological marker of neuronal differentiation, and consists of four essential phases; initiation, elongation, guidance and cessation (Haydon et al., 1987). Morphological changes from one phase to the other are likely dependent on intrinsic and extrinsic signaling molecules, which can stimulate intracellular signaling cascades to reorganize actin- and microtubule-rich structures. The initial phase of neurite outgrowth requires neurons to be attached onto permissive substrata for the next neurite elongational phase. It is known that protein synthesis is required for initiation, but not the continuation of axonal outgrowth of embryonic rat sympathetic neurons (Lein and Higgins, 1991). In contrast to axons, dendrites require protein synthesis throughout the period of extension. During the initiation phase, most cultured neurons usually produce veil-like structures called lamellipodia and finger-like structures called filopodia around their somata. When neuronal growth promoting factors, including neuritogenic ligands, exist in the extracellular environment and bind to their receptors, neurons prepare large microtubule initiation sites in their lamellipodia by rearranging assembled microtubules (Spiegelman et al., 1979; Summers and Kirschner, 1979; Smith, 1994). The microtubule initiation sites become the neuritogenesis initiation sites, which can be observed under high resolution phase contrast microscopy. This makes it possible to take time-lapse images of each phase of neurite outgrowth in living cells without specific

staining procedures. Primary initial neurites sprout from the neuritogenesis initiation sites and become axons or dendrites (Silva and Dotti, 2002). There are some studies that support the dependence of neurite extension on the polymerization of microtubules (Bamburg et al., 1986; Reinsch et al., 1991).

Much attention is paid to the mechanisms and factors responsible for initiating and maintaining neurite outgrowth. However, little is known about the factors that regulate the phase transitions during neurite outgrowth and how phase transitions are accomplished, although mechanisms occurring during each phase of neurite outgrowth have been studied extensively at the morphological, molecular and cytoskeletal levels (Suter and Forscher, 2000, Kalil et al., 2000, Gallo and Letourneau, 2000, Jay, 2000, Baker and Macagno, 2000, Luo, 2002, Rodriguez et al., 2003, Dehmelt and Halpain, 2004).

2.5. Molecular dynamics of neuronal growth cones

Neurite outgrowth during neural development and regeneration is an essential part of the neuronal pathfinding mechanism performed by growth cones responding to extracellular cues. Santiago Ramon y Cajal (1890) initially described the growth cone in fixed preparations of embryonic chick spinal cord and predicted it to be a moving sensor of the elongating neurite. Ross G. Harrison (1907 & 1910) and Carl Spiedel (1930s) confirmed his prediction of growth cone behavior through their observations of living growth cones in developing frog embryos (for review, see Letourneau and

Macagno, 2000). About 20 years later, Roger Sperry (1950 & 1951) suggested that intercellular communication took place between incoming axonal growth cones and target cells. Biochemical and physiological characterizations of growth-regulating molecules and cytoskeletal molecules have been performed over the past decades. Current research is mainly focused on the cytoplasmic signaling of positive and negative guidance cues.

1) **What is the neuronal growth cone?**

A neuronal growth cone is a motile sensory structure located at the tips of growing neurites, which responds to extracellular stimuli. There is a significant body of evidence supporting a sensory role for growth cone filopodia (Davenport et al., 1993; Chien et al., 1993). Growth cones continuously extend the boundaries of the neurite and require a steady influx of new structural components. They are autonomous structures that can continue to grow temporarily without any anterograde transport, even when a response requires local protein synthesis (Campbell and Holt, 2001; Zheng et al., 2001; Haydon et al., 1987). Isolated growth cones can sustain protein synthesis, indicating that they can integrate pathfinding cues during neurite outgrowth independent of the neuronal cell body (Davis et al., 1992). However, anterograde transport is necessary to sustain neurite outgrowth over longer periods of time. Myosin motors are thought to drive organelles and vesicles containing mRNA, neurotrophin receptors, and other materials

along the microtubule cytoskeleton into the tip of the extending growth cone (Wang et al., 1996; Wu et al., 1996 & 1997; Zheng et al., 2001; Morfini et al., 1997). In addition, retrograde transport from the neuronal growth cone back to the nucleus sometimes takes place with extracellular signaling molecules such as neurotrophic factors.

2) **Roles of neuronal growth cones in neurite outgrowth**

Developing and regenerating neurites require a sensory apparatus that detects and responds to extrinsic factors. Neuronal growth cones are highly specialized structures responsible for neurotransmitter release, axonal pathfinding, target cell selection and synaptogenesis (Lockerbie, 1987; Kater and Shibata, 1994). The growth cone is rich in membrane receptors making it sensitive to extracellular stimuli. Various guidance molecules including growth-conditioning factors are distributed in extracellular patches or gradients that activate these membrane receptors and induce intracellular signaling pathways involved in growth cone advance. These signaling pathways act on the growth cone cytoskeleton to cause structural rearrangement and subsequent changes in growth cone behavior; turning, collapsing, branching, merging, accelerating and slowing. Taken together, the neuronal growth cone at the terminus of the neurite is a sensory and motile structure specifically developed for neurite outgrowth.

3) **Cytoskeleton in the neuronal growth cone**

Growth cones are highly dynamic structures that depend on their cytoskeleton for intracellular transport, structural stability, substrate attachment and outgrowth. It is for these reasons that virtually all signal transduction pathways converge onto the cytoskeleton (Korey and Van Vactor, 2000). The cytoskeleton is mainly composed of actin filaments and microtubules (Forscher and Smith, 1988). There are a variety of proteins that interact with these main elements to orchestrate cytoskeletal rearrangement. The tip of the growth cone has protruding filopodia that contain bundled filamentous (F)-actin. F-actin is a thicker single microfilament into which two monofilaments form by polymerization of globular (G)-actin monomers. F-actin-rich filopodia extend proximally into the growth cone to form spokes guiding the assembly and transport of dynamic unbundled microtubules (Schaefer et al., 2002; for review, see Rodriguez et al., 2003). Between these bundled spokes are random networks of unbundled F-actin that comprise the flattened region of membrane, called the lamellipodium (Lewis and Bridgman, 1992).

The growth cone has three morphologically and kinetically distinct zones (Forscher and Smith, 1988) (Figure 5). The proximal region is known to be the central domain (C-domain), where a large bundle of stable less dynamic microtubules and actin filaments is present. The distal region of the growth cone is known to be the peripheral domain (P-domain), where actin filaments form an elaborate network and a sub-population of dynamic plus

ends of unbundled microtubules penetrates into. The peripheral domain of the growth cone is a dynamic region, where F-actin assembly and turnover are prevalent. Actin monomers are polymerized into filopodial bundles at the leading edge of the growth cone (Mallavarapu and Mitchison, 1999) and depolymerized at the proximal end (Forscher and Smith, 1988). These events cause F-actin to consistently turn over and advance the filopodia. The transition zone lies between the peripheral and central domains, and contains contractile actin bundles (actin arcs) orientated perpendicular to filopodia. Microtubules have also been found to co-localize with the actin arcs in the transition zone to form a dynamic pool of microtubules (Dent and Kalil, 2001). Interactions between microtubules and filopodial actin play a central role in advancing a growth cone across its substrate.

4) **Dynamics of cytoskeletal molecules in neuronal growth cones**

There is increasing evidence that interactions between actin and microtubules are important for neuronal pathfinding. Actin dynamics well-known in the study of axon guidance (Bentley and Toroian-Raymond, 1986) are affected by complex interactions that can result in actin retrograde flow (Lin and Forscher, 1995). When F-actin is turning over for depolymerization, myosin motors anchored to the cell membrane drive the filopodial actin bundles in a retrograde direction forming actomyosin networks (Lin et al., 1996). This results in an escalator-like effect, where net filopodial extension is dependent on the rate of actin polymerization and F-actin retrograde flow.

When actin polymerization occurs faster than F-actin retrograde flow, a filopodium advances. In contrast, a filopodium withdraws when actin polymerization is slower than F-actin retrograde flow (Lin et al., 1996). Similar actin retrograde flowing events occur in lamellipodia at the dynamic leading edge of the growth cone during neurite outgrowth as well.

The rate of neurite outgrowth is directly associated with the rate of F-actin retrograde flow which is determined by substrate-cytoskeletal coupling (Mitchison and Kirschner, 1988; Lin et al., 1994; Suter and Forscher, 1998; for review, see Suter and Forscher, 2000). Therefore, the degree of substrate-cytoskeletal coupling is a major determinant of the rate of neurite outgrowth, such that growth cones tend to move across the substrate along the path of maximum adhesion. There is the clutch hypothesis (Mitchison and Kirschner, 1988), in which enhanced levels of cell adhesion molecules (CAMs) strengthen the linkage for substrate-cytoskeletal coupling. CAMs found in the plasma membrane mediate this coupling by anchoring the actin cytoskeleton to the underlying substrate (Rutishauser, 1993). When an advancing growth cone encounters an attractive signal, CAMs are upregulated and bind F-actin more tightly to the substrate (Suter and Forscher, 1998). Thus, a potential molecular clutch is engaged that favors growth cone advance (Suter et al., 1998). This molecular clutch reduces F-actin retrograde flow and produces the anchor needed to pull dynamic unbundled microtubules forward. A previous study has directly shown microtubule movements within growth cones using the microinjection of

fluorescent tubulin (Dent et al., 1999). This suggests that local changes in microtubule organization and distribution are required for the axon to grow. However, little is known about microtubule dynamics even though motor molecule-dependent microtubule sliding has been suggested (Heidemann, 1996).

5) Intrinsic factors influencing cytoskeletal dynamics in the neuronal growth cone

The rates of microtubule advance and F-actin retrograde flow are influenced by many neuronal outgrowth factors. Actin-depolymerizing factor (ADF) / cofilin family (Gungabissoon and Bamburg, 2003; Meberg and Bamburg, 2000; Meberg et al., 1998; Carlier et al., 1997; Lappalainen and Drubin, 1997) and severing proteins such as gelsolin (Yin and Stossel, 1979) are known as intrinsic neurite outgrowth promoting factors. Activation of ADF/cofilin family by either dephosphorylation (Meberg et al., 1998) or reduced LIM-kinase activity (Arber et al., 1998) removes F-actin from the proximal end of filopodial spokes and thus stimulates F-actin turnover (Bamburg et al., 1999). This depolymerizing process is essential for microtubule advance, since F-actin bundles impose a steric blockade (Challacombe et al., 1996). Thus, ADF over-expression or experimentally disrupting F-actin turnover can increase the rate of neurite outgrowth in a microtubule-based manner (Meberg and Bamburg, 2000, Marsh and Letourneau, 1984; Bentley and Toroian-Raymond, 1986). Moreover, the

severing of F-actin by gelsolin could influence microtubule advance in a similar manner to ADF/cofilin family (Lu et al., 1997).

N-WASP, a neuronal homologue of WASP (Wiskott-Aldrich syndrome protein), could also contribute to actin recycling in growth cones and may therefore affect neurite outgrowth (Miki et al., 1996). In addition, N-WASP is an actin-polymerizing agent, as suggested by recent studies where it was shown to play a regulatory role in actin assembly by linking Cdc42 to the Arp2/3 complex (Rohatgi et al., 1999; Ma et al., 1998; Machesky and Insall, 1998). In a recent study (Udo et al., 2005) on *Aplysia* sensory neuron cultures (Rayport and Schacher, 1986), 5-HT activates the ApCdc42 complex and in turn, recruits N-WASP to reorganize the presynaptic actin network to induce the filopodial outgrowth.

Protein kinase C (PKC) is another factor that promotes neurite outgrowth by enhancing microtubule assembly (Kabir et al., 2001). Increased microtubule assembly coupled with pre-existing rates of anterograde transport may lead to increased outgrowth. Alternatively, increased microtubule assembly may just increase the number of microtubules found in the transition zone, independent of a direct effect on neurite outgrowth. In this case, the additional microtubules would increase the transport of vital materials to the distal region of the growth cone, resulting in faster growth cone advance.

Growth associated protein–43 (GAP-43) is known to enhance neurite outgrowth in a number of different neurons (Strittmatter et al., 1995; Meiri et

al., 1998; Bomze, 2001; Shen et al., 2002). Synthesis of GAP-43 in neurons is induced during neurite growth, and GAP-43 is found in the growth cone of axon. GAP-43 is a substrate of PKC and phosphatase. Both phosphorylated and dephosphorylated GAP-43 have different, independent effects on actin filament structure (Dent and Meiri, 1998). Phosphorylated GAP-43 stabilizes long actin filaments by polymerizing F-actin. In contrast, dephosphorylated GAP-43 reduces actin filament length by depolymerizing F-actin. Prebinding calmodulin potentiates this depolymerizing effect through dephosphorylation of GAP-43 by calcineurin, a calcium-associated phosphatase (He et al., 1997). Mitogen-activated protein kinases (MAPKs) are believed to enhance neurite outgrowth by increasing expression of growth promoting proteins like GAP-43 (Encinas et al., 1999; Olsson and Nanberg, 2001; Yuan et al., 2001). Activities of both GAP-43 and MAPKs are required for the initiation of cell adhesion molecule (CAM)-mediated neurite elongation.

Neuronal cell adhesion molecules (CAMs) act as linkage agents of substrate-cytoskeletal coupling and important mediators of neurite outgrowth. The classical neuronal CAMs belong to three distinct structural families; integrins, cadherins and IgCAMs. All of these molecules not only mediate adhesive functions but also promote axonal growth. If substrate-cytoskeletal coupling at high levels of neuronal CAMs is strong enough to support significant central-peripheral domain tension or attenuate F-actin retrograde flow, protrusive outgrowth may result directly from continued

actin assembly (Bozyczko and Horwitz, 1986; Tomaselli et al., 1988; Wu et al., 1996).

2.6. Neuronal growth factors

Intercellular communications coordinate growth, differentiation and metabolism of target neurons. One important mechanism by which cells communicate is by means of extracellular signaling molecules. These specific substances are synthesized and released by signaling cells and produce a particular response only in target cells that have specific receptors for the signaling molecules. Expression of those receptor molecules induces intrinsic influences on intercellular communications. Neuronal growth factors are substances that can influence neuronal survival and the extent and rate of neurite outgrowth. The roles of neuronal growth factors in the development of the nervous system, as well as in injury-induced plasticity, are of great interest. In this section, neuronal growth factors influencing neurite outgrowth and neuronal survival are reviewed.

1) Classification of neuronal growth factors

In order to build or rebuild the complex circuitries of functional and neuroarchitectural networks, many neurons communicate with each other or interact with their environments in highly specific manners. The initiation and guidance of a neurite can be accomplished by various growth factors. Here, three different groups of neuronal growth factors regulating neurite

outgrowth and neuronal survival are classified: (1) diffusible molecules such as neurotrophic factors, neurotransmitters, target-derived netrins, platelet-derived growth factor (PDGF), fibroblast growth factor (FGF), insulin-like growth factor (IGF) and epidermal growth factor (EGF), (2) extracellular matrix (ECM) molecules such as laminin, fibronectin, collagen, tenascin, poly-lysine, heparin-binding growth-associated molecules (HB-GAM), heparan sulfate and hyaluronate, and (3) cell adhesion molecules (CAMs) such as N-cadherins and two Ig-CAMs, neuronal CAMs (NCAMs) and L1.

Diffusible and ECM molecules are categorized as extrinsic factors while CAMs are considered to be intrinsic factors. Neuronal plasticity events, such as neurite outgrowth, allow neurons to adapt to the changing demands of their environment by modulating both the intrinsic membrane properties of neurons and the strength of the synaptic connections among them. Thus, effects of neurotrophins on neurite outgrowth are extrinsic, because neurotrophins always require specific receptor expression before activating various post receptor-binding signals through secondary messenger systems such as calcium, cyclic nucleotides, and inositol phospholipids. Further, genetically determined intrinsic factors should also be considered to explain the specificity of neuronal morphology. The presence of intrinsic endogenous determinants supports the fact that neuronal morphology in cell cultures can closely resemble their *in situ* neuronal architecture in most cases (Banker and Cowan, 1977; Haydon et al., 1985).

2) **Diffusible neurotrophic factors**

Generally, neurotrophic factors consist of three separate and distinct families, although additional neurotrophic factors have also been identified: (1) the neurotrophin family which consists of nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF), neurotrophin-3 (NT-3), neurotrophin-4/5 (NT-4/5), neurotrophin-6 (NT-6), and neurotrophin-7 (NT-7), (2) the glial cell-lined derived neurotrophic factor (GDNF) family consisting of GDNF, neurturin (NTN), persephin (PSP), artemin (ART), and activity-dependent neurotrophic factor (ADNF), and (3) neuropoietic cytokines which consist of interleukin (IL)-6, IL-11, leukemia inhibitory factor (LIF), oncostatin-M (OSM), ciliary neurotrophic factor (CNTF), and cardiotrophin (CT)-1 (Durany and Thome, 2004).

Neurotrophin families were originally identified by their ability to promote the survival of developing neurons. However, recent studies on these proteins indicate that they may also influence the proliferation and differentiation of neuronal progenitor cells and regulate neuronal differentiation. They are known to mediate neurite outgrowth and neuronal survival by binding to two classes of receptors, the tropomyosin-related receptor kinase (Trk) family of receptor tyrosine kinases and a member of the tumor necrosis factor (TNF)-α family of receptor (p75) (Rodriguez-Tebar et al., 1992; Barbacid, 1994; Lee et al., 1994; Huang and Reichardt, 2001). Binding of the individual neurotrophins has been shown to activate different Trk receptors and to trigger several intracellular signaling cascades

involving phospholipase Cγ (PLCγ), Ras, mitogen-activated protein kinase (MAPK), MAPK kinase (MEK), and phosphatidylinositol 3-kinase (PI3 kinase) (Sofroniew et al., 2001). NGF promotes the survival and neurite outgrowth of sympathetic and sensory neurons (Levi-Montalcini and Hamburger, 1953; Frazier et al., 1973; Unsicker et al., 1980). NGF in the olfactory bulb plays an essential role in regeneration, maintenance, and development in the olfactory systems of mammals (Miwa et al., 2002). In hippocampal neurons, neural activity can control the synaptic plasticity regulating NGF and BDNF gene expression (Lu et al., 1991; Zafra et al., 1991, Lu and Gottschalk, 2000; Lu, 2003). BDNF is known to promote the development of central noradrenergic neurons (Holm et al., 2003). The acute transplantation of BDNF-producing fibroblasts into a cervical lateral funiculus lesion promotes significant long-distance rubrospinal axonal regeneration (Liu et al., 1999). These neurotrophins are often absent in the injured mammalian adult CNS, whereas multiple growth-inhibiting factors such as myelin-associated glycoprotein (MAG), Nogo, and semaphorin 3 are usually expressed (Fournier and Strittmatter, 2001; Oertle et al., 2003).

On the other hand, GDNF families exert their effects on neuronal plasticity by binding to two classes of receptors, the high affinity ligand-binding domain GDNF family receptor (GFR-α) and a common signal transduction subunit (ret) of transmembrane receptor tyrosine kinases (Durbec et al., 1996; Jing et al., 1996; Vega et al., 1996). GDNF was originally known to be a potent survival factor for mesencephalic

dopaminergic neurons as well as motoneurons (Lin et al., 1993 &1994; Oppenheim et al., 1995; Yan et al., 1995). GDNF stimulates Schwann cell migration and axonal growth in hippocampal and cortical neurons via binding NCAM (Paratcha et al., 2003). GDNF also sustains the axonal regeneration of chronically axotomized motoneurons *in vivo* (Boyd and Gordon, 2003). Further, GDNF significantly increases integrin expression in dopaminergic neurons of substantia nigra (Chao et al., 2003). Neurturin promotes the survival of rat sympathetic neurons (Kotzbauer et al., 1996). Activity-dependent neurotrophic factor (ADNF) is a novel femtomolar-acting GDNF that protects rat hippocampal and cortical neurons from various toxic insults and promotes their neurite outgrowth (Smith-Swintosky et al., 2005).

Neuropoietic cytokines mediate the regulation of cellular proliferation and neuronal differentiation as well as various biological activities related to the induction of immune and inflammatory responses, wound healing and neuronal survival (Oppenhein and Saklatvata, 1993). All members of this cytokine family exhibit their signals by binding to a common signal transduction receptor subunit, gp130 (Simpson et al., 1997; Bravo and Heath, 2000). CNTF and CT-1 promote the outgrowth of embryonic cranial motor neurons (Naeem et al., 2002). Moreover, CNTF shows multiple biological effects during vertebrate retinal development, including regulating the differentiation of photoreceptor cells and promoting the survival and axonal growth of ganglionic cells (Goureau et al., 2004).

3) **Other diffusible factors**

A soluble peptidic factor, FGF, mediates neurotrophic effects of astrocytes on luteinizing hormone-releasing hormone (LHRH) neurons (Gallo et al., 2000). FGF also improves survival of adult human retinal neurons *in vitro* via a mechanism that may involve c-fos, c-jun and Bcl-2 expression (Liu et al., 2003). Unidentified diffusible cortex-derived growth factors, possibly including FGF and NGF, enhance neurite outgrowth from explants of the mouse posterior thalamus (Lotto and Price, 1995).

EGF enhances neuronal proliferation (Jin et al., 2002; Zhou et al., 2003), neurite outgrowth (Rosenstein et al., 2003) and neuronal survival (Jin et al., 2000 & 2001; Svensson et al., 2002). EGF is not only a factor controlling glial development, as previously shown, but also a potent differentiation factor for retinal stem cells at least *in vitro* (Angenieux et al., 2005). However, EGF fails to improve survival of adult human retinal neurons *in vitro* (Liu et al., 2003).

IGF may influence the survival, proliferation and differentiation of neural cells and play an important role in the development of the nervous system (Suttie et al., 1985; Clarkson et al., 2001; Poe et al., 2001). Muscle-derived nerve sprouting factors including IGF induce neurite outgrowth from peripheral neurons by elevating GAP-43 (Caroni and Grandes, 1990). Velvet antler polypeptides (VAPs) including IGF and NGF maintain neuronal survival, and promote neurite outgrowth during the development of rat brain (Lu et al., 2005).

Extracellular heat shock protein 70 (HSP70) promotes chick motoneuron survival although endogenous expression of HSP70 does not change during trophic factor deprivation (Robinson et al., 2005). Since hindlimb muscle cells and lumbar spinal astrocytes readily secrete HSP70 *in vitro*, they are potential sources of extracellular HSP70 for motoneurons.

S100B, a glia-derived calcium binding protein, exhibits strong neurite extension activity in cultured serotonergic neurons from mouse brain (Nishiyama et al., 2002). Thus, it is considered to be one of the most potent neurotrophic factors for serotonergic neurons (Azmitia et al., 1990; Liu and Lauder, 1992; Ueda et al., 1995). When S100B expression is dramatically reduced in the cortex and hippocampus, the number of serotonergic neurons in these regions is significantly decreased (Ueda et al., 1994).

Psycho-stimulants have also been reported to have neurotrophic effects. Amphetamine induces neurite outgrowth in rat PC-12 cells (Park et al., 2002) increasing dopamine release (Park et al., 2003) and marijuana has neuroprotective effects (Klein et al., 1991; Guzman et al., 2001; Iuvone et al., 2004). Ethanol may have distinct effects on initiation of neurite elongation and branching (Zou et al., 1993). Finally, cocaine decreases neuronal survival and inhibits the NGF-induced neurite outgrowth of rat locus coeruleus neurons (Zachor et al., 2000; Snow et al., 2001).

4) **Extracellular matrix (ECM) molecules**

ECM-composing macromolecules are glycoproteins, proteoglycans, glycosaminoglycans and collagens that are secreted and assembled locally into an organized network where cells adhere (Hay, 1981). The most abundant structural components of ECM in all tissues are the collagens (Zagris, 2001). The matrix offers a structural scaffold for cellular adhesion, migration, and morphogenesis (Letourneau et al., 1994; Zagris, 2001), and promotes proliferation and differentiation (Adams and Watt, 1993; DeSimone, 1994; Timpl and Brown, 1994; Letourneau et al., 1994). Since ECM molecules also act as a physical barrier or selective filter to soluble diffusible molecules (Adams and Watt, 1993), it can regulate various cellular processes either by specific receptors for itself or by growth factors retained in the matrix (Zagris, 2001; Kiryushko et al., 2004).

Interactions of neurons with ECM molecules are reviewed in the previous article of Letourneau and colleagues (1994). Neuron-ECM interactions may generate signals that can directly regulate polymerization of cytoskeletal components and associations of cytoskeleton and plasma membrane. Thus, their interactions consequently produce the extension and adhesion of lamellipodia and filopodia accompanying mechanical force. Morphology and orientation of early neurites are mainly regulated by the outgrowth promoting molecules of ECM: (1) adhesive glycoproteins such as laminin (Giancotti, 1997), tenascin (Jones and Jones, 2000) and fibronectin (Biran et al., 2001; Akers et al., 2001), (2) glycosaminoglycans such as

hyaluronate (Sherman et al., 2002) and heparan sulfates (Bovolenta and Fernaud-Espinosa, 2000), and (3) the heparin-binding growth-associated molecule (HB-GAM) (Rauvala and Peng, 1997). All of these are strongly expressed in the developing brain and induce neuritogenesis both *in vitro* and *in vivo*. Interaction between regrowing axons and astroglial-associated fibronectin in mouse white matter is critical to promote neuritic regeneration (Tom et al., 2004). Neurite outgrowth triggered by ECM molecules is a result of both modulation of cellular adhesion to ECM and activation of cell surface receptors initiating intracellular signaling cascades. Strength of adhesion to the ECM substrates is positively correlated with the amount and complexity of neurite outgrowth by developing neurons (Chamak and Prochiantz, 1989; Letourneau, 1975). Specific ECM receptors have extensively been identified (Adams and Watt, 1993; Letourneau et al., 1994; Zagris, 2001; Kresse and Schönherr, 2001): (1) integrins for laminin and fibronectin, (2) integrins, CAMs, fibronectin and lectican for tenascin, (3) N-syndecan for HB-GAM, and (4) CD44 and RHAMM for hyaluronate. The most common adhesive interactions between neurons and ECM molecules are mediated by ECM receptors called integrins, which recognize a specific short linear amino acid sequence (e.g. Arginine-Glycine-Aspartate) in extracellular matrix proteins (Wildering et al., 1998). Intracellularly, integrins are bound to cellular actins through cross-linking proteins called actinins (Letourneau et al., 1994).

5) **Cell adhesion molecules (CAMs)**

It was initially suggested that accurate axonal targeting and synaptic formation in the CNS may be driven by changes in adhesivity of individual growth cones (Sperry, 1963). CAMs are adhesive molecules found in the nervous system (Kiryushko et al., 2004). CAMs not only regulate mechanical adhesions between cell-cell and cell-ECM, but they also trigger intracellular signaling cascades resulting in neurite outgrowth, cell migration and/or myelination (Riehl et al., 1996; Ronn et al., 1998; Schmid et al., 2000; Thelen et al., 2002).

Cadherins including N-cadherin are transmembrane glycoproteins, which have five cadherin domains mediating calcium-dependent homophilic interaction (Kiryushko et al., 2004). Cadherins also participate in heterophilic binding with integrins (Cepek et al., 1994) and FGF receptors (Williams et al., 1994). They can interact with various intracellular proteins such as catenins, mediating the interaction between cadherins and the actin cytoskeleton (Hirano et al., 1992). Cadherins are important molecules required for axonal outgrowth in retinal ganglionic neurons (Riehl et al., 1996).

L1-CAM is an integral membrane glycoprotein that consists of six Ig-like domains and a highly conserved cytoplasmic domain (Kiryushko et al., 2004). L1 is known to participate in homophilic interactions and to bind with multiple heterophilic ligands including other CAMs (NCAM, TAG-1/axonin-1, contactin/F3/F11), proteoglycans (neurocan and phosphacan), ECM

molecules (laminin and tenascin), integrins, FGF receptors and receptor tyrosine phosphatases (Brummendorf and Rathjen, 1995). The cytoplasmic domain of L1 contains at least three regions that interact with the cytoskeleton-associated proteins (Crossin and Krushel, 2000). L1 promotes axon growth and guidance critical to neuronal development (Schmid et al., 2000) and potentiates integrin-dependent cell migration along ECM molecules (Thelen et al., 2002).

NCAM is a cell surface adhesion component that also exists extracellularly, in cerebrospinal fluid, or associated with ECM components (He et al., 1987), L1 (Brummendorf and Rathjen, 1995), TAG-1/axonin-1 (Brummendorf and Rathjen, 1995), and FGF receptors (Kiselyov et al., 2005). It has a heparin-binding domain that mediates heterophilic interactions with heparan sulfate on the cell surface (Kallapur and Akeson, 1992; Letourneau et al., 1994). Homophilic adhesion mediated by NCAM is suggested as a multi-binding interaction between some of its five Ig domains (Ranheim et al., 1996). NCAM is an essential molecule for axonal outgrowth of hippocampus neurons (Cremer et al., 1997).

2.7. Molluscan neuronal growth factors

Mammalian growth factors have often been known to be inactive when tested on invertebrate neurons (Wong et al., 1981; Chiquet et al., 1988). However, some snail neurons have been shown to respond to mammalian NGF (Ridgway et al., 1991), ciliary neurotrophic factor (CNTF) (for review,

see Bulloch and Syed, 1992) and gonadotropin-releasing hormone (mGnRH) (Goldberg et al., 1993). NGF induced sprouting of motoneurons and interneurons from *Lymnaea* (Ridgway et al., 1991), in contrast to an earlier report, which showed that it had no effect on *Helisoma* neurons (Wong et al., 1981). Addition of mGnRH arrested the neurite outgrowth of dissociated embryonic *Helisoma* neurons in culture (Goldberg et al., 1993). Homologues of vertebrate substrate and growth factors have been found in invertebrates (Mattson and Kater, 1988; Chiquet et al., 1988; Muskavitch and Hoffman, 1990; Miller and Hardley, 1991). Therefore, there appears to be a marked conservation of growth factors during evolution.

1) **Putative neuronal growth factors in *Helisoma***

Helisoma conditioned medium (CM) produced by central ganglionic tissues from the adult CNS has long been known to be capable of promoting neurite outgrowth in cultures (Wong et al., 1984). However, the mechanisms by which molluscan growth-conditioning factors affect neurite outgrowth are one of the major unanswered questions regarding neuronal plasticity. Williams and Cohan (1994) suggest that the *Helisoma* brain-conditioned factors in CM may not be involved in growth cone formation, but rather in neurite elongation. This may be accompanied by the redistribution of neuronal cytoskeleton elements, such as intrusion of extending microtubules into the actin filament-depolymerizing zone in peripheral growth cones. Thus, their study suggests that growth cone formation is

intrinsic while neurite outgrowth is extrinsic. In a study using *Helisoma* embryonic neurons, neurite elongation was promoted by extrinsic factors in the CM, but the initiation of neurite outgrowth did not require exogenous growth-promoting factors (Goldberg et al., 1988). It has also been shown in *Lymnaea* that neurite outgrowth and synaptic specification are probably mediated by different trophic factors (Munno et al., 2000).

The release of neurite outgrowth promoting factors is dependent on new protein synthesis and spontaneous neuronal electrical activity associated with calcium channels, but not with sodium channels (Berdan and Ridgway, 1992). It has also been suggested that neurite outgrowth of adult *Helisoma* neurons can be induced via release or activation of conditioning factors modulated by the hypertonic, stress-elevated amino acid, L-glutamate (Bulloch and Ridgway, 1989). However, a previous study with embryonic cultures suggests that brain-conditioned factors from adult *Helisoma* could halt neuronal development (Goldberg et al., 1988). In this study, the time of onset of morphological differentiation was delayed by trophic factors indicating the presence of inhibitory soluble factors in the CM.

Although the contents of *Helisoma* CM have not been analyzed in detail, some unidentified conditioning factors in the *Helisoma* CM were reported to be protease-, trypsin- and heat-sensitive, and were shown to bind to the poly-lysine substratum (Barker et al., 1982; Wong et al., 1983 & 1984). An interesting feature of the conditioning factors is their exceptional affinity for cellulose nitrate (Millipore filter, 0.22 μm) but not for cellulose

triacetate (Gelman filter, 0.22 μm) (Wong et al., 1981). The addition of mammalian sera, NGF or fibronectin individually to the minimal defined medium (DM) cannot induce outgrowth in the same manner that CM does, suggesting that each of these classes of molecules on their own is not enough to cause neurite outgrowth. An anti-fibronectin preabsorption test in the CM-inducing neurite outgrowth confirms the presence of fibronectin-like molecules in the CM of *Helisoma* (Mattson and Kater, 1988). *Helisoma* CM is known to contain an unidentified neurite outgrowth promoting factor (~300 kDa), which is a laminin-like extracellular matrix molecule (Miller and Hadley, 1991; Williams and Cohan, 1994). The collagen substrate produces *Helisoma* neurons with thin, relatively straight neurites attached only at the growth cones while a poly-lysine substrate produces thicker neurites that branch profusely (Wong et al., 1981). Soluble choline metabolism-enhancing factors were also found in the same CM, indicating an interaction between neuronal growth and metabolism (Barker et al., 1982).

2) **Putative neuronal growth factors in other invertebrates**

The existence of a molluscan insulin-related neuropeptide (MIP) which promotes neurite outgrowth, was first found in *Lymnaea* CM (Ebberink et al., 1987; Kits et al., 1990). When isolated cerebral ganglia with median lip nerves (MLN) are incubated overnight, the neuroendocrine light green cells (LGCs) release MIP into the CM (Ebberink et al., 1987). The presence of

NGF-like molecules in *Lymnaea* CM is also supported by anti-NGF preabsorption experiments (Ridgway et al., 1991).

Sheath cells from CNS connectives and arterial cells from the anterior aorta of *Aplysia* enhance neurite outgrowth from co-cultured neurons (Montgomery et al., 2002). This study suggests that sheath and arterial cells produce substrate-bound and diffusible factors to promote neurite outgrowth, respectively. *Aplysia* hemolymph (ApHM)-coated dishes are known to exhibit extensive neurite outgrowth (Munno et al., 2000). Conditioning factors in molluscan CM are well conserved between two different molluscan species, *Lymnaea* and *Aplysia* (Munno et al., 2000).

The ECM of leech ganglionic capsules contains a protease-sensitive factor, which can be extracted with urea (Chiquet and Nicholls, 1987). Using this factor as a substrate in defined medium induces the rapid neurite outgrowth of identified leech neurons. Nitric oxide also controls microglial migration that releases neuronal growth factors in leech (Duan et al., 2005).

2.8. Cross-talk between neurotrophic factors and neurotransmitters

Diffusible neurotrophic factors and neurotransmitters are intercellular signals that determine the details of neuritic form, and influence neuronal survival and synapse formation (for review, see Mattson, 1989). Under normal conditions, levels of neurotrophic factors and neurotransmitters can be controlled through intercellular communication between neurons and their support cells. In addition, uncontrolled levels of neurotrophic factors

and neurotransmitters may lead to neurotoxicity or a tonic inhibitory state (Lipton and Kater, 1989).

A growing literature suggests that many neuronal growth factors do not operate individually, but interact with other factors to influence brain development (Dreyfus, 1998). This interaction is evident in the cerebellum, the basal forebrain-hippocampus and the locus coeruleus-hippocampus. Cross-talk between neurotransmitters and neurotrophic factors may be a common occurrence during neuronal development and communication between neurons and glial cells (Leith et al., 1990; Azmitia et al., 1990; Liu and Lauder, 1992; Ueda et al., 1995; Knipper and Rylett, 1997; Dreyfus, 1998; Barres and Barde; 2000; Nishiyama et al., 2002; Mattson et al., 2004; Rumajogee et al., 2005; Edenfeld et al., 2005; Garcia-Ovejero et al., 2005). For example, 5-HT stimulates the expression of BDNF in the adult rat brain, and BDNF enhances neurite outgrowth and neuronal survival of 5-HT neurons (Zetterstrom et al., 1999; Russo-Neustadt et al., 1999). In addition, stimulation of 5-HT_{1A} receptors enhances expression of the calcium-binding protein, S100B (Haring et al., 1993). Conversely, 5-HT levels are influenced by S100B expression in hippocampus (Whitaker-Azmitia et al., 1990). 5-HT can stimulate cultures of neonatal astrocytes or embryonic radial glia and astrocytes to release trophic factors into the medium, which stimulate or inhibit growth of serotonergic neurons depending on culture conditions (Whitaker-Azmitia and Azmitia, 1989). It has also been known that 5-HT

neurons can interact selectively with glia via cell surface determinants (Leith et al., 1990).

2.9. Effects of neurotransmitters on neurite outgrowth in vertebrates

Besides trans-synaptic communication, neurotransmitters have been suggested to play important roles in neuronal differentiation. The neuronal growth cone is not a static structure but changes morphology in response to a variety of extracellular guidance cues such as diffusible factors and ECM molecules. Thus, some morphological changes in regenerating neurons could be attributed to the modulatory roles of neurotransmitters in rearranging cytoskeleton elements such as microtubules, microfilaments and intermediate filaments.

1) Effects of 5-HT on neurite outgrowth in vertebrates

5-HT may have an organizing function in the developing nervous system, which includes effects on neurite outgrowth (Lauder, 1990). *In vivo* studies of neuronal development in the rat have indicated that 5-HT may be a factor that delays the onset of neuronal differentiation in specific brain regions (Liu and Lauder, 1991). Likewise, 5-HT initially appeared to act as an inhibitory signaling molecule influencing the neurite outgrowth of raphe neurons (Janakait et al., 1988; Whitaker-Azmitia and Azmitia, 1986) and cortical neurons (Sikich et al., 1990). This inhibitory effect may be enhanced by co-culturing embryonic raphe neurons with mesencephalic glia (Liu and

Lauder, 1992). In the goldfish retina, 8-OH-DPAT, a 5-HT_{1A} receptor agonist, inhibits neurite outgrowth, increases cAMP levels (Urbina et al., 1996) and impairs the trophic effect of taurine (Lima et al., 1994). However, this inhibitory effect cannot be explained without the involvement of 5-HT_7 receptors that are positively coupled to adenylate cyclase, because activation of the 5-HT_{1A} receptor is known to decrease cAMP levels. 5-HT covalently bonded with α_2-macroglobulin inhibits NGF-promoted neurite outgrowth of embryonic sensory and cerebral cortical neurons (Liebl and Koo, 1993). However, 5-HT has also been shown to cause both inhibitory and stimulatory effects on neurite outgrowth of embryonic monoaminergic neurons (Liu and Lauder, 1991). 5-HT enhances neurite outgrowth of thalamic neurons from mouse embryos (Lotto et al., 1999). This facilitatory effect is blocked by the sodium channel blocker, tetrodotoxin (TTX). Interestingly, the selective 5-HT_{1B} agonist, CGS-12066A, is capable of mimicking the effect of 5-HT. Thus, 5-HT has been shown to affect the fine-tuning of thalamocortical connections through 5-HT_{1B} receptors associated with the sodium channel (Bennett-Clark et al., 1994; Cases et al., 1996; Lotto et al., 1999). Similar facilitatory effects of 5-HT on neurite outgrowth were found in another study using ventrobasal thalamic neurons (Lieske et al., 1999). Interestingly, only the concentration of 25 μM 5-HT significantly promoted neurite outgrowth, whereas the other concentrations (10, 50 and 100 μM) did not.

2) **Effects of other neurotransmitters on neurite outgrowth in vertebrates**

Dopamine has been shown to have both inhibitory and stimulatory effects on neurite outgrowth in different systems. For instance, dopamine inhibits neurite outgrowth of cultured chick retinal neurons (Lankford et al., 1988) and decreases non-primary neurite arborization of avian retinal neurons (Lankford et al., 1987) during a period of development when a D_1 dopamine receptor subtype is transiently expressed (Ventura et al., 1984). Conversely, stimulation of dopamine D_2 receptors substantially increases neurite branching and neurite length of rat cortical neurons *in vitro* (Todd, 1992). However, dopamine has also been shown to stimulate neurite outgrowth in embryonic rat striatum cultures through activation of D_1, but not D_2 receptors (Schmidt et al., 1998). In addition, the possible involvement of D_3 receptors in the induction of neurogenesis including neurite outgrowth has recently been suggested in the adult rat substantia nigra (Kampen and Robertson, 2005).

Glutamate is well known to cause degeneration of hippocampal neuroarchitecture (Mattson et al., 1988; Mattson and Kater, 1989). Glutamate halts dendritic growth cones but has no effect on axonal growth cones (Mattson et al., 1988). The glutamate-induced dendritic regression may be reduced by Co^{++} and trifluoperazine, suggesting that calcium influx and/or PKC activation mediate glutamate's actions (Mattson et al., 1988). However, glutamate can also promote neurite sprouting through its action

on NMDA receptors in rat cerebellar granule cells (Pearce et al., 1987; Balazs et al., 1988).

γ-Aminobutyric acid (GABA) can block the regressive effect of glutamate on dendrites of hippocampal pyramidal neurons (Mattson and Kater, 1989). Thus, inhibitory GABA seems to promote dendritic elongation in the presence of glutamate (Mattson et al., 1988). This effect suggests that in general, electrically excitatory agents have a negative regulatory effect on neurite outgrowth, while electrically inhibitory agents mainly promote neurite outgrowth.

Acetylcholine inhibits neurite outgrowth in rat retinal ganglion cells (Lipton et al., 1988), chick retinal neurons (Lankford et al., 1988) and hippocampal pyramidal neurons (Mattson, 1988). This inhibition can be released by blocking nicotinic acetylcholine receptors on rat retinal ganglionic neurons (Lipton et al., 1988; Lipton, 1988). It is interesting that the antagonism of a neurotransmitter receptor can have regulatory effects on neurite outgrowth.

2.10. Effects of neurotransmitters on neurite outgrowth in invertebrates, including *Helisoma trivolvis*

Several studies support the regulatory roles of neurotransmitters in neurite outgrowth (for review, see Kesteren and Spencer, 2003). The effects of neurotransmitters on neurite outgrowth are neuron-selective (Haydon et al., 1984 & 1987; McCobb et al., 1988; Murrain et al., 1990) so that a

specific neurotransmitter is involved in the modulation of only a certain type of neuron. In other words, a specific neuron requires the appropriate receptor expression in order to receive a neurotransmitter signal. Therefore, different types of neurons may exhibit characteristic morphological and functional features. For example, embryonic neurons have smaller cell bodies with a few faster outgrowing neurites while adult neurons have larger cell bodies and more complicated neuritic arbors (Goldberg et al., 1991). Buccal ganglionic neuron B5 exhibits a more complicated neuritic branching pattern, and wider growth cones than does another buccal ganglionic neuron B19 (Mattson, 1988). Thus, growth cone width and neurite outgrowth pattern could be morphological keys to form ultimate cytoarchitectual structures of a neuron through various intracellular second messengers stimulated by neurotransmitters.

1) **Effects of 5-HT on neurite outgrowth in invertebrates**

5-HT was the first neurotransmitter found to modulate neurite elongation and growth cone motility of isolated *Helisoma* neurons (Haydon et al., 1984 & 1987; Goldberg et al., 1991). Neurite outgrowth of the identified dopaminergic pedal ganglionic neuron P1 from *Helisoma* is inhibited by 5-HT, but not by dopamine (McCobb et al., 1988). A pharmacological study (Diefenbach et al., 1995) on intact *Helisoma* embryos has shown that 5-HTP treatment increases 5-HT content in ENC1 and inhibits neurite outgrowth, whereas *p*CPA reduces 5-HT content in ENC1

and promotes neurite outgrowth. In cell culture studies, 5-HT inhibitory effects have been localized to growth cones and filopodia in specific neurons (Cohan et al., 1987; Goldberg, 1998). As a consequence of this inhibition, serotonin's actions lead to growth cone collapse and the prevention of synaptogenesis (Haydon et al., 1987).

However, the responses of neurites to 5-HT are not exclusively inhibitory. In *Helisoma* embryonic neurons, 5-HT reinitiates neurite elongation in non-elongating neurites, while it arrests neurite outgrowth in elongating neurites (Goldberg et al., 1991). Similar biphasic effects of 5-HT have also been investigated in unidentified adult buccal ganglionic neurons from *Helisoma* (Goldberg et al., 1991) and in the antennal lobe neurons from the silk moth (Kim et al., 2003). In previous studies on *Helisoma* adult neurons, only actively elongating neurons or neurites were investigated so that the only outgrowth-inhibitory effects of 5-HT were observed. Therefore, possible facilitatory 5-HT actions may have gone undetected in the rapid neurite elongating phase. In order to overcome these problems, the effects of 5-HT on outgrowing and sessile neurites should be examined.

5-HT acts on a novel serotonergic receptor on neuron B19 that associates with a GTP-binding (G_s) protein and initiates the exchange of GDP with GTP (Price and Goldberg, 1993). Activation of the G_s protein results in dissociation of its β-γ and α subunits, and then the α subunit stimulates membrane-bound adenylate cyclase (AC) to elevate cytosolic cyclic AMP. This intracellular messenger directly activates a class of cyclic

nucleotide-gated sodium channels, leading to sodium influx, membrane depolarization, and activation of voltage-gated calcium channels (Price and Goldberg, 1993). The resulting elevation of intracellular calcium acts through a calcium/calmodulin-dependent pathway to inhibit neurite elongation and growth cone motility (Cohan et al., 1987; Mattson et al., 1988; Polak et al., 1991; Kater and Mills, 1991; Davenport et al., 1996; Torreano and Cohan, 1997; Rehder and Cheng, 1998; Goldberg, 1998). There are optimum levels of intracellular calcium that promote normal neurite elongation and growth cone motility (Mattson and Kater, 1987; Kater and Mills, 1991; Goldberg, 1995). These two components of neurite outgrowth appear to have different sensitivities to intracellular calcium levels (Mattson and Kater, 1987). Moreover, it has been suggested that any extracellular signal that regulates intracellular calcium has the potential to regulate neurite outgrowth (Kater and Mills, 1991). Application of cyclic AMP to both neuron B5 and B19 results in an increase in intracellular calcium and an inhibition of neurite outgrowth (Mattson et al., 1988). Neuron B5 seems not to be affected by 5-HT, but potentially by other transmitters coupled to the cAMP second messenger system (Haydon et al., 1987; Murrain et al., 1990).

2) **Effects of other neurotransmitters on neurite outgrowth in invertebrates**

There have only been a handful of studies on the effects of neurotransmitters other than 5-HT on neurite outgrowth of *Helisoma* neurons.

These studies have investigated the effects of dopamine, glutamate, acetylcholine and somatostatin (Bulloch, 1987; McCobb et al., 1988; Jones and Bulloch, 1988; McCobb and Kater, 1988).

Dopamine (DA) has been shown to inhibit growth cone motility and neurite elongation of neuron B19 (McCobb et al., 1988; McCobb and Kater, 1988). Similar to 5-HT, this inhibitory effect is linked to membrane depolarization and can be blocked by hyperpolarizing current (McCobb et al., 1988; McCobb and Kater, 1988). Therefore, it has been suggested that any source of electrical excitation should result in the inhibition of neurite outgrowth. In *Lymnaea*, neurite outgrowth of non-target neurons is severely restricted and retarded in the presence of exogenous dopamine (Spencer et al., 1996). Furthermore, dopamine alone is not sufficient to initiate and support neurite outgrowth in target neurons (Spencer et al., 1996). It has been reported that endogenously released dopamine from a presynaptic neuron attracts growth cones (Spencer et al., 1998).

Glutamate may promote neurite outgrowth in adult *Helisoma* neurons (Jones and Bulloch, 1988) and somatostatin has also been known as a stimulatory agent for the regulation of neurite outgrowth (Bulloch, 1987). Application of acetylcholine (ACh) to neuron B19 blocks the inhibitory effects on neurite outgrowth that occurred with 5-HT alone (McCobb et al., 1988). This suggests that ACh can block the electrical excitatory effect of 5-HT on neuron B19 and that ACh can prevent the rise in growth cone Ca^{++} level.

It is evident that different neurotransmitters affect neurite outgrowth differently and that the inhibition or facilitation of neurite outgrowth will depend upon many factors, including second messenger systems, Ca^{++} levels, electrical stimulation, neuritic phase of outgrowth, and intrinsic receptor expression. Therefore, many studies have focused on identified neurons of invertebrate systems so that roles of individual neurotransmitters in neurite outgrowth can be examined and compared between individual neurons.

2.11. Possible autoregulation of neurite outgrowth by 5-HT

If neurotransmitters regulate neurite outgrowth, neighboring neurons should be considered as logical sources of the signaling neurotransmitters. Moreover, it is also possible that an outgrowing neuron locally releases its own neurotransmitter to regulate its own neurite outgrowth. Based on previous studies, such autoregulation of neurite outgrowth likely occurs. The autoregulation of neurite outgrowth has initially been considered in serotonergic neurons from both invertebrate and vertebrate studies. The first possibility of 5-HT autoregulation in neurite outgrowth was reported in an invertebrate serotonergic neuron, *Helisoma* pedal ganglionic neuron P5, where neurite outgrowth was suppressed by exogenously applied 5-HT (Haydon et al., 1984). Another *Helisoma* serotonergic cell, cerebral ganglionic neuron C1, may also use 5-HT in an autoregulatory fashion during development. Neuron C1 sprouted neurites following axotomy and

rapidly regenerated to innervate buccal neuron B19 (Murphy et al., 1985). Moreover, exogenous 5-HT (10 μM) arrested the axonal regeneration of neuron C1 in cerebro-buccal ganglia cultures (Murrain et al., 1990). Thus, this cell may provide an initial step to examine a putative autoregulation of neurite outgrowth in serotonergic systems. A similar inhibitory effect of exogenous 5-HT on neurite outgrowth was found in the serotonergic raphé neurons of vertebrates (Whitaker-Azmitia and Azmitia, 1986). Furthermore, the development of serotonergic neurons has been shown to be controlled by 5-HT during critical periods of development (Liu and Lauder, 1991).

A couple of studies were actually performed to control endogenous 5-HT levels and support the possibility of 5-HT autoregulation in neurite outgrowth. In a separate study using *Drosophila* neurons, a mutation which knocked down the expression of 5-HT, resulted in increased arborization of 5-HT fibers in the gut of *Drosophila* (Budnik et al., 1989). Similarly, axonal outgrowth and the number of branching neurites in *Helisoma* serotonergic ENC1 were inversely proportional to 5-HT concentration in the embryo (Diefenbach et al., 1995). However, those studies for the down-regulation of endogenous 5-HT still cannot rule out a possibility that a serotonergic neuron releases 5-HT and regulates the neurite outgrowth of other neighboring serotonergic neurons.

A relatively recent study on the cerebral giant cells (CGC) from *Lymnaea,* which are thought to be homologous to *Helisoma* serotonergic neuron C1s (Koert et al., 2001), strongly implicates autoregulation of neurite

outgrowth. Here, 5-HT release from a CGC was detected using a sniffer cell, and exogenously applied 5-HT was found to have an inhibitory effect on the neurite outgrowth of CGCs in culture. However, this study also did not show the direct involvement of 5-HT autoreceptors in the autoregulation of neurite outgrowth since some other neurotransmitters in the serotonergic neuron can be involved in the outgrowth regulation.

Taken together, an autoregulatory role of 5-HT in neurite outgrowth is unclear so far. However, evidence is accumulating that the cessation of neurite outgrowth and the resultant final morphology of an individual serotonergic neuron may be controlled by 5-HT released from that particular neuron.

2.12. Putative 5-HT receptors that regulate neurite outgrowth

The pharmacological profiles of the molluscan 5-HT receptors in *Helisoma* that regulate neurite outgrowth are unknown. It is still not clear how molluscan 5-HT receptors are different from mammalian 5-HT receptors. Here, an interesting question can be asked whether hypothesized autoregulatory 5-HT receptors involved in the autoregulation of neurite outgrowth are similar to the classical autoreceptors that inhibit 5-HT biosynthesis, neuronal firing and release from vertebrate serotonergic neurons (Cerrito and Raiteri, 1980; Rogawski and Aghajanian, 1981; Göthert and Schlicker, 1983; Westerink et al., 1990; Stamford et al., 2000; Ahn et al., 2005). If neurite outgrowth of serotonergic neurons can be modulated with a

change of 5-HT levels, then the control of 5-HT levels through a negative feedback loop via activation of presynaptic autoreceptors will be considered to be a potential target for the autoregulatory neurite outgrowth. To answer the above questions, extensive pharmacological studies are necessary. Pharmacological characterization of a novel molluscan 5-HT receptor mediating the inhibition of neurite outgrowth has been suggested in *Helisoma* neuron B19 (Price and Goldberg, 1993). The new class of 5-HT receptor in neuron B19 has a relatively high affinity for 5-HT and is positively coupled to the cAMP second messenger system. Nonetheless, it has not been determined whether this novel cAMP-related 5-HT receptor also plays a role in the regulation of neurite outgrowth in serotonergic neurons. An interesting model for autoregulation of 5-HT biosynthesis was developed through repression of MAP kinase stimulation of the tryptophan hydroxylase promoter (Wood and Russo, 2001). Theses authors found that activation of 5-HT_1 autoreceptors lowered tryptophan hydroxylase mRNA levels and suppressed the activity of MAP kinases that might be associated with the stimulation of tryptophan hydroxylase promoter (Wood and Russo, 2001). Since MAP kinases are closely associated with neuronal differentiation and neurite outgrowth, this finding leads to the suggestion that the signaling mechanisms involved in 5-HT autoreception may be linked to the signaling mechanisms involved in neurite outgrowth.

An extensive review of the molluscan 5-HT receptor subtypes from previous studies (Walker, 1984 & 1985; Hen, 1992; Humphrey et al., 1993;

Tierney, 2001; Hoyer et al., 2002; Barbas et al., 2003) might allow us to determine some of the properties of the putative 5-HT receptors in *Helisoma* that may be involved in the hypothesized autoregulation of neurite outgrowth. Although studies in molluscs have provided abundant information on the numerous physiological actions of 5-HT, little is known about the pharmacology and diversity of the 5-HT receptor system in molluscs. There was an attempt to analyze the excitatory actions of 5-HT on molluscan neurons based on the mammalian 5-HT receptor classification (Bokisch et al., 1983; Bokisch and Walker, 1986). This kind of study may be helpful to understand the pharmacological profiles of possible molluscan 5-HT receptor subtypes.

The isolated penis retractor muscle of the pulmonate snail, *Archachatina marginata,* probably contracts through a new class of molluscan 5-HT receptor that has a unique pharmacological profile compared to the mammalian 5-HT_1, 5-HT_2 and 5-HT_3 receptors (Innocent & Olufemi, 1992). In *Helix aspersa*, there is a fast 5-HT activating ligand-gated channel that differs significantly from known vertebrate 5-HT_3 receptors (Green et al., 1996) and can be classified as a new class of 5-HT receptor in molluscs. A gene for G-protein coupled 5-HT receptor (5-HT_{1lym}) has been cloned in *Lymnaea* (Sugamori et al., 1993). The 5-HT_{1lym} receptor exhibits a mixed 5-HT_1-like receptor profile that cannot be precisely categorized with existing mammalian classification nomenclature. Another type of G-protein coupled 5-HT receptor (5-HT_{2lym}) similar to the mammalian 5-HT_2 receptor

family has been cloned from *Lymnaea* (Gerhardt et al., 1996). This 5-HT_{2lym} receptor reveals a pharmacological profile most likely resembling those of *Drosophila* 5-HT_{2Dro} and mammalian 5-HT_{2B} and 5-HT_{2C} receptors. It has also been suggested in *Lymnaea* that 5-HT_2- and 5-HT_3-like receptors are involved in the responses of serotonergic CGCs and buccal motoneurons to 5-HT (Walcourt-Ambakederemo & Winlow, 1994 & 1995). The cloning of cDNA coding for an *Aplysia* G-protein coupled 5-HT receptor (5-HT_{ap1}) was reported (Angers et al., 1998). Its deduced amino acid sequence is similar to those of mammalian 5-HT_1 and 5-HT_7 receptors. Moreover, 5-HT_{ap1} receptor exhibits the high-affinity binding for lysergic acid diethylamide (LSD) and is coupled to adenylate cyclase. Recently, two putative 5-HT receptors in *Helisoma*, 5-HT_{1hel} and 5-HT_{7hel} receptors have been cloned in the present laboratory (Mapara, 2001). These gene products share characteristics of the seven transmembrane G-protein coupled receptors. In addition, these cloned receptors are closer to the mammalian 5-HT receptors which couple to adenylate cyclase than those stimulating phospholipase C. By *in situ* hybridization, mRNA for these receptors was expressed in *Helisoma* ciliated embryonic cells although there is no evidence for the expression in adult *Helisoma* brain (Parries et al., 2003).

The mammalian 5-HT receptor subtypes associated with cyclic nucleotides are 5-HT_1, 5-HT_4, 5-HT_6 and 5-HT_7 receptor families, while 5-HT_2 and 5-HT_3 receptor families are associated with phospholipase C (PLC) and ligand-gated cation channels, respectively. A signal transduction

mechanism has not clearly been demonstrated for the 5-HT_5 receptor family. It is postulated that the inhibitory action on mammalian neurons may be mediated through 5-HT_1 receptor subtypes, while the excitatory actions of 5-HT may be mediated through 5-HT_2 receptor subtypes (Peroutka and Snyder, 1981 & 1982). 5-HT_1 receptors in vertebrates are coupled to G-proteins and inhibit adenylate cyclase, reducing cAMP levels (Middlemiss and Tricklebank, 1992; Boess and Martin, 1994; Peroutka, 1994; Martin and Humphrey, 1994). Therefore, autoreceptors in mammalian 5-HT systems are suggested to belong to the 5-HT_1 receptor family. It is interesting that hyperpolarization through 5-HT_{1A} receptors may be induced by opening potassium channels (Cooper et al., 1996). 5-HT_{1A} receptors play a critical role in mediating inhibition of cell firing in the midbrain raphé nuclei (Sprouse and Aghajanian, 1986; Stamford et al., 2000), where neurite outgrowth is regulated by 5-HT. However, 8-OH-DPAT (8-hydroxy-2-dipropylaminotetralin), a 5-HT_{1A} receptor agonist, has been reported to induce an increase in the activity of adenylate cyclase in goldfish retina, although inducing a decrease in the activity of adenylate cyclase in the hippocampus of rat (Urbina et al., 1996). Moreover, the increased cAMP levels induced by 8-OH-DPAT causes the inhibition of neurite outgrowth, and WAY 100135 [N-(2-(4-(2-methoxyphenyl)-1-piperazyl)ethyl)-N-(2-pyridinyl) cyclohexane carboxamide trihydrochloride], a 5-HT_{1A} receptor antagonist, significantly blocks the inhibition of neurite outgrowth (Lima et al., 1994; Urbina et al., 1996). These different results may be due to the non-

selective binding of 8-OH-DPAT between 5-HT_{1A} and 5-HT_7 receptor subtypes.

Taken together, it appears that molluscan 5-HT receptors involved in the regulation (or autoregulation) of neurite outgrowth may be similar to either the mammalian 5-HT_1 or 5-HT_7 receptors which are coupled to G-proteins and adenylate cyclase.

3. Hypothesis and objectives of proposed studies

The final morphological properties of a given neuron arise from the integrated actions of various intrinsic and extrinsic signals. In other words, signals are responsible for controlling each of the essential stages of neurite outgrowth. Diffusible trophic factors such as neurotransmitters are known to be important regulators of neuronal cytoarchitecture and cell death. 5-HT is perhaps the best-studied neurotransmitter in terms of effects on neuronal differentiation and neuronal survival. 5-HT may delay the onset of cellular differentiation in specific brain regions. Moreover, 5-HT may influence neuronal cytoarchitectures depending on concentrations of the neurotransmitter, types of neuron and states of neurite outgrowth. Thus, tonic inhibition of neurite outgrowth induced by small amounts of 5-HT in the *Helisoma* CM may also take place.

Due to intrinsic cell-specificity in outgrowth responses to 5-HT, serotonergic neurons may use a different pathway from those used by non-serotonergic neurons. Autoregulation, whereby the activity of a neuron is controlled by its own neurotransmitter, has been suggested to be a contributing mechanism in the regulation of neurite outgrowth in many systems. The potentially important 5-HT regulatory mechanism has not been tested yet using a single serotonergic neuron culture. Establishment of a single neuron culture model for the study of neurite outgrowth is very critical, since mass cultures that contain several neurons and glial cells cannot rule out cross-talk between serotonergic neurons and glial cells.

The projects in the present thesis focus on direct roles of 5-HT in regulating neurite outgrowth of the identified serotonergic neuron C1 from the freshwater snail, *Helisoma trivolvis*. This is an area of research that has been relatively unexplored due to some difficulties in technical approaches. The following two major questions are addressed to investigate the effects of 5-HT on neurite outgrowth;

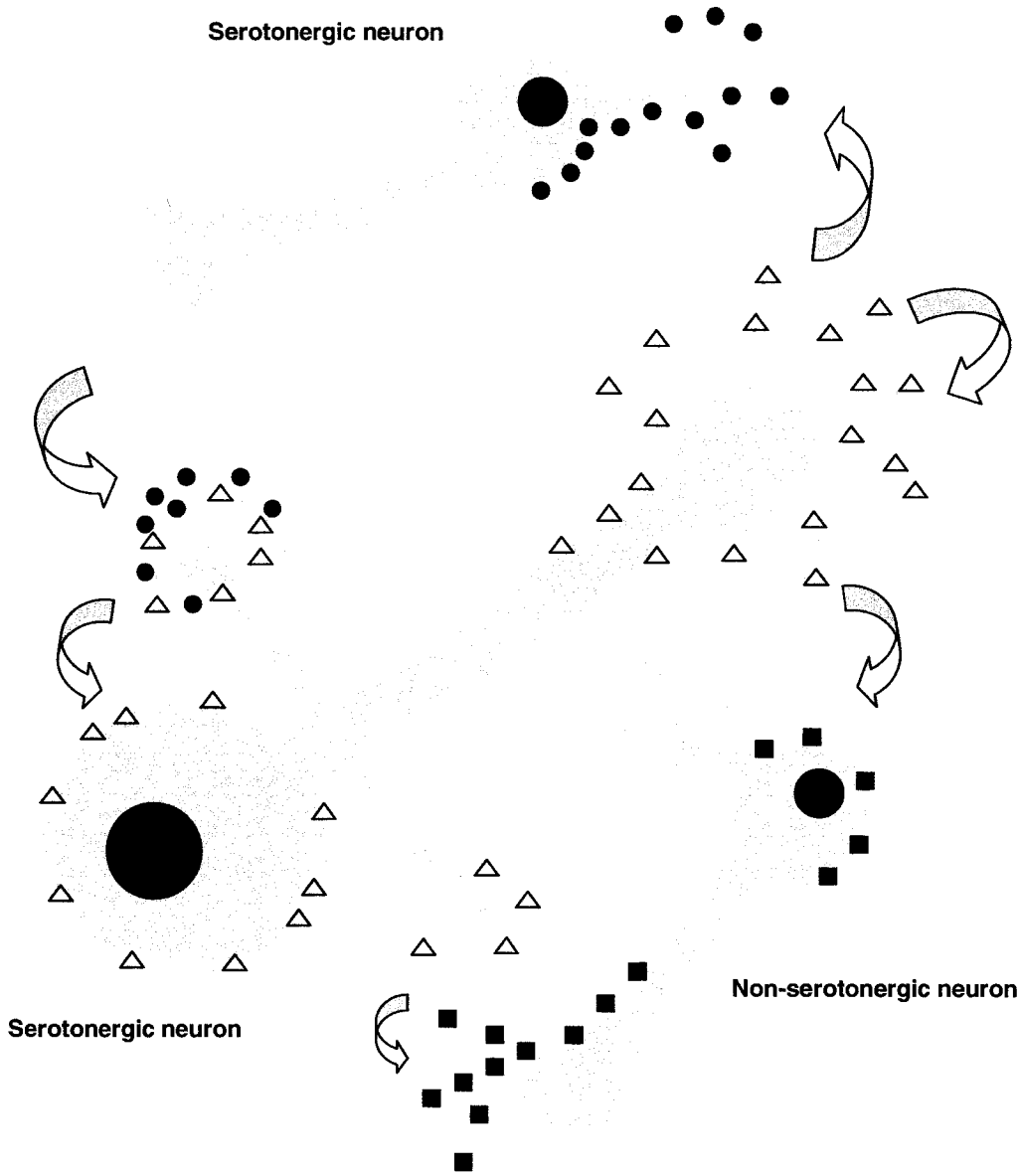
1) Does the presence of 5-HT in brain-conditioned medium exhibit tonic inhibitory effects on the phase transition of neurite outgrowth from initiation to elongation?
2) Does 5-HT differentially affect the elongation rates of slow-growing and fast-growing neurites?

In order to answer the above two questions in detail, the following hypotheses were tested in the present studies;

1) The onset of neurite elongation of neuron C1 is delayed by 5-HT, a tonic inhibitory diffusible factor in brain-conditioned medium.
2) Excessive exposure of neuron C1 to 5-HT is neurotoxic.
3) The neurite outgrowth of neuron C1 is modulated by 5-HT in a dose-dependent manner.
4) The regulatory effects of 5-HT on neurite outgrowth are biphasic and depend on the phase of outgrowth displayed by each neurite.
5) A second messenger system such as Ca^{++} mediates 5-HT regulatory effects on neurite outgrowth.

The purpose of the present studies is to understand neurotoxic effects of 5-HT on neuronal survival and multiple-regulatory actions of 5-HT on both the initiation and elongation of regenerating neurites in an identified molluscan serotonergic neuron. My understanding of the 5-HT regulation of neurite outgrowth and neuronal survival from the present studies, can provide a greater opportunity to understand the formation of neural networks during the development of nervous systems, as well as the reformation of original neural networks during neuronal regeneration.

Figure 1. General classes of serotonin receptors on serotonergic and non-serotonergic neurons. Heteroreceptors (green square) occur on non-serotonergic neurons and mediate responses to 5-HT. Homoreceptors (red circles) occur on serotonergic neurons and mediate responses to 5-HT that is released from other serotonergic neurons. Autoreceptors (yellow triangles) occur on or near the presynaptic terminals (or soma) of serotonergic neurons and mediate responses to 5-HT released from the same neuron. Autoreceptors often do not produce changes in membrane potential. Instead, they function to control internal processes including synthesis and release of neurotransmitter. It is believed that autoreceptors are part of a regulatory system that controls the amount of neurotransmitter that is released. Arrows indicate the direction of 5-HT release.



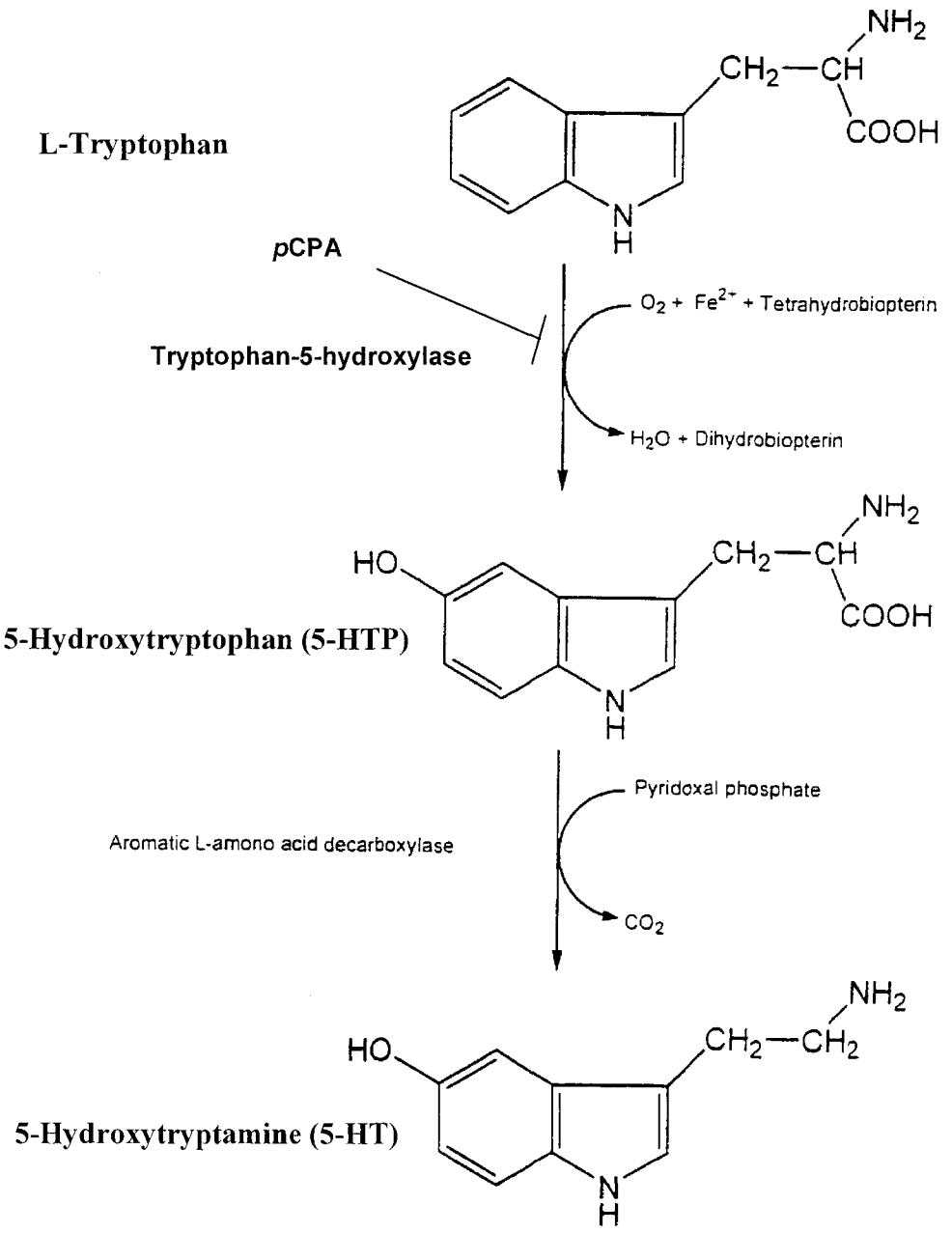


Figure 2. The pathway for 5-HT synthesis. Tryptophan-5-hydroxylase is the important rate-limiting enzyme. *p*CPA (*p*-chlorophenylalanine) is a well-known specific inhibitor for the tryptophan-5-hydroxylase.

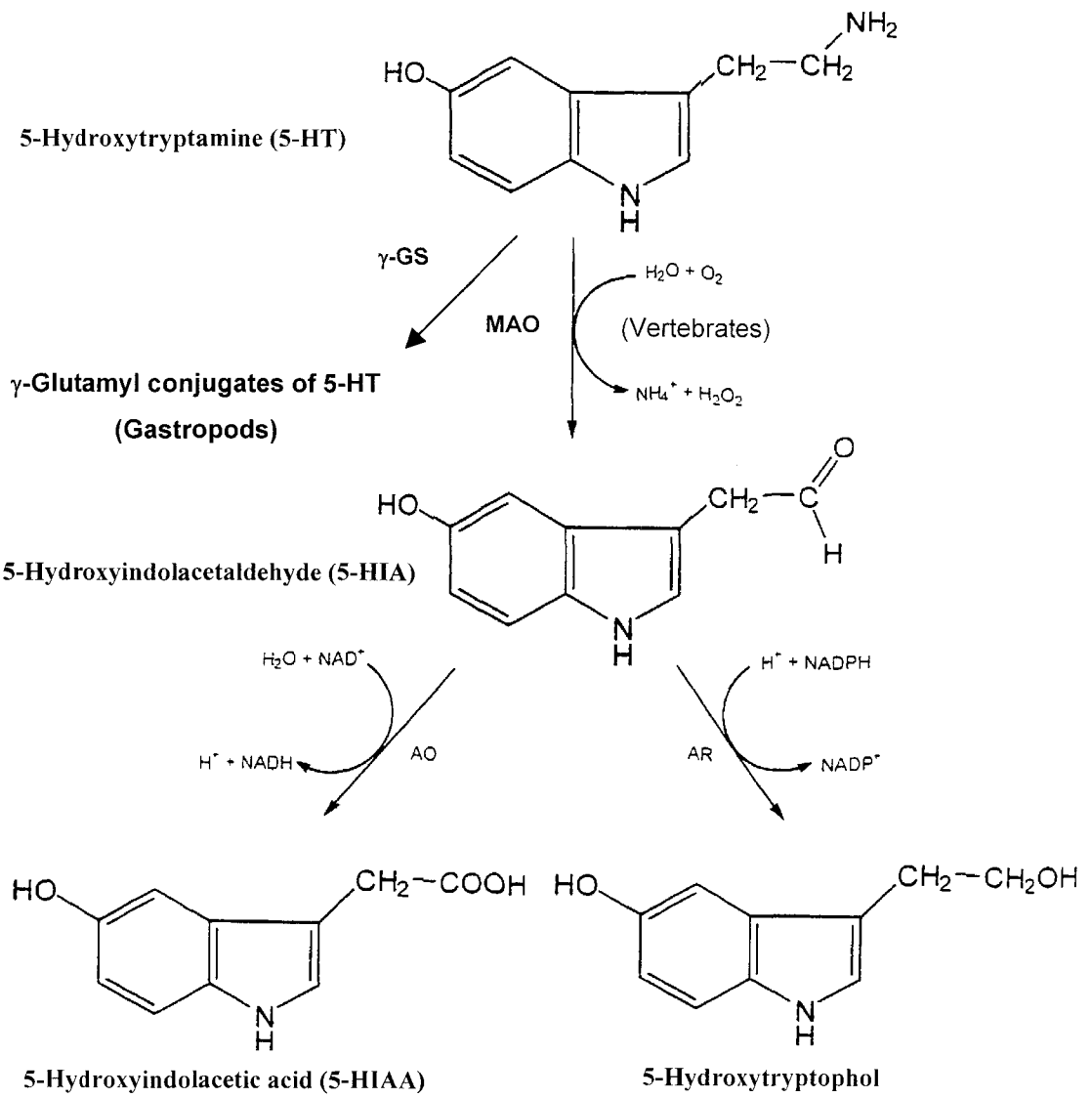


Figure 3. The pathways for 5-HT inactivation. The inactivation mechanisms of amines in gastropods is considerably different from the well-known monoamine oxidation that occurs in vertebrates. γ-Glutamyl conjugates are detected as 5-HT metabolites in *Helisoma trovolvis*. (AO, aldehyde oxidase; AR, aldehyde reductase; MAO, monoamine oxidase; γ-GS, γ-glutamylamine synthetase)

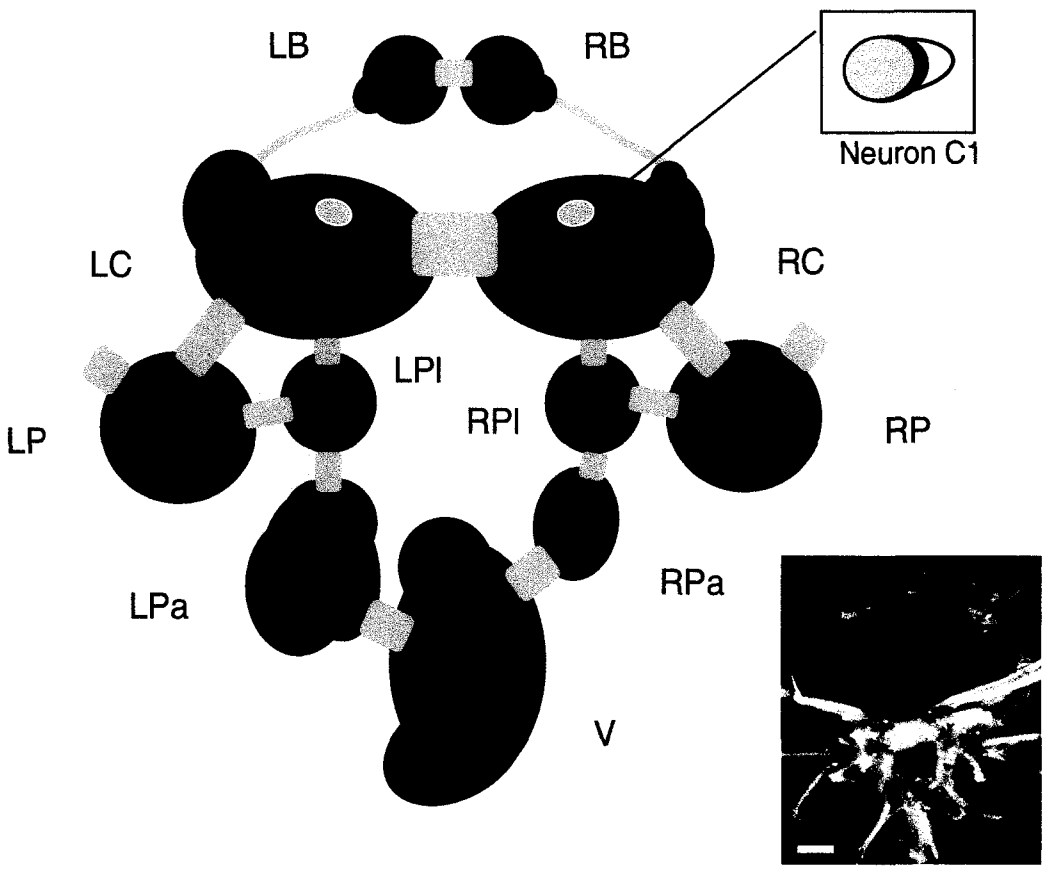
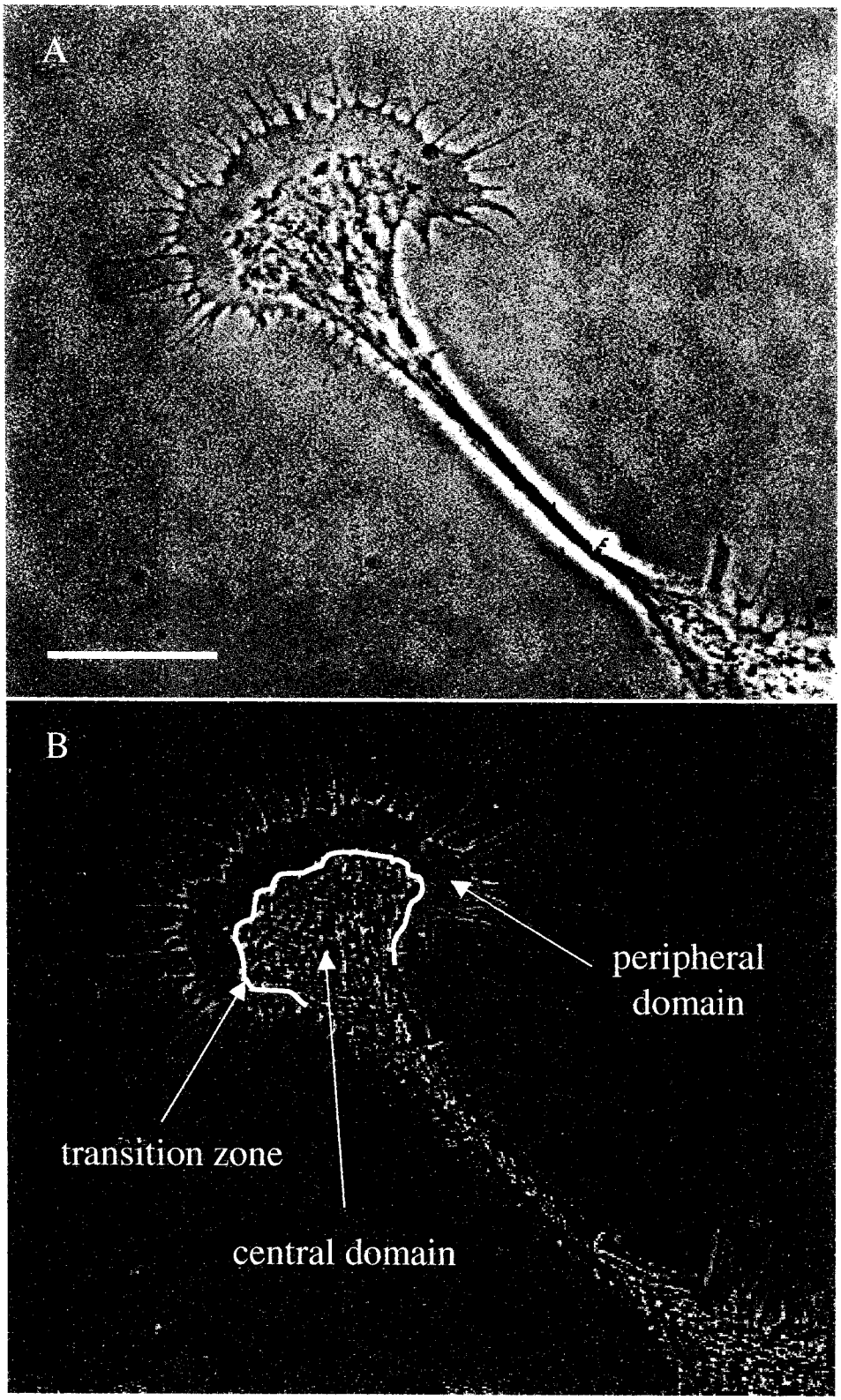


Figure 4. The central nervous system of *Helisoma trivolvis,* a freshwater pulmonate (LB & RB, left and right buccal ganglion; LC & RC, left and right cerebral ganglion; LP & RP, left and right pedal ganglion; LPl & RPl, left and right pleural ganglion; LPa & RPa, left and right parietal ganglion; V, visceral ganglion). Top right inset, location of the serotonergic neuron C1; Bottom right corner inset, actual picture of a dissected-out brain. Scale bar indicates 1 mm.

Figure 5. Two domains of the neuronal growth cone in serotonergic neuron C1 of *Helisoma trivolvis*. A. phase contrast micrograph. B. differential interference contrast (DIC) micrograph of the same growth cone. Central and peripheral domains are separated by the transition zone. Scale bar indicates 30 μm.



MATERIALS AND METHODS

Animals. An inbred laboratory-reared snail colony (Oregon Red albino strain) of *Helisoma trivolvis* was maintained at the University of Alberta. These fresh water pond snails were raised in flow-through aquaria containing an oyster shell substratum and de-chlorinated water to keep at a water temperature of 22 - 26 °C under a 12 hr light/dark cycle (lights on 7:00 am). Animals were fed a diet of trout pellets (NU-WAY, United Feeds, Calgary, Canada) and Romaine lettuce *ad libitum*. Adult snails with a vertical shell diameter of 10 - 20 mm were used, except in the case of single neuron isolation and culture for which only snails with a vertical shell diameter of 15 ± 1 mm were used.

Cell Culture. Procedures for isolation of identified single neurons have been previously described (Haydon et al., 1985). In order to isolate and culture the cerebral serotonergic neuron C1, some modifications were applied. Snails were deshelled carefully and placed in a 25 % Listerine / artificial pond water solution (0.025 % Insta Ocean, Aquarium Systems, Mentor, Ohio) for 15 minutes. Under aseptic conditions, the snails were rinsed twice with *Helisoma* saline (51.3 mM NaCl, 1.7 mM KCl, 4.1 mM $CaCl_2$, 1.5 mM $MgCl_2$, 5.0 mM Hepes; pH 7.35) and pinned onto a dissection Sylgard well containing *Helisoma* saline and 5 % gentamycin. Cerebral and buccal ganglia with intact cerebrobuccal connectives (CBCs) were isolated from the other ganglia and then placed in 0.2 % trypsin for 25 minutes, followed by 0.1 % trypsin inhibitor for 15 minutes. Following

trypsinization, ganglia were rinsed in defined medium [DM: 50 % Liebovitz-15 (Gibco,Burlington, ON) medium containing 40 mM NaCl, 1.7 mM KCl, 4.1 mM $CaCl_2$, 1.5 mM $MgCl_2$, 5 mM HEPES, 50 μg/ml gentamycin and 0.015 % L-glutamine (Sigma, St. Louis, MO), pH 7.45 ~ 7.5] for 1 hour. Ganglia were then pinned securely down with the dorsal surface exposed, and neuron C1 was identified by its size, location and coloration. After neuron C1 was located, the CBCs were crushed to sever the axon of neuron C1. An electrolytically sharpened tungsten microknife was used to apply a lateral slit on the connective sheath below neuron C1, without making contact with its cell body. The cell body was gently pushed out through the slit by applying pressure on the opposite side of the neuron. Once neuron C1 was exposed, the neuropil was gently scrambled using the tungsten microknife to detach the soma from its distal neurites. Neuron C1 was then withdrawn into a fire-polished, coated (Sigma-coat) glass micropipette (diameter 150 ~ 200 μm) which was attached to a micrometer syringe (Canlab) on a micromanipulator (Märzhäuser).

An isolated single neuron C1 was plated onto a 0.05 % poly-L-lysine coated (MW 70,000 ~ 150,000, Sigma) 35 mm plastic (Falcon 3001) or carbon-covered glass bottomed cell culture dish. Each dish contained 2 ml of conditioned medium (CM) prepared according to the previously described standard protocol (s-CM; Wong et al., 1981) or three modified protocols (m-CM1, m-CM2 and m-CM3). The s-CM was made by incubating two isolated *Helisoma* brains per ml of DM for 72 hours. To make m-CM, isolated brains were first rinsed for 24 hours in DM, and then incubated 3 times in fresh DM each for 96 hours at two brains per ml.

The medium collected after the 1st, 2nd and 3rd incubation period was called m-CM1, m-CM2 and m-CM3, respectively (Figure 6). The incubations were carried out at room temperature in a dark humidified culture chamber containing a disinfectant (Roccal) placed in a small plastic dish. During the incubation, various neurite outgrowth-conditioning factors are released from brains to the medium (Wong et al., 1981 & 1984; Ridgeway et al., 1991). To prevent released trophic factors from binding to the substratum, a glass tissue culture dish coated with Sigma-coat was used for brain-conditioning. The CM was filtered with a low protein-affinity filter (0.22 μ, Nalgene).

Morphological Determination of Each Phase of Neurite Outgrowth such as Initiation or Elongation. The ability of neurons to initiate neurite formation and onset of neurite elongation was assessed by a microscopic analysis of neuronal morphology. Under high resolution using a low-light time-lapse video microscopy system (Axiovert 135 & Q Imaging Retiga EXi), morphology of individual neuron C1s in culture was monitored for 60 minutes every 24 hour after plating with or without drug treatments. Neuron C1 initially formed lamellipodia surrounding its soma and projected numerous filopodia. Around 24 hours after plating onto a culture dish with CM, neuron C1 began to form short neuronal processes through lamellipodia rearranging assembled microtubules (Smith, 1994; For review of microtubule array in the lamellipodium, see Kalil et al., 2000) and generating a large microtubule initiation center (Spiegelman et al., 1979). Since these short initial neurites could elongate or withdraw depending on extrinsic factors,

neuritogenesis was limited up to formational steps of initial short neurites. Fortunately, neuritogenesis can be visualized by phase-contrast and differential interference contrast (DIC) microscopy (Spiegelman et al., 1979, Goldberg and Burmeister, 1989). Therefore, onset of neurite elongation was morphologically determined by observing a turning point from neuritogenesis to continuous neurite extension for 60 minutes (positive value μm/hr). Both phase contrast (20x or 40x) or DIC (40x or 100x) microscopy were complementally used to observe specific neuronal structures such as lamellipodia, filopodia, growth cones or neurites.

Neurite Elongation Measurements. Neurite elongation experiments were performed immediately after neurons were determined to be in the elongation phase of neurite outgrowth. High resolution phase-contrast images were taken with a low-light time-lapse video microscopy system (Axiovert 135 & Q Imaging Retiga EXi). Elongating neurites of individual neuron C1s in culture were monitored for 2 hours. The first 1-hour measurements were used to identify baseline elongation rates of neurites before drug application. Concentrated drug solutions were applied directly into 2 ml of cell-bathing CM around two opposing edges of the culture dish yielding a final desired concentration. Using image processing software (Adobe Photoshop 7.0 & Norther Eclipse), the amount of neurite elongation was measured for each individual neurite from the images taken every 20 minutes. The leading edge of the lamellipodium was used to indicate the neurite terminus at each time point. Neurites that did not grow or

those that intersected other neurites were not included in the analysis. Carbon particles on the glass bottom or scratches on the plastic culture dish were used as reference points to accurately measure the distance of elongation between each 20-minute interval. Outgrowth rates were expressed in micrometer per hour (μm/hr).

Morphological Determination of Neuronal Cell Death. Both phase contrast (40x objective) and DIC (40x and 100x objectives) microscopy (Axiovert 135) were used to detect morphologically neuronal cell death 24 hours after cell-plating with 5-HT treatments. Cell-swelling, size of nucleus, membrane integrity and cellular granulation were used as determinative criteria for cell death. Viabilities of all tested neurons for cell death were confirmed using 12-minute trypan blue staining (0.1 mg/ml in *Helisoma* CM) immediately after morphological investigations (Choi et al., 2005; Uliasz & Hewett, 2000; Liu et al., 1999; Bursztajn et al., 1998; Rosa et al., 1997; Abeta, 1995).

Preparation of Antibodies to 5-HT$_{1hel}$ and 5-HT$_{7hel}$ Receptors. Antibodies to the 5-HT receptors 5-HT$_{1hel}$ (503 amino acid long) and 5-HT$_{7hel}$ (479 amino acid long) cloned from *Helisoma* were raised against specific peptides derived from intracellular loop sequences which showed a high antigenicity (Parries et al., 2003). Peptide 5-HT$_{1hel}$ (residues 409 – 423, YSRTREKLELKRERK) and Peptide 5-HT$_{7hel}$ (residues 246 – 261, YFKIWRVSSKIAKAEA) were prepared by Washington Biotechnology (Baltimore, MD). Synthesized peptides were coupled

to keyhole limpet hemocyanin (KLH), and used to immunize rabbits. Individual sera were collected when antibodies gave a positive reaction against those peptide antigens at a titer of >100,000 in an Enzyme-Linked Immunosorbent Assay (ELISA).

Immunochemistry for Serotonin and its Receptors in Neuron C1. Phenotypic expression of serotonergic neuron C1 was demonstrated in cerebral ganglia and single neuronal cultures. Wholemount 5-HT immunofluorescence made it possible to localize all the serotonin-immunoreactive neurons including neuron C1 in the cerebral ganglia. The wholemount immunofluorescence protocol of Goldberg and Kater (1989) was fundamentally employed. Isolated cerebral ganglia were immersed in *Helisoma* Ringers saline (51.3 mM NaCl, 1.7 mM KCl, 4.1 mM $CaCl_2$, 1.5 mM $MgCl_2$ and 5 mM HEPES, pH 7.3) and pinned to silicon rubber-coated (General Electronic, RTV627) culture dishes. A prefixation treatment of 0.05 % pronase (Sigma) in the saline was performed for 15 ~ 30 minutes and then ganglia were fixed in cold Zamboni's fixative [4 % paraformaldehyde and 7.5 % picric acid in phosphate-buffered saline (PBS), pH 7.3 ~ 7.5] for 12 hours, rinsed 4 times in PBS for 20 minutes, and then rinsed in tris-buffered saline (TBS) for 60 minutes. A postfixation treatment of 0.2 % trypsin (Sigma) was performed for 30 ~ 60 minutes. Both enzymatic treatments were administered to digest the connective tissue sheath that envelops the cerebral ganglia. These treatments facilitated tissue penetration without compromising serotonin-like immunoreactivity (Croll and Lo, 1986; Goldberg and Kater, 1989).

The primary antibody (rabbit anti-serotonin, Sigma) was diluted 1:500 in 0.01 M PBS containing 1 % horse serum and 0.4 % Triton X-100 for 72 hour incubation, and then rinsed 3 times in 0.01 M PBS. The secondary antibody, either fluorescein isothiocyanate (FITC)-conjugated goat anti-rabbit IgG (Sigma) or tetramethyl rhodamine isothiocyanate (TRITC)-conjugated goat anti-rabbit IgG (Sigma) was diluted 1:400 in 0.01 M PBS containing 1 % horse serum and 0.4 % Triton X-100 for 2 hour incubation. After all incubations, the wholemount preparations were then rinsed 3 times in 0.4 % Triton X-100 in PBS for 20 minutes. The wholemount preparations were left at room temperature throughout all procedures except the fixation and the primary antibody incubation which were carried out at 4°C. Preparations were dehydrated, cleared and mounted in methyl salicylate (Sigma) and viewed under epifluorescence or confocal microscopy (Molecular Dynamics Multiprobe 20001 laser scanning system with Nikon Diaphot TMD microscope or Leica TCS-SP2 Multiphoton laser scanning system).

5-HT immunocytochemistry for the single-cultured neuron C1 was used to verify consistent phenotypic expression in cultured cells. After cultured neuron C1s displayed neurite outgrowth, immunocytochemistry was performed using a modification of the wholemount protocol of Goldberg and Kater (1989). Cultured cells were fixed in 4 % paraformaldehyde in 0.01 M PBS (pH 7.5) for 10 minutes and rinsed three times in 0.01 M PBS. After rinsing, 10 % horse or goat serum in 0.4 % Triton X-100 was applied for 30 minutes. Individual primary antibodies, either rabbit anti-serotonin (Sigma), rabbit anti-5-HT_{1hel} or rabbit 5-HT_{7hel} receptor were diluted 1:1000 in 0.01 M PBS containing 1 % horse serum and 0.4 % Triton

X-100 for 72 hour incubation, and the secondary antibody, either FITC-conjugated goat anti-rabbit IgG (Sigma) or TRITC-conjugated goat anti-rabbit IgG (Sigma) was diluted 1:500 in 0.01 M PBS containing 1 % horse serum and 0.4 % Triton X-100 for 2 hour incubation. After three brief rinses in 0.01 M PBS, preparations were dehydrated, cleared and mounted in methyl salicylate (Sigma) and viewed under epifluorescence or confocal microscopy (Molecular Dynamics Multiprobe 20001 laser scanning system with Nikon Diaphot TMD microscope or Leica TCS-SP2 Multiphoton laser scanning system).

For both wholemount and culture preparations, control experiments were also carried out where the primary antibody was excluded. Additional control experiments for the 5-HT receptor antibody experiments were carried out where the pre-immune serum from the same rabbit as the one used to generate the primary antibody was used instead of the primary antibody. In addition, a preabsorption control was carried out to take an account for non-specific KLH binding because KLH was used as a carrier to generate the 5-HT receptor antiserum. All these protocols did not affect the results of immunostaining detected for either 5-HT_{1hel} or 5-HT_{7hel} receptor.

Labeling for Cytoskeletal Molecules in Cultured Neuron C1. Since tubulin and actin labeling procedures require efficient extraction of cytosolic fraction and permeabilization, saponin would be a good candidate detergent rather than TX-100 (Lewis and Bridgman, 1992). Thus, the labeling procedure was modified based on the cytoskeleton-immunofluorescence protocol of Suter and Forscher

(2001). Cultured cells were initially fixed in 4 % paraformaldehyde in 0.01 M PBS (pH 7.5) for 10 minutes, which was supplemented with 400 mM sucrose. Additional fixation for 10 minutes was performed in 4 % paraformaldehyde plus 0.1 % saponin in 0.01 M PBS. After three rinses in 0.01 M PBS plus 0.01 % saponin for 10 minutes, preparations were incubated with Phalloidin-TRITC (1:500) (Sigma) for 3 hours. Subsequently, three rinses in 0.01 M PBS plus 0.01 % saponin were carried out for 10 minutes, and 3 % bovine serum albumin (BSA) in 0.01 M PBS was applied for 30 minutes to block non-specific binding. Primary mouse anti-tubulin (Sigma) was diluted 1:1000 in 0.01 M PBS containing 1 % horse serum and 0.01 % saponin plus 3.3 mg/ml BSA for overnight incubation. After three brief rinses for 10 minutes, the secondary FITC-conjugated anti-mouse (Sigma) was diluted 1:500 in 0.01 M PBS containing 1 % horse serum and 0.01 % saponin for 2-hour incubation. Preparations were rinsed, dehydrated, cleared and mounted in methyl salicylate (Sigma) and viewed under epifluorescence or confocal microscopy (Leica TCS-SP2 Multiphoton laser scanning system).

HPLC Analysis for Biogenic Monoamines. Chemical separations can be accomplished using high-performance liquid chromatography (HPLC) by using the fact that certain compounds have different migration rates given a particular column and mobile phase. To measure serotonin content in CM, HPLC with electrochemical detection (HPLC-ED) was employed. The mobile phase consisted of 920 ml double-distilled water containing 6.6 g NaH_2PO_4 (0.06 M),

197.2 mg SOS, 137.7 mg EDTA and 2 mM NaCl. Acetonitrile (80 ml) was added to the mobile phase and the pH was adjusted to 2.9 with *o*-phosphoric acid before filtering (0.25 μm) and degassing. After whole brains were removed from the CM at the end of the incubation period, 20 μl 4N $HClO_4$ containing 250 mg EDTA and 22 mg ascorbic acid was added to the CM (180 μl). The CM sample was centrifuged at 6,500 g (about 10,000 rpm) for 4 minutes at 4 °C. An aliquot (20 μl) of the supernatant was injected into the columns (Waters uBondapak Symmetry Guard C18 and Phenomenex Spherisorb ODS2, 250 × 3.2 mm, 5 μm, set at 30 °C) using an automated injection system. The flow-rate was 300 μl/min and running time was around 50 minutes per sample. The pumping and sample management was performed by a Waters Alliance 2690 XE system. A Waters 2465 electrochemical detector with the applied potential set at 0.60 V was employed. For each run of samples, various concentrations of serotonin in perchloric acid were also run as standard solutions to calculate serotonin content.

Immunoblotting for Tryptophan Hydroxylase. Whole snail brains were removed and incubated for 24 hours under the same conditions as those used for CM preparation. At the end of each incubation period, brains were rinsed with ice-cold TBS containing 1 mM sodium orthovanadate and 10 mM sodium fluoride. Brains were then mulched using dissection micro-scissors and homogenized with a Dounce mini-homogenizer in 100 μl of modified Laemmlli sample buffer (40 mM Tris–HCl pH 6.8, 1 % sodium dodecyl sulfate (SDS), 4 % 2-mercaptoethanol, 10 % glycerol and 0.002 % bromophenol blue). The samples were clarified by

microcentrifugation for 5 minutes, and the supernatant was boiled for 2 minutes and then clarified again for 10 minutes of high-speed microcentrifugation. Protein quantifications were performed through a Bradford dye-binding assay (1976). Samples were loaded and separated by SDS-PAGE on 8 % polyacrylamide gels containing 0.1 % SDS. Electrophoretically separated proteins were transferred overnight at 4°C to PVDF membranes (2 μm) in 25 mM Tris buffer (pH 8.3) plus 192 mM glycine and 16 % methanol containing 0.1 % SDS using a Trans-Blot cell apparatus (Bio-Rad, USA). Membranes were blocked for 1 hr in TBS plus 0.1 % Tween-20 (TTBS) containing 5 % non-fat milk and incubated overnight in the primary antibody solution prepared in TTBS containing 5 % bovine serum albumin. A monoclonal antibody for tryptophan hydroxylase (1:1000) from mouse (Sigma-Aldrich, Oakville, ON, Canada) was used. Membranes were washed three times with TTBS and then incubated for 1 hour with a peroxidase-conjugated secondary antibody (Sigma) for mouse monoclonal IgG (1:2000) in blocking buffer at room temperature with gentle agitation. Immunoreactivity was detected by a Immun-Star HRP Enhanced Chemo-Luminescence (ECL) Western blot kit (Bio-Rad, USA). To quantify a specific immunoblotted band, membranes were scanned by Odyssey infrared imaging system (LI-COR Inc., Lincoln, Nebraska, USA) or ChemiDoc imaging system (Bio-Rad, USA). Equal loading and transferring was checked on gels or membranes by applying densitometry for Ponceau S (0.2 % in dH_2O) stain over total bands. Membranes incubated in blocking buffer without the primary monoclonal anti-tryptophan hydroxylase were considered as controls. These membranes did not present any labeling.

Ratiometric Calcium Imaging on Neuritic Growth Cones. A fura-2 AM cell-loading suspension was prepared by dissolving 50 μg of fura-2 AM (Molecular Probe F-1221) in 5 μl of DMSO containing 20 % pluronic F-127 (suspension concentration; 10 μg fura-2 AM / μl). Pluronic is an anti-foaming agent blocking polymerization and increases cell permeability. In order to get a fine enough suspension for adequate cell loading, the preparation was sonicated for 20 minutes and then centrifuged for 10 minutes. The fura-2 AM loading suspension was administered directly into the cultures to a final concentration of 10 μM fura-2 AM. Individual cultures were loaded for 45 minutes, washed briefly, and imaged without delay. A 45-minute incubation at room temperature was sufficient for effective loading of fura-2 AM into cells, including neurite growth cones. Membrane-permeable acetoxymethyl esters of the dye are hydrolysed by non-specific esterases present in most animal cells, producing a hydrophilic free acid which is trapped and concentrated inside the cells. The fura-loaded cultured neurons were imaged with a differential interference contrast (DIC) 100 × oil-immersion objective (Fluor, N.A. 1.3) on an inverted microscope (Zeiss, Axiovert 135) with sequential excitation at 340 nm and 380 nm from a Hg-Xe arc lamp (Hammatsu, Japan) and Lambda 10-3 microprocessor-controlled multi-filter wheel system (Sutter). Emission fluorescence at 510 nm was collected using a Retiga Ex digital CCD camera (Q-Imaging) fire-wired to a Pentium IV PC computer with Northern Eclipse imaging software (Empix Imaging Inc.). The Northern Eclipse Ca^{++} imaging application IonWaveN was used to capture and

analyze produced images. Calcium imaging data was presented as the 340/380 ratio of fluorescence (F ratio), which provides a relative measure of cytoplasmic free calcium concentration (Grynkiewicz et al., 1985). Vehicle *Helisoma* saline or 5-HT solutions (10 μM) were applied into the culture dish using a gravity-driven perfusion system at an approximate rate of 1 ml/min (Warner).

Statistical Data Analysis. Results were presented as mean ± standard error of the mean (S.E.M.) unless otherwise stated. Statistical significance caused by individual treatments was determined by parametric (analysis of variance, ANOVA) or non-parametric (Kruskal-Wallis analysis for median comparison) tests followed by multiple comparisons. Further, non-parametric Fisher's exact test was applied for the percentage of elongating neurons.

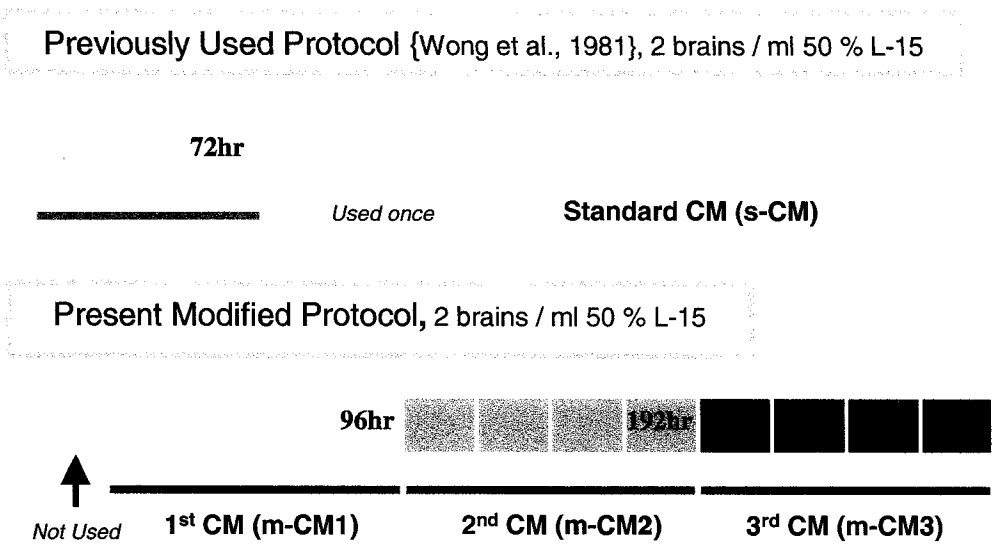


Figure 6. Standard and modified protocols to prepare conditioned medium (CM). Standard CM (s-CM) was prepared by incubating 2 isolated *Helisoma* brains per ml of DM for 72 hours. To make modified CM (m-CM), isolated brains were rinsed for 24 hours in DM, and then incubated in fresh DM for 96 hours 3 times at 2 brains per ml. The medium collected after the 1st, 2nd and 3rd incubation period was called m-CM1, m-CM2 and m-CM3, respectively. Each box of both diagramed protocols indicates 24 hour incubation.

RESULTS

Neurotransmitters are widely known to regulate growth cone motility and neurite extension, and to act as axon guidance cues (Zheng et al., 1996; Tessier-Lavigne, 1994; Kater and Mills, 1991; Haydon et al., 1987). 5-HT is the first-known diffusible agent that plays an important role in neurite outgrowth (Haydon et al., 1984). Neurite outgrowth consists of four essential phases; initiation, elongation, guidance and cessation (Haydon et al., 1987). Morphological changes based on those phases are likely influenced by 5-HT, which may induce intracellular signaling cascades to reorganize cytoskeletal molecules (Zhou and Cohan, 2001; Torreano et al., 2005). Here were tested two hypothesized regulatory roles of 5-HT on neurite outgrowth of serotonergic neuron C1; tonic inhibition of the onset of neurite elongation and differential regulation of neurite elongation in populations of neurites elongating at different rates.

1) Tonic inhibitory role of 5-HT on the onset of neurite elongation in the identified serotonergic neuron C1

Morphological determination of the initiation and elongation stages of neurite outgrowth in neuron C1

Neurite outgrowth is a dynamic, morphological process that is modulated by 5-HT (Haydon et al., 1987). To distinguish between the various stages of neurite

outgrowth, the cell morphology of neuron C1 was characterized over 24 hours in culture using a low-light, time-lapse video microscope. Approximately 10 hours after attaching to the substratum, neuron C1 formed a veil-like lamellipodium surrounding its soma that was irregular in shape, and often projected numerous filopodia. This lamellipodium underwent continual changes in shape over several hours before neuritogenesis (Figure 7. A-H). Under phase contrast microscopy, neurite formation, or neuritogenesis, was first recognized by the formation of a microtubule initiation center (Figure 7. I & J), which is the phase dark structure thought to arise from a cytoskeletal rearrangement of microtubules into a concentrated parallel cluster (Spiegelman et al., 1979; Summers and Kirschner, 1979; Smith, 1994; Zhou and Cohan, 2001; Silva and Dotti, 2002; Torreano et al., 2005). About 24 hours after plating the isolated neuron C1 onto a poly-lysine coated glass-bottomed culture dish, short neuronal processes, named initial neurites, emerged from the lamellipodia (Figure 7. I & J). The initiation phase of neurite outgrowth includes all of the above neuritogenetic processes. In the presence of modified conditioned medium (m-CM), newly formed initial neurites underwent continuous elongation and had growth cones at the tips of neurite shafts (Figure 7. K-O). Thus, the onset of neurite elongation was determined 24 hours after plating neuron C1 onto a substratum-coated dish by taking a measurement of the neurite length over a 60-minute time period (Haydon et al., 1984). Neurites with any positive value of neurite length during the 60-minute time period were considered to be in an elongation phase. After development of a huge growth cone, neurite branching occurred (Figure 7. O). In contrast, neuron

C1s grown in the presence of standard conditioned medium (s-CM), formed small transient neurites following neuritogenesis, rather than continuously elongating neurites. Those transient neurites did not have a positive value of total elongating length for 60 minutes, but retreated, dispersing the central domain of their growth cones and shafts (Figure 8). Neurons in this case were not considered to be in the elongation stage of neurite outgrowth.

Effect of medium-containing protocol on probability and onset of neurite elongation

s-CM was prepared according to the standard protocol that was previously developed in order to culture neurons isolated from ganglia of *Helisoma* (Wong et al., 1981). However, we cannot rule out the possibility that the s-CM may contain a tonic inhibitory factor that interferes with neurite outgrowth. Thus, it is important to determine whether s-CM is optimal for neurite outgrowth of neuron C1 or whether a modified conditioning procedure is warranted.

To investigate the effects of conditioned media on elongation probability and onset of neurite elongation, I examined the morphological characteristics of neuron C1s cultured in either s-CM or a m-CM (see Figure 7. for morphological criteria of neurite initiation and elongation). Neuron C1s cultured in s-CM initiated neurite outgrowth and immediately formed lamellipodia and filopodia. Neuritogenesis was not always observed and in a few cases, only transient neurites were produced. Neurite elongation occurred in only 58 % (n=50) of

neuron C1s cultured in the s-CM (Figure 9. A). In contrast, most of neuron C1s cultured in the m-CM1 (89 %, n=35) and the m-CM2 (92 %, n=39) exhibited neurite elongation. The number of neurons that exhibited neurite elongation when cultured in m-CM3 (73 %, n=37), was not significantly different from the number cultured in s-CM ($p = 0.12$). Moreover, the 24 hour CM discarded during the preparation procedure for the m-CM1 induced only 6 % of neuron C1s (n=32) to elongate their neurites. This value was also significantly different from that of the s-CM. Non-parametric Fisher's exact tests were statistically applied to see significances in the elongation probability ($p < 0.05$).

On the other hand, inhibition of the onset of neurite elongation was observed in neuron C1s cultured in s-CM, whereas all three m-CMs resulted in spontaneous elongation of cultured neuron C1s immediately after neuritogenesis (Figure 9 B & C). m-CM2 was more effective in promoting the phase transition from neurite initiation to neurite elongation when compared with other culture media, with over 50% of neuron C1s (n=22 out of 39) entering the neurite elongation phase within the first 24 hour in culture (Figure 9. B). Since the onset of neurite elongation was monitored on a 24 hour schedule, I determined the median number of days in culture for neuron C1 to start neurite elongation in the various culture media and compared these median values to test statistical significance between the groups (Figure 9. C). There were significant differences in onset of elongation periods in all m-CM groups versus the s-CM group ($p < 0.05$, Kruskal-Wallis test with Dunnett's multiple comparison tests). For example,

neuron C1s in the s-CM required 5 days to start neurite elongation while neuron C1s in m-CM2 required only 1 day for the onset of neurite elongation.

Presence of 5-HT and dopamine in s-CM and m-CMs.

In comparison with m-CMs, the s-CM attenuated the elongation probability and inhibited the onset of neurite elongation of neuron C1 (see Figure 9). Therefore, these results led to the hypothesis that diffusible molecules that specifically inhibit neurite outgrowth of neuron C1 may be synthesized in snail brains, and are released into the first 24 hour conditioned medium. Excitatory neurotransmitters are usually diffusible trophic factors that inhibit neurite outgrowth at lower levels and affect cell survival at higher levels (Lipton and Kater, 1989). In several biochemical studies, very high levels (up to 40 μg / g) of 5-HT were detected in mollusc brains (Gerschenfeld, 1973; Gerschenfeld et al., 1978; Gadotti et al., 1986) and were reported to inhibit neurite outgrowth (Haydon et al., 1987). Thus, 5-HT is a possible neurotransmitter candidate that may produce the tonic inhibitory effect on neurite outgrowth in neuron C1.

To test the hypothesis that isolated snail brains could produce and release endogenous 5-HT into s-CM, the presence of 5-HT in the s-CM was analysed. High-performance liquid chromatography (HPLC) with electrochemical detection was used to test for the presence of monoamines in the various conditioned media. Chromatograms showed the presence of 5-HT (130 ng/brain) and dopamine in snail brains (data not shown here) and s-CM (72 hour incubation;

Figure 10. inset). After a 24 hour conditioning period, the 5-HT level was 2.4 ± 0.4 μM (mean ± SEM), after which it was attenuated to 1.8 ± 0.2 μM (mean ± SEM) and 0.8 ± 0.1 μM (mean ± SEM) at the 48 hour and 72 hour time points respectively. 5-HT was not found in the conditioned medium prepared after 96 hours of incubation (Figure 10). In addition, all conditioned media prepared on newly modified protocol showed the absence of 5-HT (data not shown). Taken together, relatively low concentrations of 5-HT were present in s-CM, but there was no detectable 5-HT in m-CMs.

Inhibitory effects of lower 5-HT levels on neurite outgrowth and neurotoxic effects of higher 5-HT levels on neuronal survival; Investigations for cellular morphological changes of neuron C1

In order to test whether 5-HT caused the tonic inhibitory effects on neurite outgrowth of neuron C1 in s-CM, the same concentration of 5-HT found in the conditioned medium was tested in neurite outgrowth assays. Moreover, since excitatory neurotransmitters may have different dose-dependent effects on neuronal morphology (Lipton and Kater, 1989), various doses of 5-HT were tested on neurite outgrowth.

To test whether 5-HT was responsible for the tonic inhibitory role in controlling a phase transition from neurite initiation to neurite elongation, four different concentrations of 5-HT (1 μM, 10 μM, 50 μM and 100 μM) were bath-applied into m-CM2 (control). Because the m-CM2 was found as the optimal

culture condition for neurite outgrowth of neuron C1 (Figure 9), bath-applications of exogenous 5-HT into the m-CM2 was principally used to test the effects of 5-HT concentration on neurite outgrowth. Morphological characteristics of 24 hour cultured neurons C1 (see Figure 7 & 8) were used to determine what phase of neurite outgrowth the cells were in after addition of each dose of 5-HT into the m-CM2.

Most control neuron C1s cultured in the m-CM2 (58 %, n=12) were already in the elongation phase of neurite outgrowth within 24 hours (see Figure 9. B). One morphological example of a 24-hour control neuron C1 with several elongating neurites and growth cones is shown in Figure 11. A. Neuron C1 with elongating neurites was immunostained using anti-tubulin for microtubules (Figure 11. B). Immunoreactivity for microtubules was stronger in neurites than in the soma. Addition of 1 μM 5-HT (n=11) affected the outgrowth state of neuron C1 after 24 hours of culture. This neuron C1 still remained in the initiation phase of neurite outgrowth making a veil-like lamellipodium with many filopodia on the site of neuritogenetic microtubule initiation center (Figure 11. C-F). Neuron C1s in the neurite initiation phase showed microtubule immunoreactivity all over the cell, but the soma and neuritogenetic initiation center had stronger immunoreactivity than the lamellipodium (Figure 11. C). 10 μM 5-HT (n=10) prevented neuron C1 cultures from developing a huge lamellipodium and induced many protruding filopodia within 24 hours after cell plating (Figure 11. G-I). There was no morphological sign of neurite initiation or elongation in the 24 hour cultured neuron C1s when treated with a high concentration of 5-HT (50 μM; n=9). At high

concentrations of 5-HT, some neurons remained alive based on morphological criteria, while others entered a stage of cell death rather than the initial phase of neurite outgrowth (Figure 11. J-L, Morphological cell death was confirmed through a trypan blue exclusion assay, Figure 12). 100 μM 5-HT (n=20) also caused neuronal cell death, which was determined by morphological criteria such as cell-swelling, the presence of a mega-nucleus and membrane blebbing (Figure 11. M-O). Since disintegration of membranes (blebbing-fried egg shape) and cell-swelling are common morphological features found in necrotic cells (Levin et al., 1999; Clarke, 1999; Jellinger, 2001), the higher concentrations of 5-HT probably induced necrosis of neuron C1.

In order to confirm these neurotoxic effects of 5-HT, cell viability was investigated with a trypan blue exclusion assay of neurons 24 hours after cell plating onto the 5-HT treated m-CM2 dishes (Figure 12). In trypan blue staining (0.1 mg / ml), dead cells appeared darker blue compared with clear living cells. The lower concentrations of 5-HT (1 and 10 μM) had no effect on neuronal cell death (n=11 & 12, relatively; *$p > 0.05$, Fisher's exact test) compared with the control m-CM2 group. However, 50 μM and 100 μM 5-HT caused cell death in 55.6 % (n=9) and 84.6 % (n=13) of neuron C1s respectively. These data confirm the neurotoxic effects of concentrated 5-HT.

Similar to the s-CM, 5-HT also lowered elongation probability and inhibited onset of neurite elongation of neuron C1 without immediate spontaneous elongation observed with the m-CM2.

Taken together, the above results suggest that s-CM inhibited neurite outgrowth responses. 5-HT is present in s-CM and exogenous 5-HT negatively affects the cellular morphological changes of neurite outgrowth. However, more studies were required to quantitatively support that 5-HT found in the s-CM can produce the inhibition of neurite outgrowth response. Thus, the same doses of 5-HT used before were tested here to quantify its effects on the onset of neurite outgrowth.

To examine a putative direct role of 5-HT found in the s-CM in affecting elongation probability and onset of neurite elongation, morphological investigations for each phase of neurite outgrowth were performed. Neuron C1s cultured in the m-CM2 were considered the control group to be compared with 5-HT treated neuron C1s in m-CM2. Controls (n=12) showed a 92 % elongation probability, which was not significantly different from that of the lower concentrations of exogenous 5-HT such as 1 μM (82 %, n=11) and 10 μM (70 %, n=10). However, the elongation probability of neuron C1s in higher concentrations of 5-HT such as 50 μM (33 %, n=9) and 100 μM (10 %, n=20) were significantly lower ($p < 0.05$, Fisher's exact tests) (Figure 13. A).

On the other hand, 58 % (n=7 out of 12) of control neuron C1s (m-CM2 group) exhibited an onset of neurite elongation within the first 24 hour, while only

about 10 % of neuron C1s started the elongation within the first 24 hour (Figure 13. B) in lower concentrations of 5-HT (1 μM, n=1 out of 11 & 10 μM, n=1 out of 10). Moreover, the onset of neurite elongation did not occur within the first 24 hours in higher doses of 5-HT. Comparisons of median days in culture required for neuron C1 to begin elongating showed a significantly later onset of neurite elongation in all 5-HT treated groups as compared with the controls ($p < 0.05$, Kruskal-Wallis test with Dunnett's multiple comparison tests) (Figure 13. C).

Effect of pCPA on 5-HT concentration in s-CM

The data described above suggest that the 5-HT has a tonic inhibitory effect on neurite outgrowth. Therefore, inhibition of endogenous 5-HT synthesis would be expected to lower 5-HT level in the s-CM and rescue neurite outgrowth. Tryptophan hydroxylase is the rate-limiting enzyme in the biosynthesis of 5-HT, and is contained only within those specific neurons, which synthesize, store, and release 5-HT (Knapp and Mandell, 1972). Treatments with either *para*-chlorophenylalanine (*p*CPA) or *para*-ethynylphenylalanine (*p*EPA) inactivated tryptophan hydroxylase in previous studies (Joh et al., 1975; Diefenbach et al., 1995; Stokes et al., 2000; Pflieger et al., 2002). Thus, *p*CPA is known as an irreversible competitive inhibitor of tryptophan hydroxylase.

To examine whether 5-HT levels in s-CM can be attenuated by inhibiting the rate-limiting enzyme, a specific irreversible tryptophan hydroxylase inhibitor, *p*CPA, was applied in two different ways; 0.02 % *p*CPA was added directly into

the defined medium with snail brains to prepare the s-CM or 0.1 mg *p*CPA was injected intramuscularly (IM) into the foot of snails 6 hours before ganglia were removed to condition media (see Diefenbach et al., 1995). Both treatments attenuated 5-HT levels during conditioning periods, although the direct bath application resulted in a more effective reduction of 5-HT within 24 ~ 48 hours compared with IM injection ($p < 0.05$, one-way ANOVA) (Figure 14).

Effects of pCPA on tryptophan hydroxylase expression in snail brains

Tryptophan hydroxylase assays in previous studies showed that *p*CPA inhibited the enzyme activity completely (Jequier et al., 1969; Ichiyama et al., 1970; Gal et al., 1970; Joh et al., 1975). After treatment of *p*CPA, a significant decrease in the number of tryptophan hydroxylase-immunoreactive cells was observed in the rat raphe nuclei (Raison et al., 1996). Based on densitometric measurements through immunoautoradiographs, tryptophan hydroxylase expression appears to be differentially regulated by *p*CPA depending on subpopulations of raphe serotonergic cells (Raison et al., 1996). However, there is no evidence that the amount of tryptophan hydroxylase in snail brains can be directly affected by the enzyme inhibitor, *p*CPA. If 5-HT biosynthesis in snail brains is rate-limited by the amount of tryptophan hydroxylase as well as its activity, the hypothesized *p*CPA-reduced amount of tryptophan hydroxylase may rescue the neuron C1 from the 5-HT-inhibited transition into elongation phase.

To test the effect of *p*CPA on tryptophan hydroxylase expression, the protein content of tryptophan hydroxylase in 24 hour-incubated snail brains was investigated by Western blot analysis (see Richard et al., 1990). The tryptophan hydroxylase content (55 kDa) in 24 hour-incubated snail brains was attenuated by both treatments (0.02 % bath-application or 0.1 mg IM injection, n=6) of *p*CPA (Figure 15. A). In contrast, 5,7-dihydroxytryptamine (5,7-DHT), a known 5-HT depleting agent, did not affect the tryptophan hydroxylase content in 24 hour-incubated snail brains, which is similar to controls ($n=6$, $p < 0.05$, one-way ANOVA with Tukey's multiple comparison tests) (Figure 15. A). Interestingly, tubulin expression was greatly increased in both *p*CPA (0.02 %) and 5,7-DHT (0.02 %) bath applications ($n=3$, $p < 0.05$, one-way ANOVA with Tukey's multiple comparison tests) (Figure 15. B), possibly relating to the neuritogenic effects of these treatments (see below). Thus, tubulin expression was not a good control in this experiment to monitor equal amounts of sample loading, and some other protein bands were considered to be loading controls.

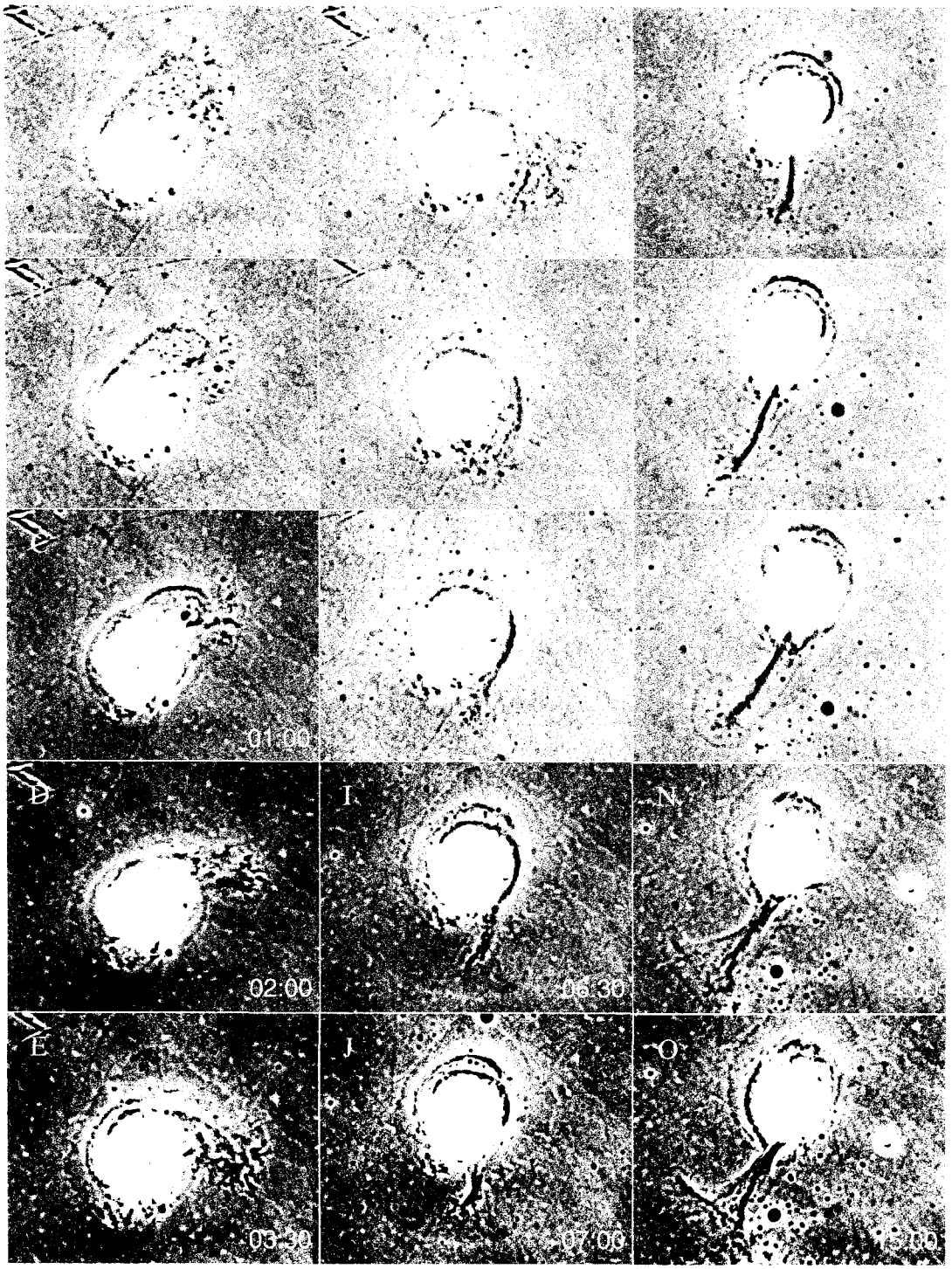
Effects of pCPA on the probability and onset of neurite elongation

Taken together, the experiment results suggest that *p*CPA reduces the 5-HT content in s-CM at least in part by down-regulating tryptophan hydroxylase levels in snail brains. To investigate whether the *p*CPA-lowered 5-HT level in s-CM can rescue neuron C1s from the inhibited neurite outgrowth responses, the elongation probability and onset of neurite elongation were measured with s-CM

prepared after *p*CPA treatments. Because *p*CPA has been known as a specific inhibitor of tryptophan hydroxylase, both bath-treated (0.02 %) and IM-injected (1 mg/ml) *p*CPA groups were initially expected to increase the probability of neurite elongation by reducing 5-HT level. However, both *p*CPA treatments did not significantly increase the probability of elongation (79 %, n=14 in the bath-treated group; 73 %, n=15 in the IM-injected group, $p > 0.05$, Fisher's exact tests), as compared with the s-CM control group (61 %, n=18 in the s-CM control) (Figure 16. A).

On the other hand, both *p*CPA treatments induced the onset of neurite elongation 1 ~ 2 days earlier than the onset of neurite elongation observed in s-CM (Figure 16. B). A significant difference in median days in culture required to initiate elongation ($p < 0.05$, Kruskal-Wallis test with Dunnett's multiple comparison tests) was observed between control and bath-treated *p*CPA groups, although the IM-injected *p*CPA group was not statistically different from controls (Figure 16. C). These data support that *p*CPA can rescue neuron C1s from the inhibited neurite outgrowth responses presumably by lowering 5-HT level in s-CM.

Figure 7. Morphological characteristics of the initiation and elongation stages of neurite outgrowth in neuron C1. At the beginning of neurite outgrowth, a round veil-like lamellipodium (A) was formed surrounding the soma. The cytoskeletal elements such as microtubules were shown darker around the soma (Spiegelman et al., 1979; Smith, 1994), which would be rearranged to form a neurite (A-J). No sooner than neuritogenesis was completed (J), neurite elongation started immediately under the optimum condition (K-O). Thus, the onset of elongation can be decided with at least 60 minute measurement of the neurite length from one specific point to the leading edge (Haydon et al., 1984) after neuritogenesis. Neurites with any positive value were considered as in elongation phase. Scale bar indicates 60 μm. The time-course of this experiment is displayed on each image (in hours:minutes).



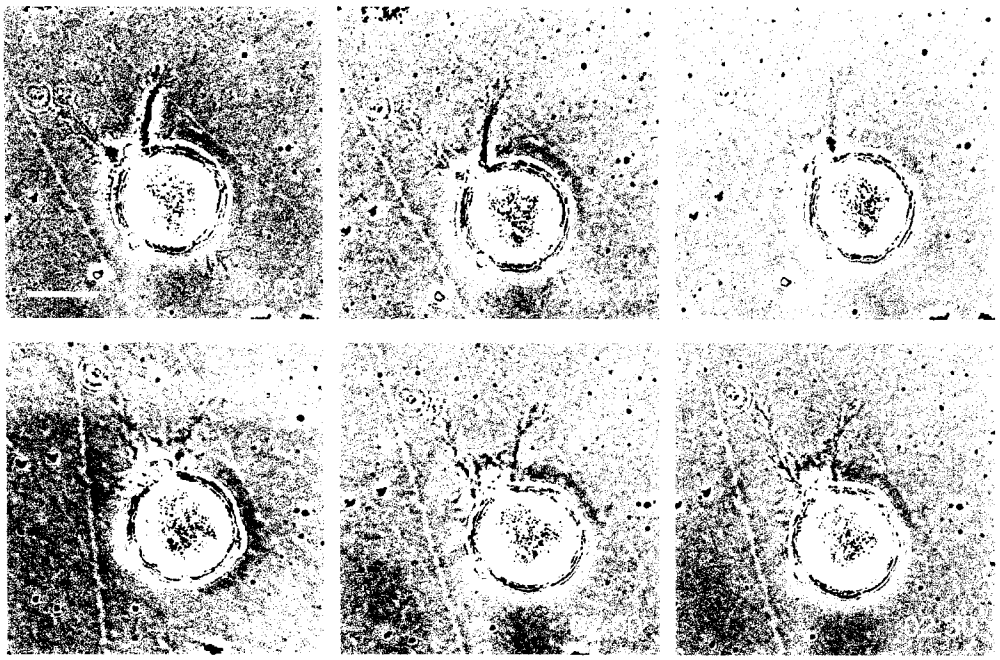
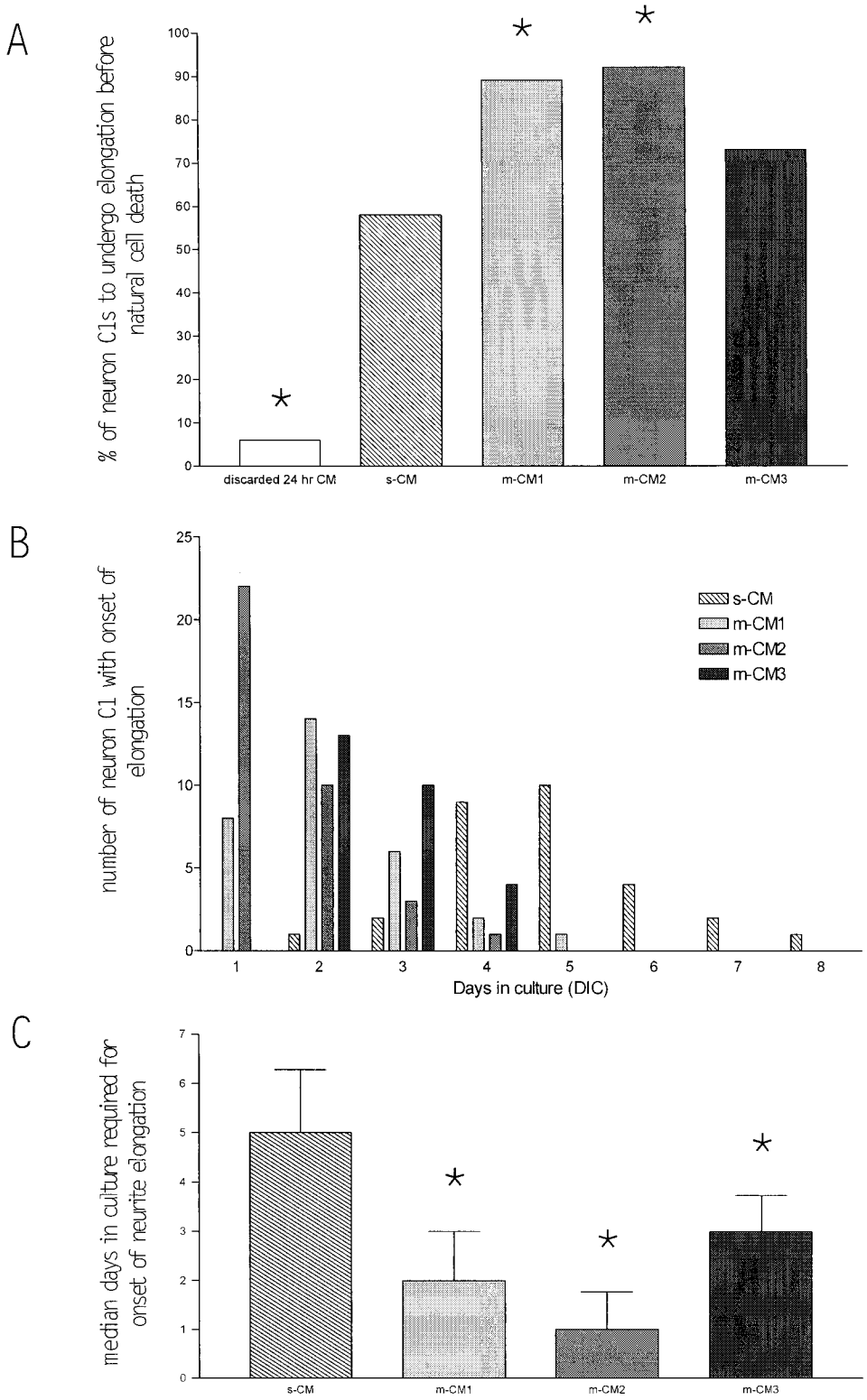


Figure 8. Absence of neurite elongation in neuron C1s plated in standard CM. As soon as neuritogenesis (A) occurred following cytoskeleton rearrangement, neurites detected their circumstances and retreated quickly (B-D). There was dispersion of the growth cone central domain and neurite shafts (E & F). Scale bar indicates 60 μm. The time-course of this experiment is displayed on each image (in hours:minutes). Phase contrast 40x, oil immersion.

Figure 9. Effects of m-CMs on elongation probability and onset of neurite elongation of neuron C1. A. The percentage of neuron C1s to undergo neurite elongation before natural cell death indicates the elongation probability under certain conditions. Two m-CMs such as m-CM1 and m-CM2 were more optimized media for neurite elongation than s-CM (*$p < 0.05$, Fisher's exact test) because more cells in m-CMs completed neurite elongation ultimately. B. Occasions of cultured neuron C1 with onset of neurite elongation were shown in frequency histogram. C. Median days in culture required for neurite elongation of neuron C1 was significantly reduced with the different m-CMs in comparison to the s-CM (*$p < 0.05$, Kruskal-Wallis test with Dunnett's multiple comparison tests). All three m-CMs appeared to recover the spontaneous neurite elongation right after neuritogenesis from the inhibition of neurite elongation with s-CM.



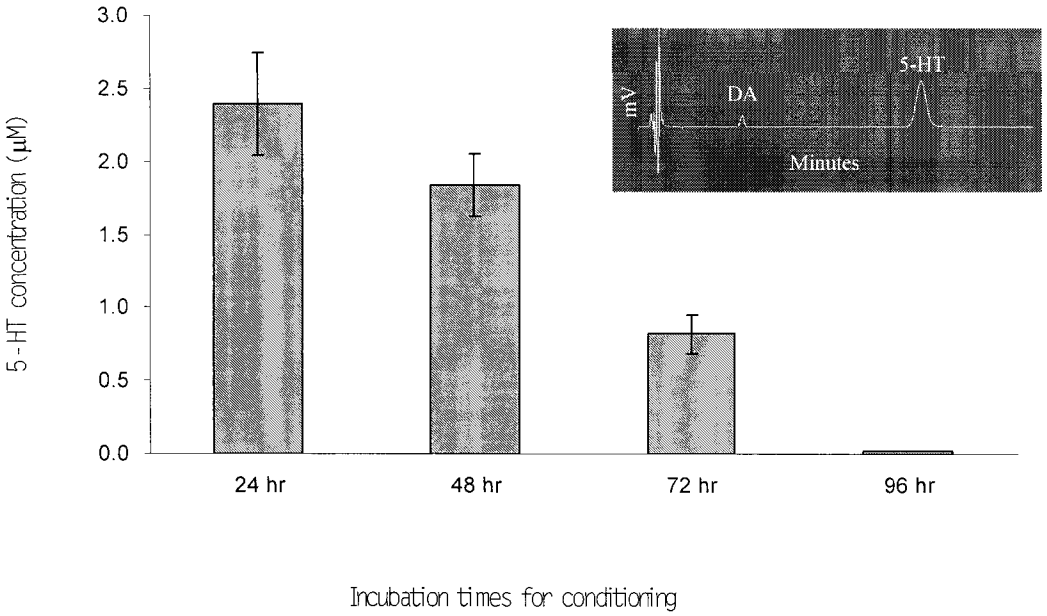
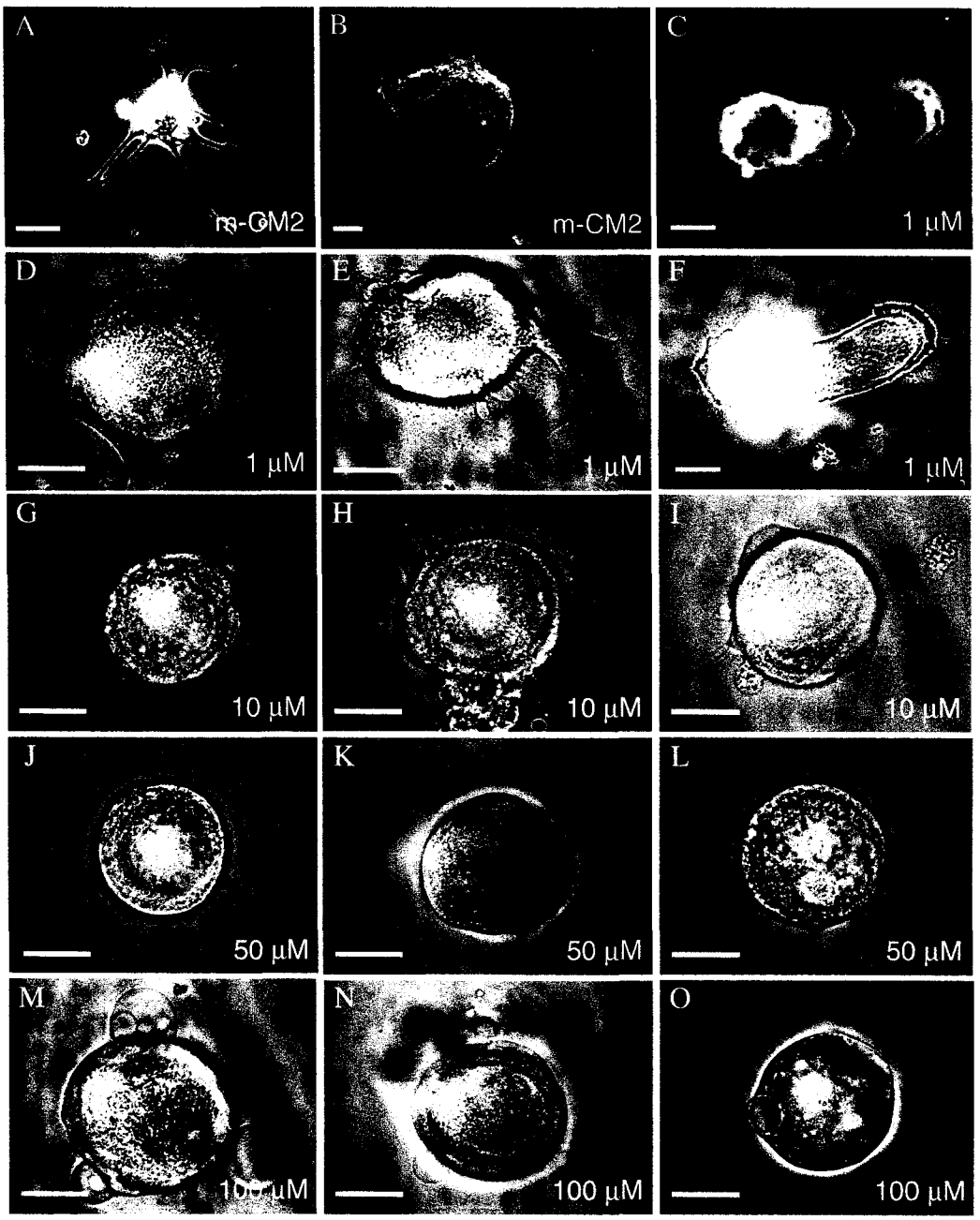


Figure 10. HPLC (high performance liquid chromatography) analyses for the presence of 5-HT in s-CM. Chromatograms showed the existence of monoamines such as 5-HT and dopamine (DA) in each brain-incubated medium (inset). 5-HT was detected during the first 24 hour (2.4 ± 0.35 μM; mean ± SEM). The level of the indoleamine neurotransmitter was reduced gradually to about 0.8 μM in the 72 hour conditioning period.

Figure 11. Effects of 5-HT on neurite outgrowing morphology. Morphological characteristics of 24 hour cultured neuron C1s were used to determine what phase of neurite outgrowth they were in after various concentrations of 5-HT treatments into m-CM2 (control). A. Most control neuron C1s (56 %) in m-CM2 elongated their neurites within 24 hours (see Figure 7. B). B. 3-D reconstructed confocal microscopic image for a control elongating neuron C1 showed the more rearranged microtubule into elongating neurites (green-tubulin; red-phalloidin) in comparison with relatively less immunostained tubulin in the cell body. C-F. 24 hour cultured neuron C1s with 1 μM 5-HT did not show neurite elongation, but stayed in the neurite initiation phase making a veil-like lamellipodium (C, the same cell in F, showed the immunoreactivity of tubulin in the 3-D reconstructed confocal microscopic image for the microtubule initiation center on the edge of lamellipodium). G-I. 10 μM 5-HT induced tiny lamellipodia with many protruding filopodia within 24 hours after cell plating. J-L. There was no morphological sign for neurite initiation or elongation since treated with 50 μM 5-HT. Many of the neurons appeared to be unhealthy or dead. M-O. Higher concentration (100 μM) of 5-HT revealed manifest cytotoxic effects on cultured neuron C1s causing cell-swelling, mega-nucleus and membrane blebbing (fried-egg shape). Disintegration of membranes (blebbing) and cell-swelling are distinct characteristics in necrotic cell death. Overall morphologically characterizing, lower concentrations of 5-HT delayed onset of neurite elongation showing tonic inhibitory effects while higher concentrations of 5-HT induced cytotoxic effects. All scale bars indicate 40 μm.



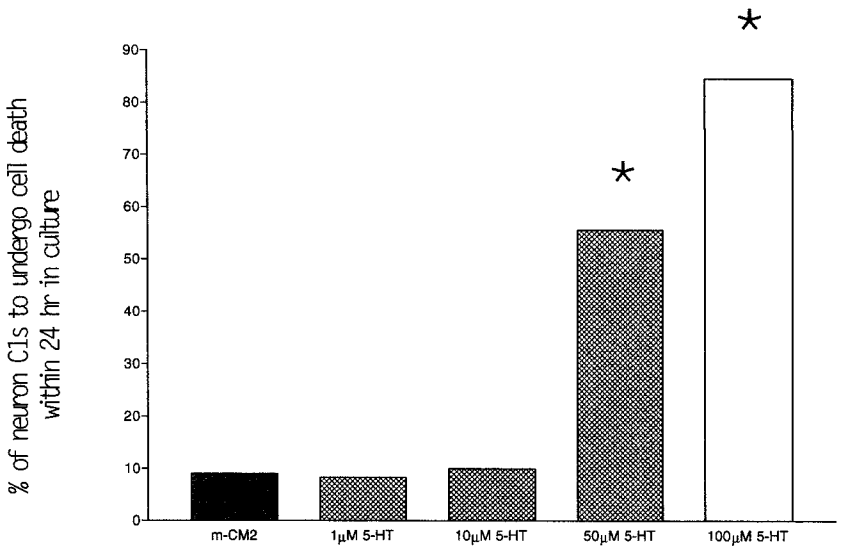
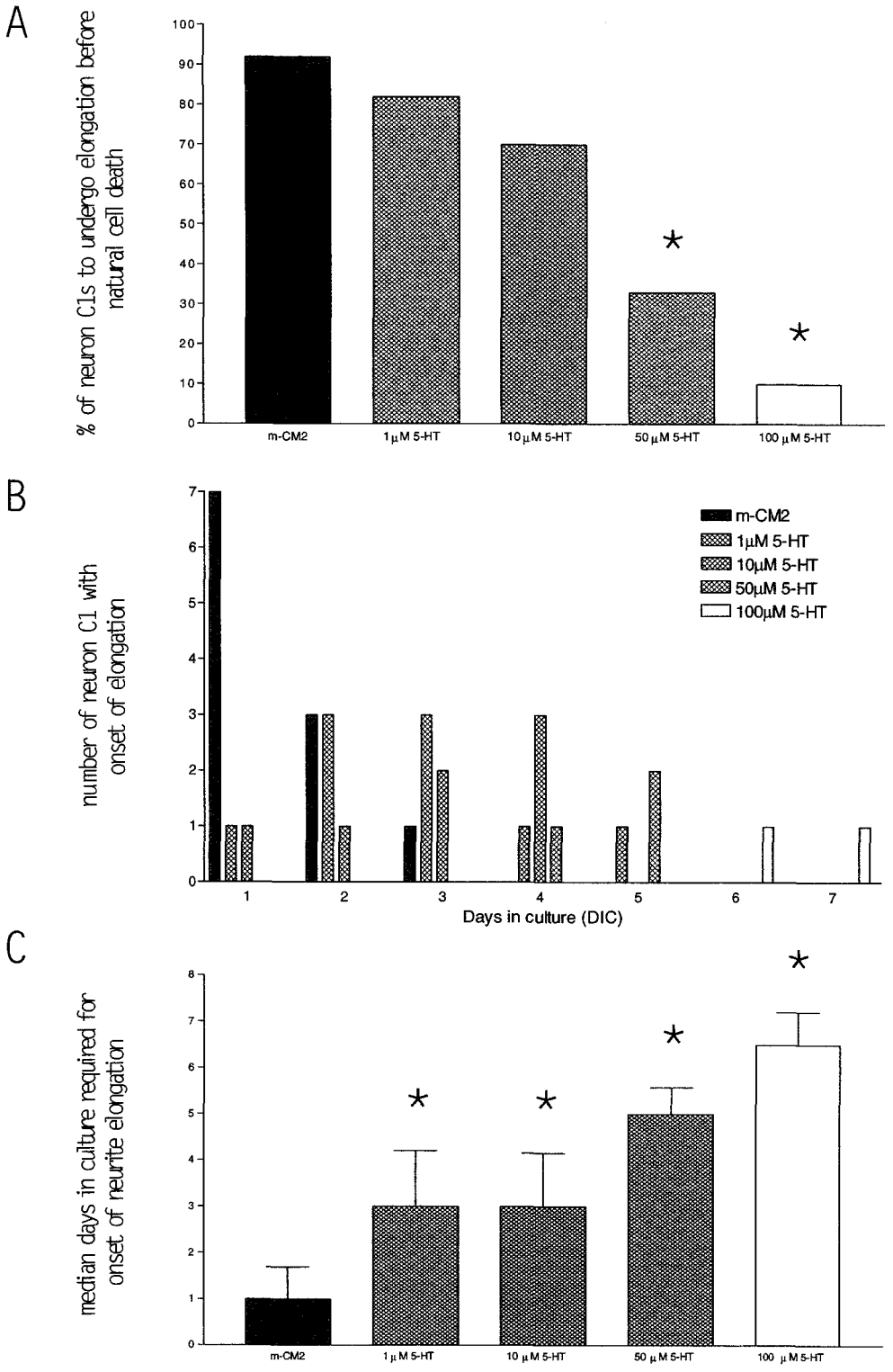


Figure 12. Cell death 24 hours after 5-HT treatments. The lower concentrations of 5-HT (1 and 10 μM) had no effects on neuronal cell death (n=11 & 12, respectively) compared to the control m-CM2 group. However, the higher concentrations of 5-HT (50 and 100 μM) caused significant cell deaths, compared to m-CM2 (n=9 & 13, respectively; *$p < 0.05$, Fisher's exact test) as observed in the morphological tests (see Figure 10). Viabilities of all neurons were confirmed with trypan blue staining (0.1 mg / ml).

Figure 13. Effects of 5-HT on elongation probability and onset of neurite elongation of neuron C1. A. The percentage of neuron C1s to undergo elongation before natural cell death indicates elongation probability under certain conditions. The higher doses of 5-HT (50 and 100 μM) reduced the elongation probability while the lower concentrations of 5-HT (1 and 10 μM) did not affect significantly (*$p < 0.05$, Fisher's exact test). B. Occasions of cultured neuron C1 with onset of neurite elongation were shown in frequency histogram. Trend comparisons to the control group (m-CM2) shows longer lag phase for neurite elongation observed in the 5-HT treated groups. C. Median days in culture required for neurite elongation of neuron C1 was significantly longer with all 5-HT treated m-CM2 groups in comparison to the control m-CM2 (*$p < 0.05$, Kruskal-Wallis test with Dunnett's multiple comparison tests). Neuron C1 under the optimum culture condition (m-CM2) for neurite outgrowth displayed its own earlier neurite elongation than under various concentrations of 5-HT added into the same m-CM2. Thus, 5-HT significantly inhibited the phase transition from initiation to the onset of elongation in a dose-dependent manner.



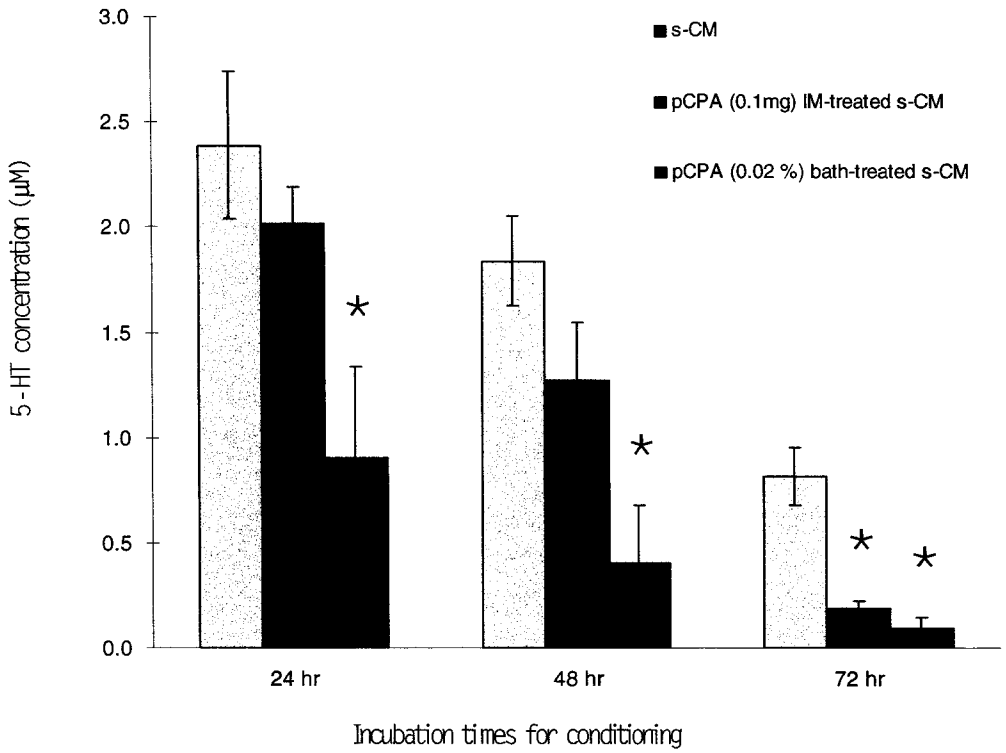


Figure 14. Effects of *p*CPA on the level of 5-HT in s-CM during the medium-conditioning periods. HPLC analysis was performed to detect 5-HT levels in each time-period CM. Treatments of *p*CPA (0.02 % directly bath-treated at the initial conditioning period or 0.1 mg intramuscular-injected into snails about 6 hours before starting to condition CM) reduced 5-HT levels during conditioning periods (*$p < 0.05$, one-way ANOVA).

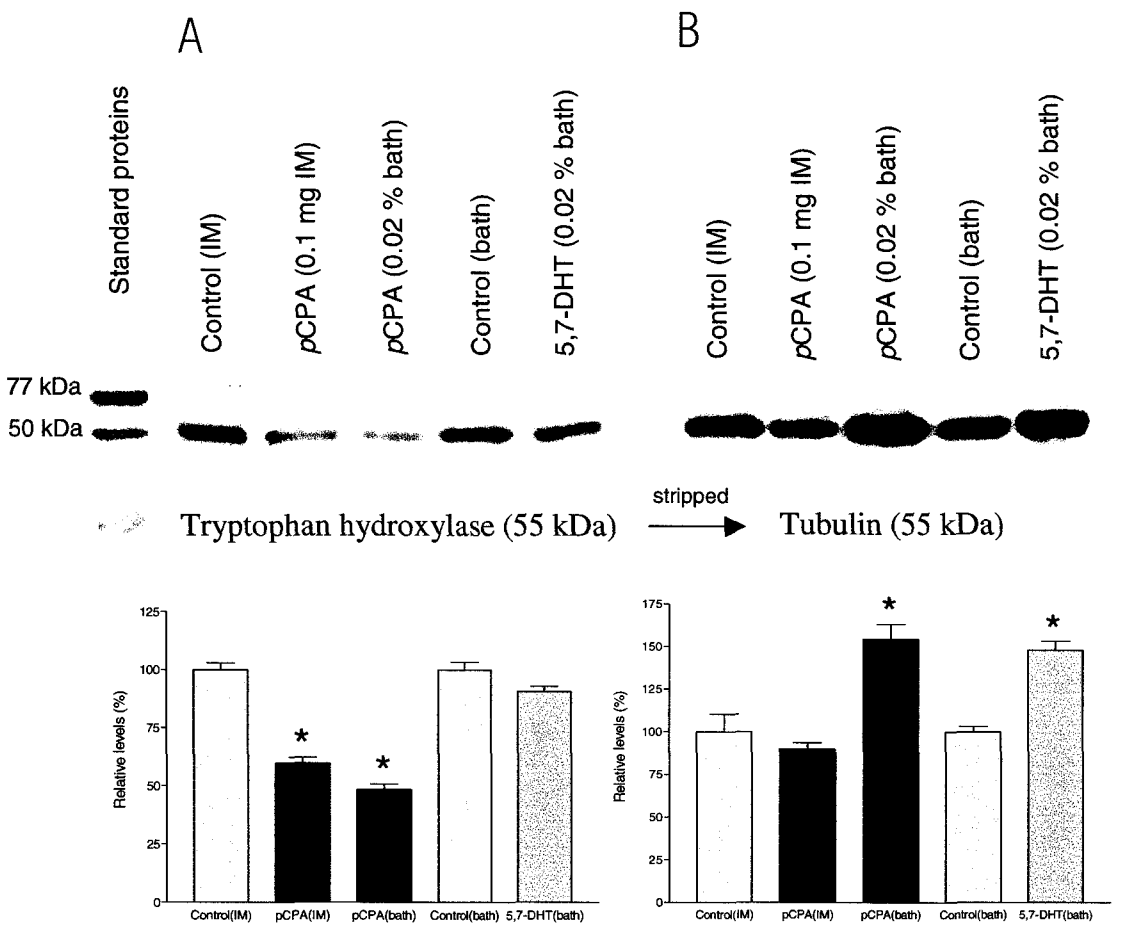
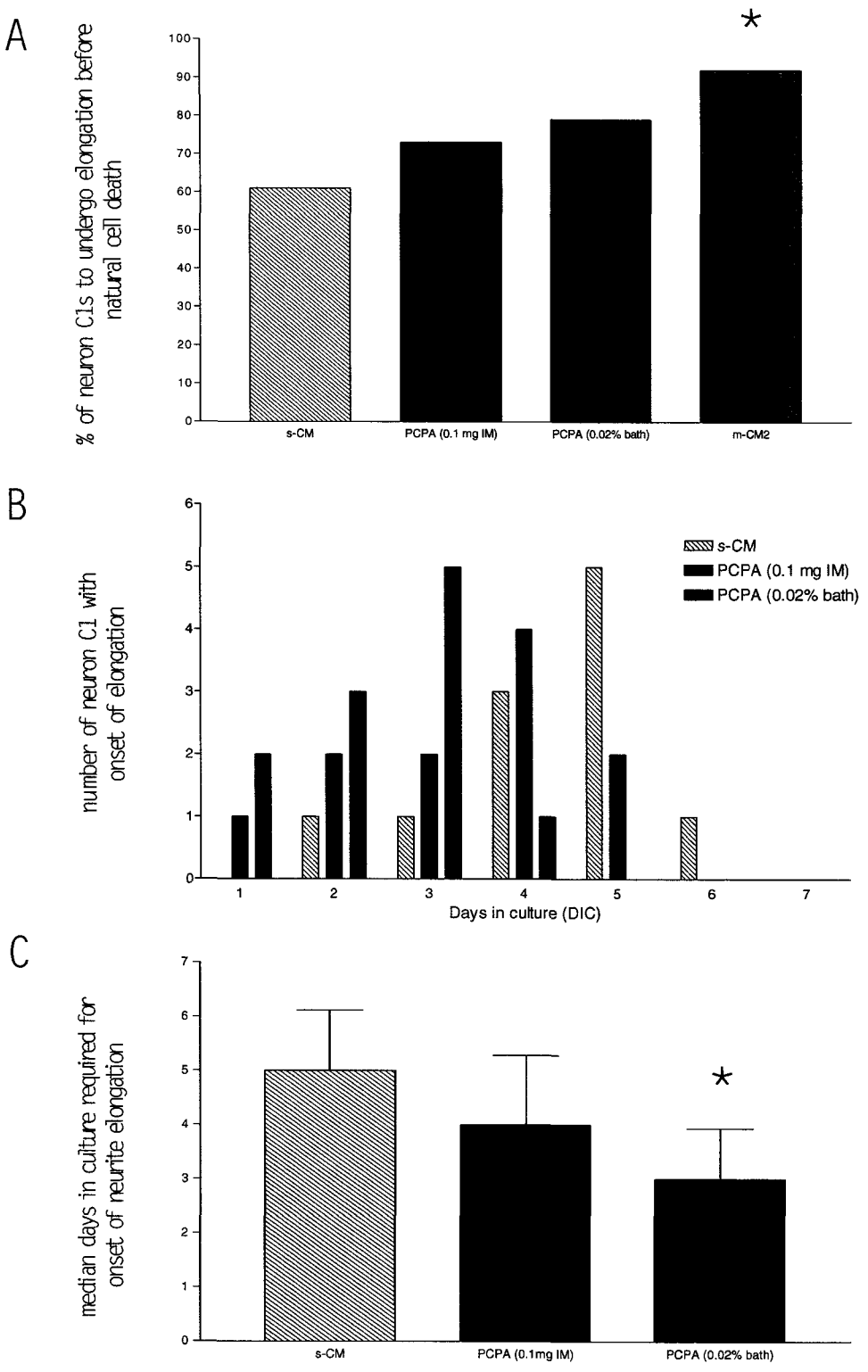


Figure 15. Western immunoblotting analysis (horse radish peroxidase chemiluminescence detection on X-ray film) for tryptophan hydroxylase content in snail brains incubated in the defined medium for 24 hours. A. Both treatments of *p*CPA (0.02 % directly bath-treated or 0.1 mg intramuscular-injected) attenuated the enzyme expression, but bath application of 5,7-DHT (0.02 %) similar to controls did not show the attenuation on tryptophan hydroxylase content (n=6, *$p < 0.05$, one-way ANOVA with Tukey's multiple comparison tests). B. Total expression of tubulin after stripping-out anti-tryptophan hydroxylase were accessed. Both *p*CPA and 5,7-DHT bath treatments caused significantly enhanced expression (n=3, *$p < 0.05$, one-way ANOVA with Tukey's multiple comparison tests). Equal loading and transferring of samples were verified by densitometric measurements after Ponceau S stain over total bands.

Figure 16. Effects of *p*CPA on the lowered elongation probability and the inhibited elongation onset of neuron C1 during the culture in s-CM. A. Both *p*CPA treatments (0.02 % directly bath-treated and 0.1 mg intramuscular-injected, Diefenbach et al., 1995) might lead a little higher probability of neurite elongation compared with the negative control s-CM. However, their recoveries did not attain the significant probability level of optimal m-CM2 (*$p > 0.05$, Fisher's exact tests). B. Frequency histogram was provided with number of cultured neuron C1 with onset of neurite elongation. Bath-treated *p*CPA (0.02 %) group showed earlier elongation than other groups. C. In median days in culture (dic) comparisons, only bath-treated *p*CPA (0.02 %) group had a significant recovery from the inhibited onset of neurite elongation observed in the s-CM control group (*$p < 0.05$, Kruskal-Wallis test with Dunnett's multiple comparison tests). Because 5-HT inhibited the phase transition from initiation to elongation (see Figure 12. C), *p*CPA was likely to switch the initiation easily to elongation phase reducing 5-HT levels.



2) Differential effects of 5-HT on different elongation-rate neurites

Isolated serotonergic neuron C1 from the cerebral ganglion retained its own serotonergic properties during single neuron culture in the m-CM2.

Phenotypic changes have sometimes been reported when culturing neuronal cells (Schoenen et al., 1989; Adler and Black, 1985). Thus, it is important to confirm whether cultured neuron C1 still expresses the serotonergic phenotype. To demonstrate that the dissection and cell culture do not change the serotonergic phenotype of neuron C1, tissue-wholemount immunostaining, immunoblotting and immunocytochemistry were applied to cerebral ganglia, isolated neuron C1s and cultured neuron C1s, respectively.

Wholemount 5-HT immunostaining confirmed that there were at least five different serotonergic neuronal clusters in each cerebral ganglion, with neuron C1 located in cluster I (Figure 17. A & B). Neuron C1 was surrounded by several smaller satellite serotonergic cells and extended its neurite ipsilaterally into the buccal ganglion (Figure 17. B). Western blot analysis for tryptophan hydroxylase was performed with extracts of 100 isolated neuron C1s. Protein expression of tryptophan hydroxylase was detected in a 55 kDa band (Figure 17. C). In addition, 5-HT immunoreactivity was also detected in the soma, neurites and growth cones of neuron C1 after surgical isolation and plating in culture under outgrowth-promoting conditions (Figure 17. D). However, the intensity of 5-HT immunoreactivity in neurites and growth cones was variable.

Classification of neuron C1 neurites according to rate of elongation

It is hypothesized that extrinsic neurotransmitters cause differential effects on extending neurites. There is a review (Lipton and Kater, 1989) suggesting that excitatory neurotransmitters may have two different dose-dependent effects on neuronal morphology; neurite outgrowth is inhibited at lower levels whereas cell death is caused at higher levels. In addition, different neurites may display various elongation rates, even in the same neuron. Thus, differential effects of neurotransmitters may not be revealed if averages of the entire population of neurites are taken. Since analysis of individual neurites indicated that such differential effects were likely, elongation rates from individual neurites of neuron C1 were first measured for 60 minutes, and then all elongating neurites were placed into categories based on their own elongation rates (Figure 18). By examining the distribution of elongation rates, I observed three distinct neurite groups from cultured neuron C1s; slow-growing neurites, fast-growing neurites, and collapsing neurites. Slow-growing neurites grew at a rate of 1 ~ 20 μm per 60 minutes while fast-growing neurites grew at a rate of >20 μm per 60 minutes. Collapsing neurites had negative values in total outgrowth over 60 minutes.

The frequency distribution of neurite elongation rates differed depending on days in culture (Figure 19). In 24-hour cultures of neuron C1, a similar distribution to the overall pattern for all 24 ~ 72 hour neurites (see Figure 18) was observed, including three distinct neurite groups (Figure 19. A). However, the occurrence of

collapsing neurites was very low, compared to the other groups. In 48-hour cultures of neuron C1, fast outgrowth was primarily observed, with a lower frequency of slow outgrowth (Figure 19. B). In 72-hour cultures of neuron C1, the occurrence of collapsing neurites and slow-growing neurites increased, while few neurites displayed fast outgrowth (Figure 19. C).

Morphological examples of the three classes of neurites are demonstrated using time-lapse video microscopy (Figure 20). Slow-growing neurites often had wider growth cones compared with fast-growing neurites (Figure 20. A & B). Collapsing growth cones were typically small and club-shaped, with reduced or indistinct lamellipodia and filopodia (Figure 20. C).

Functional relationship between growth cone width and rate of neurite elongation

There is a diversity of growth cone sizes and elongation rates, but no clear evidence for a functional relationship between the two. In order to test for functional relationship between morphological characteristics of neurites and elongation rates, the average width of growth cones was analyzed in neurites elongating in various rates after 24 ~ 72 hours of culture (Figure 21). The distribution of growth cone width over neurite elongation rate showed a statistically significant correlation between some slow-growing neurites and fast-growing neurites with a specific growth cone width ($p < 0.01$, one-way ANOVA with Newman-Keuls test for all pairs of groups). The growth cone width around 30 ~ 40 μm was observed only in some slow-growing neurites which was at a

rate of elongation around 11 ~ 20 μm per 60 minutes. Further, the growth cone width around 15 ~ 20 μm was observed only in some fast-growing neurites which was at a rate of elongation around 36 ~ 40 μm per 60 minutes. However, the growth cone width around 20 ~ 30 μm was observed in both slow-growing (6 ~ 10 μm/hr) and fast-growing neurites (21 ~ 35 μm/hr), and thus their correlation was not comparable ($p > 0.05$, one-way ANOVA with Newman-Keuls test for all pairs of groups).

Differential effects of 5-HT on elongation of three different classes of neurites

5-HT is mainly known as a neurotransmitter that inhibits neurite elongation and collapses growth cones in some specific identified molluscan neurons (Haydon et al., 1984 & 1987; Koert et al., 2001; Torreano et al., 2005). Nonetheless, little is known about how 5-HT affects serotonergic neurons, and the possibility of autoregulation of neurite outgrowth. 5-HT induced differential effects on neurite outgrowth of dissociated embryonic neurons, whereby elongating neurites responded differently to 5-HT from non-elongating neurites (Goldberg et al., 1991). However, these differential effects were not examined in a single identical neuron with different elongation-rate neurites. Thus, the differential effects of 5-HT might still be due to the responses of different cell types from mass cultures. To test the hypothesis that 5-HT can differentially affect neurite outgrowth depending on elongation rates of neurites from the same neuron, baseline elongation rates (μm/hr) were first measured from individual

neurites of cultured neuron C1s over 60 minutes. Those same neurons were then treated with various concentrations of 5-HT (1, 10, 50 and 100 μM), respectively and analyzed for neurite elongation rates over the same 60 minutes.

In slow-growing neurites from 24 ~ 72 hour cultures, 5-HT showed a biphasic effect on the regulation of neurite elongation in a dose-dependent manner (Figure 22). In general, lower concentrations of 5-HT (1 and 10 μM) facilitated neurite elongation while higher concentrations of 5-HT (50 and 100 μM) inhibited neurite elongation ($p < 0.05$, one-way ANOVA with Dunnett's multiple comparison test). In these experiments, 1 μM 5-HT caused a prominent facilitation in neurite elongation, switching slow-growing neurites into fast-growing neurites, while 100 μM 5-HT induced slow-growing neurites to collapse.

In fast-growing neurites from 24 ~ 72 hour cultures, 5-HT produced inhibitory effects on neurite elongation in a dose-dependent manner (Figure 23). Fast-growing neurites turned into slow-growing neurites immediately after the treatment of 10 μM 5-HT. Moreover, higher concentrations of 5-HT (50 and 100 μM) caused all or most neurites to collapse ($p < 0.01$, one-way ANOVA with Dunnett's multiple comparison test).

In collapsing neurites which had negative values from baseline outgrowth measurements, all concentrations of 5-HT except 1 μM sustained the present collapsing state. However, 1 μM of 5-HT significantly made the present collapsing state worse, sometimes leading these neurites into the irreversible cessation phase after washing out this concentration of 5-HT ($p < 0.05$, one-way ANOVA with Dunnett's multiple comparison test) (Figure 24). Although 100 μM of

5-HT tended to retrieve the outgrowth from collapsing neurites, statistics did not show any significance of the phenomenon.

Effects of 5-HT on the population of different elongation-rate neurites at different stages of culture

In the present results (see Figure 19), frequency distribution of different elongation-rate neurites showed a specific population pattern depending on the day in culture. To investigate whether neuron C1s respond differentially to 5-HT depending on different stages of culture, neurite outgrowth measurements was made at 24, 48 and 72 hours.

In slow-growing neurites from 24 hour cultured neuron C1s, 5-HT produced a similar biphasic effect on neurite elongation (Figure 25) to that seen in the combined 24 ~ 72 hour data. However, the facilitatory effect of 1 μM 5-HT on neurite elongation was likely smaller in these 24 hour cultures, and none of the above concentrations of 5-HT caused neurites to collapse. In fast-growing neurites from 24 hour culture, 10 μM of 5-HT did not show a significant inhibitory effect while higher concentrations of 5-HT (50 and 100 μM) inhibited neurite elongation (Figure 26). Moreover, only 100 μM 5-HT caused neurites to collapse ($p < 0.01$, one-way ANOVA with Dunnett's multiple comparison test).

Most neurites in 48 hour cultures of neuron C1 were in a fast-growing phase, and thus 5-HT effects were only investigated on those fast-growing neurites. Effects of 5-HT on elongation of fast-growing neurites in 48 hour cultures (Figure

27) showed similar patterns to the overall 5-HT effects on elongation of fast-growing neurites over 24 ~ 72 hours. 10 μM of 5-HT did show a significant inhibitory effect on neurite elongation and higher concentrations of 5-HT, such as 50 and 100 μM, caused neurites to collapse ($p < 0.01$, one-way ANOVA with Dunnett's multiple comparison test).

In contrast to 48 hour cultures, the slow-growing phase was prevalent in 72 hour cultures of neuron C1, and thus 5-HT effects were only investigated on those slow-growing neurites. There were no observations of facilitatory effects of lower concentrations of 5-HT (1 and 10 μM) as shown in overall 24 ~ 72 hour cultures, but higher concentrations of 5-HT (50 and 100 μM) induced collapse of neurites ($p < 0.01$, one-way ANOVA with Dunnett's multiple comparison test) (Figure 28).

Taken together, these data demonstrated that 5-HT could change the individual population of different elongation-rate neurites depending on the stages of culture.

Effects of 5-HT on intracellular Ca^{++} *levels in growth cones*

It is not surprising that calcium is a regulator of neurite outgrowth for many different organisms and cell types. Increases in intracellular Ca^{++} levels cause a reversible inhibition of neurite outgrowth in *Helisoma* neurons (Cohan et al., 1987; Polak et al., 1991; Davenport et al., 1996). From the calcium hypothesis for neurite outgrowth (Kater and Mills, 1991), elongation-permissive ranges of

intracellular calcium concentrations, $[Ca^{++}]_i$, are suggested. Furthermore, a specific optimal $[Ca^{++}]_i$ is likely to be associated with the maximal rate of neurite elongation. This optimal $[Ca^{++}]_i$ level is present in all growth cones, but it varies between different neurons (Torreano and Cohan, 1997). Thus, the intracellular buffering abilities of neurons are important in determining the response to a change in $[Ca^{++}]_i$. In the present study, it was hypothesized that 5-HT induces differential changes in $[Ca^{++}]_i$ from neurites at different elongation rates.

To test how 5-HT affects $[Ca^{++}]_i$ to regulate neurite elongation, 10 μM of 5-HT was used in the fura-2 intracellular calcium imaging. Because this concentration of 5-HT alternatively showed either facilitatory or inhibitory effects depending on different elongation-rate neurites in the present studies (see Figure 22 & 23), the single-dose of 5-HT treatment may introduce differential effects on $[Ca^{++}]_i$ levels. In order to observe the hypothesized differential effects of 5-HT, the elongation rate of various neurites has to first be determined. However, separating neurites into classes based on elongation rates right before the calcium imaging was limited by the 45 minute fura-2 loading procedure. The limitation was due to a negative impact on the elongation-rate measuring process caused by the fura-2 loading for 45 minutes. To overcome this problem, two rate-distinct neurite groups with significantly different growth cone widths (smaller growth cones; n=4, 19.8 ± 2.1 μm and larger growth cones; n=5, 42.3 ± 3.4 μm) were used in $[Ca^{++}]_i$ measurements, instead of identifying slow- and fast-growing neurites for 60 minutes before calcium imaging. This indirect determination of two different elongation-rate neurite groups was based on a significant functional

correlation between growth cone width and elongation rate (see Figure 21). Therefore, I used this correlation to measure $[Ca^{++}]_i$ in growth cones whose widths I specifically knew were correlated with neurite elongation. Besides neurites, cell bodies (n=4) of serotonergic neuron C1s were imaged for $[Ca^{++}]_i$ measurements.

First of all, there were increased Ca^{++} levels (F ratio 340/380) in the cell body of the serotonergic neuron C1s after 5-HT treatment (Figure 29. A). There was no significant change in Ca^{++} levels (F ratio 340/380) in the smaller growth cones of the serotonergic neuron C1 (Figure 29. B). In contrast, Ca^{++} level (F ratio 340/380) in the larger growth cones of serotonergic neuron C1 was increased after addition of 5-HT, higher than the 5-HT-induced Ca^{++} level change of the cell body (Figure 29. C). There was an interesting difference in calcium responses (F ratio 340/380) observed between peripheral and central domains of the larger growth cones (Figure 29. C). After the addition of 5-HT, Ca^{++} levels in the central domains of the larger growth cones was increased much higher than those in the peripheral domains. There was a calcium diffusion from an adjacent part of the neurite shaft into the central domain of the larger growth cone, and the diffusion rate was about 30 – 40 μm/minute. Thus, the calcium entry site for the diffusion was about 100 μm away from the transition zone between the central and peripheral domains (Figure 29. C insets).

Differences in immunocytochemical expression of newly cloned Helisoma 5-HT receptors between slow- and fast-growing neurites of cultured neuron C1s.

Newly cloned *Helisoma* 5-HT receptors such as 5-HT_{1hel} and 5-HT_{7hel} were immunostained using corresponding antibodies to localize 5-HT receptor expression in neuron C1. Antibodies to the receptors 5-HT_{1hel} and 5-HT_{7hel} were raised against specific peptides (5-HT_{1hel} residues 409 – 423, YSRTREKLELKRERK and 5-HT_{7hel} residues 246 – 261, YFKIWRVSSKIAKAEA) derived from intracellular loop sequences which showed a high antigenicity (Parries et al., 2003). Instead of Normarski differential interference contrast (DIC) or phase contrast images, phalloidin-labeling was applied to show the neuronal morphology in detail. All images were obtained from the 3-dimensional reconstruction after confocal laser scanning microscopy. Immunoreactivity of 5-HT_{1hel} receptors was found in some fast-growing neurites with smaller growth cones (~ 15 - 20 μm) as well as the cell body (Figure 30. A). However, Immunoreactivity of 5-HT_{7hel} receptors was observed only in the cell body area (Figure 30. B). Immunoreactivity of 5-HT_{1hel} receptors was not expressed in slow-growing neurites with larger growth cones (~ 30 - 40 μm) (Figure 30. C). Moreover, immunoreactivity of 5-HT_{7hel} receptors was never expressed in any of both fast- and slow- growing neurites (Figure 30. C).

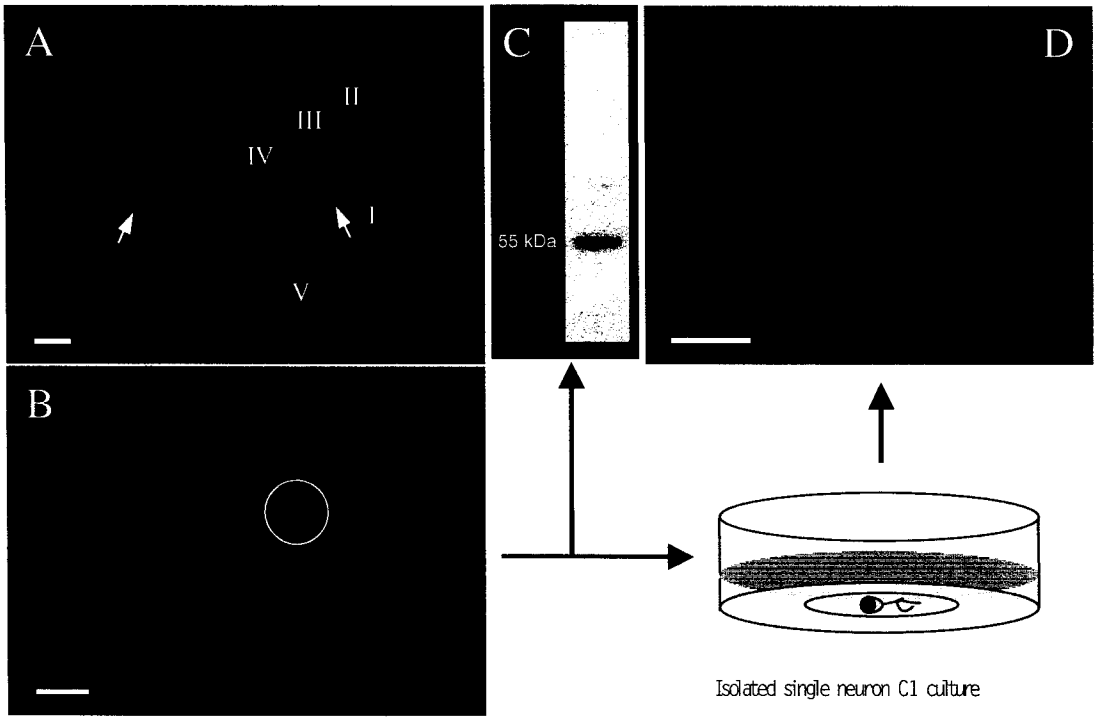


Figure 17. Phenotypic expression of 5-HT in serotonergic neuron C1 of the freshwater snail, *Helisoma trivolvis*. A. 5-HT immunoreactivity in the cerebral ganglia. At least five serotonergic neuronal clusters (I, II, III, IV and V) were expressed in each ganglion . Each arrow localizes neuron C1 in cluster I of each hemi-ganglion. B. Identified serotonergic neuron C1 with many other smaller satellite serotonergic neurons was in neuronal cluster I. This single cell was isolated using a micro-tungsten knife and cultured in m-CM2. C. Immunoblotting for tryptophan hydroxylase in neurons C1 (n=100). D. 5-HT immunoreactivity in a cultured neuron C1. Scale bars indicate 100 μm.

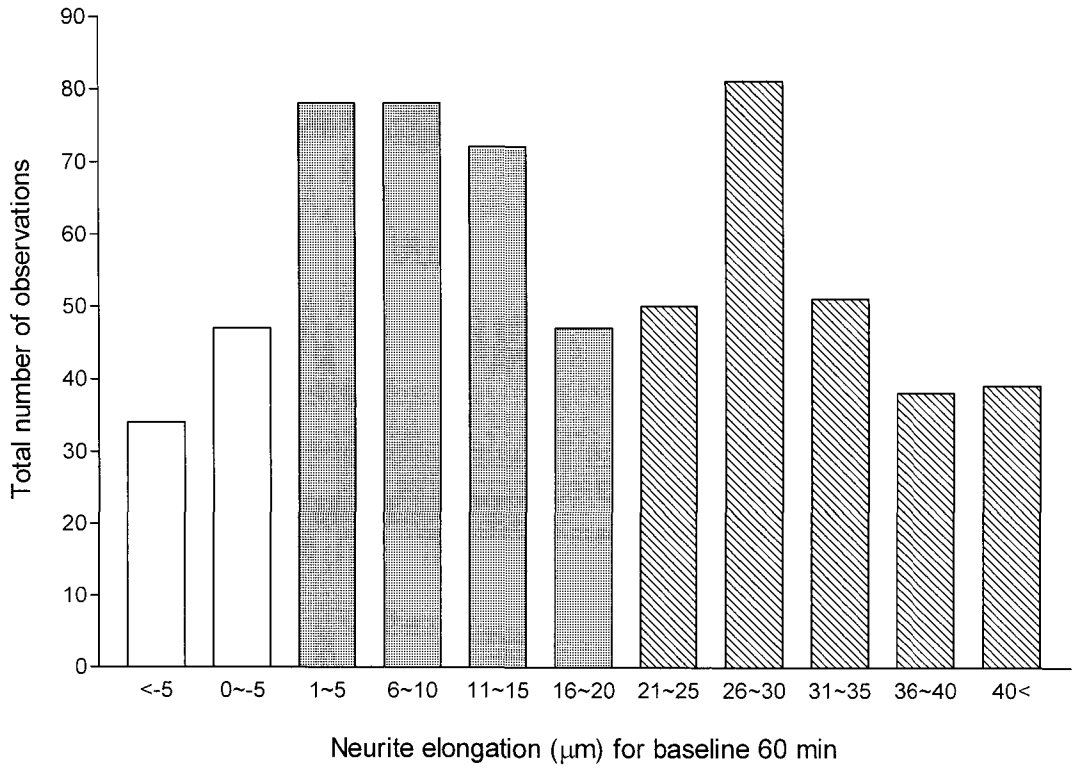


Figure 18. Overall frequency distribution of neurite elongation rates from 24 ~ 72 hour neuron C1 cultures. The elongation rates were measured every 20 minute for 1 hour. Neurites were designated as either slow-growing neurites (stippled bars), fast-growing neurites (striped bars), or collapsing neurites (clear bars).

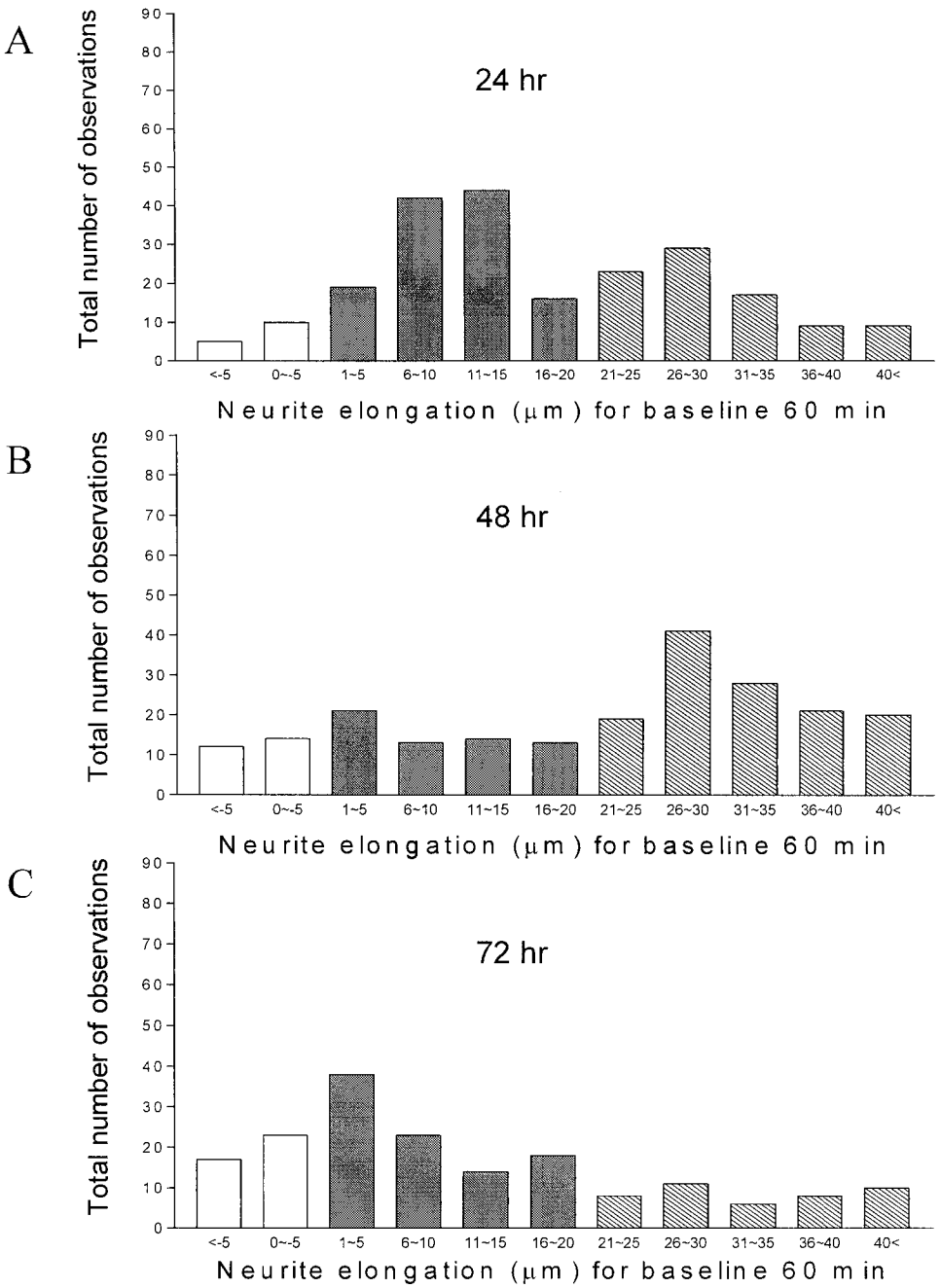


Figure 19. Frequency distribution of elongation rates at different days in culture (stippled bars, slow-growing neurites; striped bars, fast-growing neurites; clear bars, collapsing neurites). A. 24 hour cultures. B. 48 hour cultures. C. 72 hour cultures.

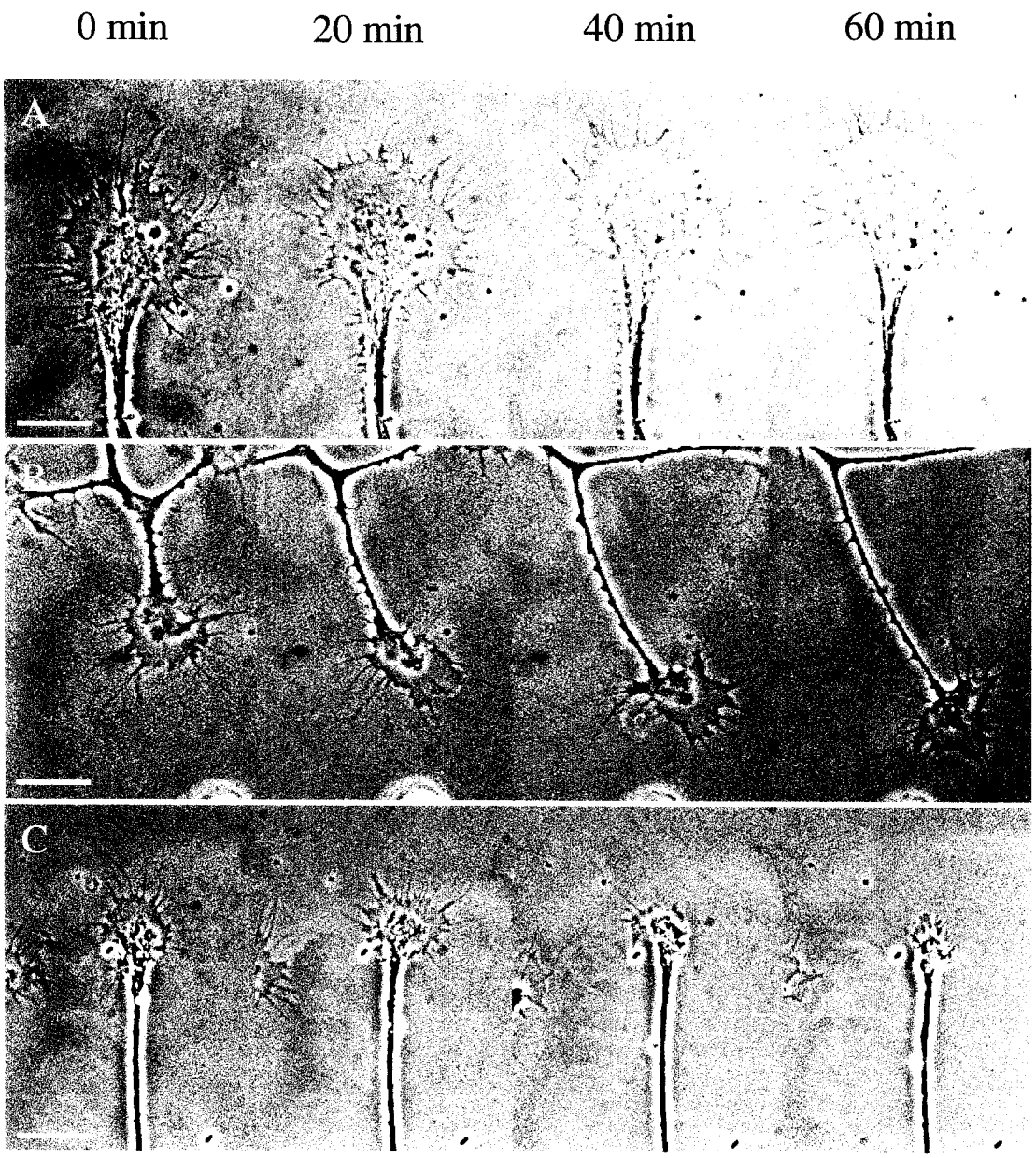


Figure 20. Morphology of neurites elongating at different rates. A. Slow-growing neurite (1 ~ 20 μm per hour). B. Fast-growing neurite (> 20 μm per hour). C. Collapsing neurite (< 0 μm per hour). Scale bars indicate 20 μm.

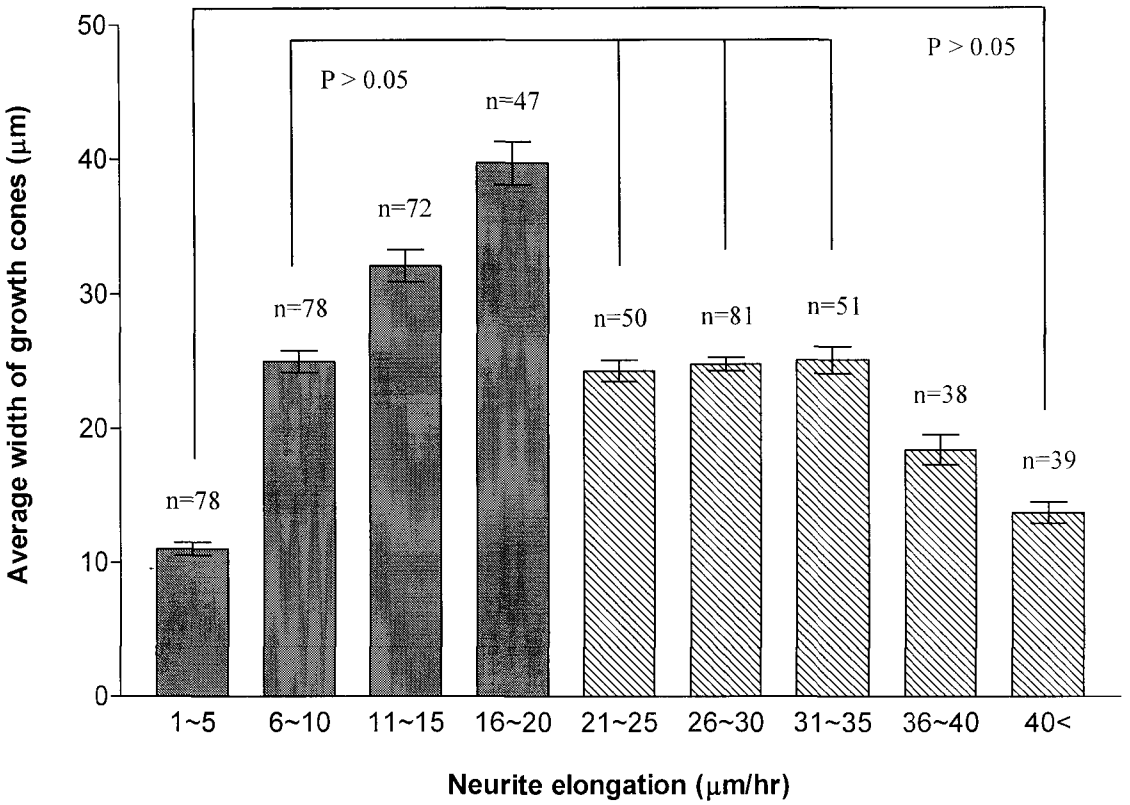


Figure 21. Width of growth cones as a function of elongation rates in neurites from 24 ~ 72 hour neuron C1 culture (dark bars, slow-growing neurites; striped bars, fast-growing neurites) ($p < 0.01$, one-way ANOVA with Newman-Keuls test for all pairs of groups).

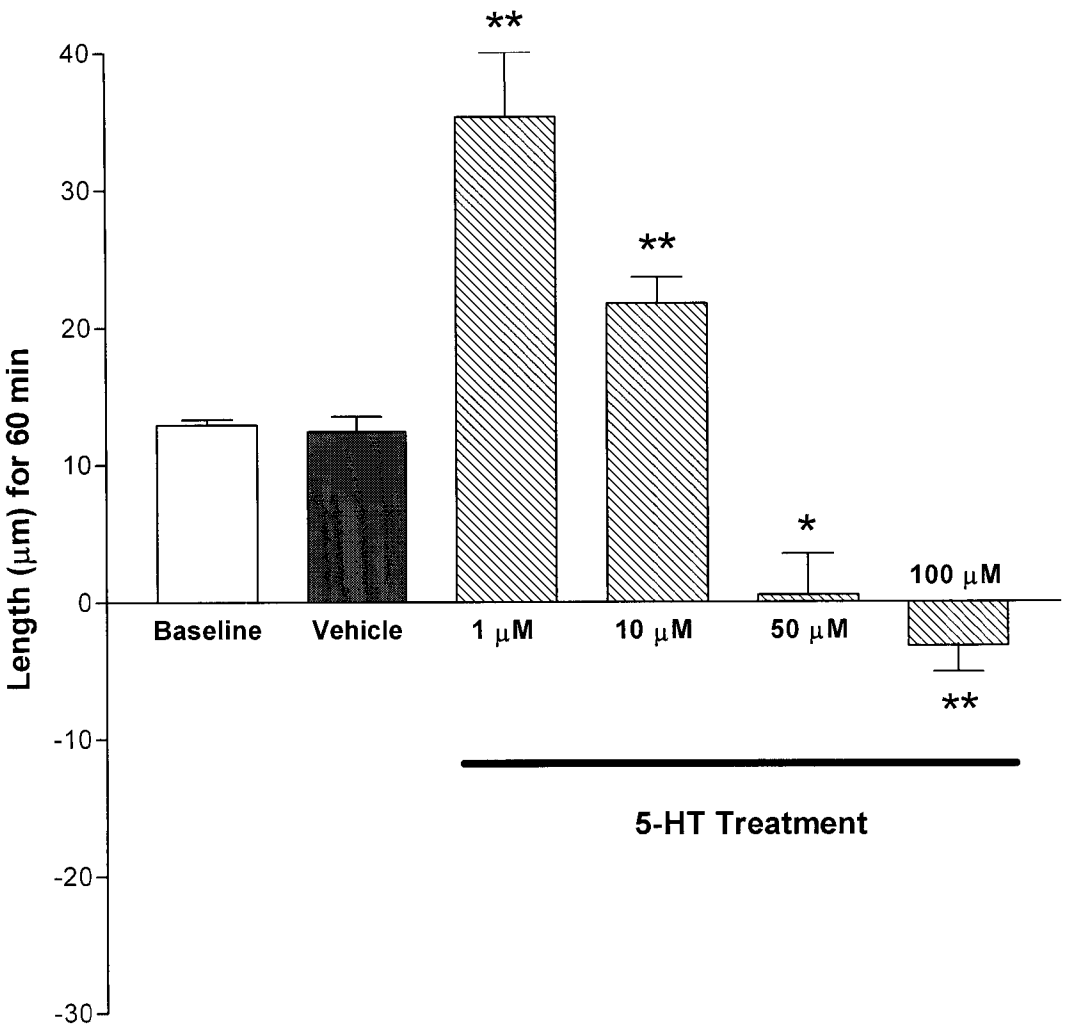


Figure 22. Effects of 5-HT on the elongation rates of slow-growing neurites (≤ 20 μm per hour) from 24 ~ 72 hour cultures. 5-HT showed a dose-dependent biphasic effect on neurite elongation rate (n=144, *$p < 0.05$, one-way ANOVA with Dunnett's multiple comparison test, **$p < 0.01$, one-way ANOVA with Dunnett's multiple comparison test).

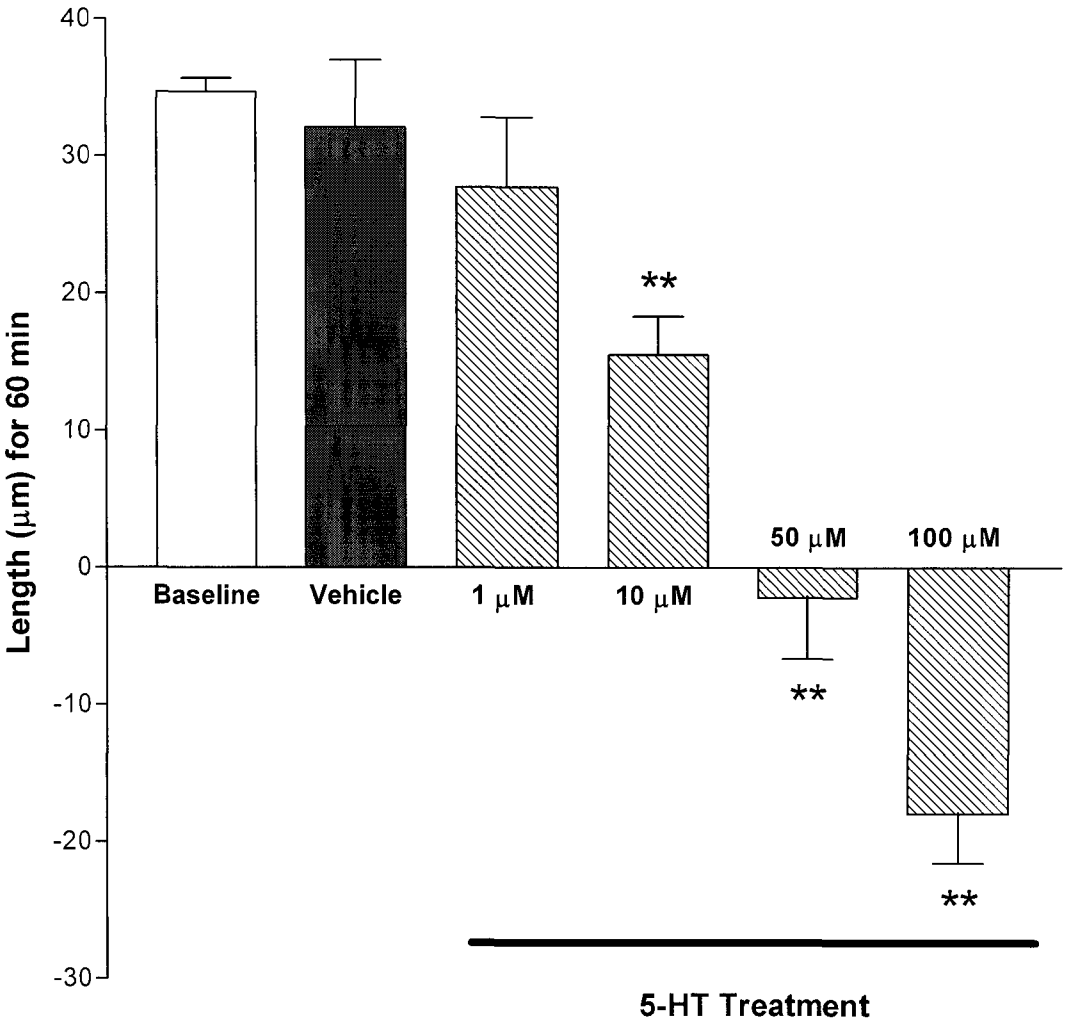


Figure 23. Effects of 5-HT on the total elongation rates of fast-growing neurites (> 20 μm per hour) from 24 ~ 72 hour cultures (n=161, **p < 0.01, one-way ANOVA with Dunnett's multiple comparison test).

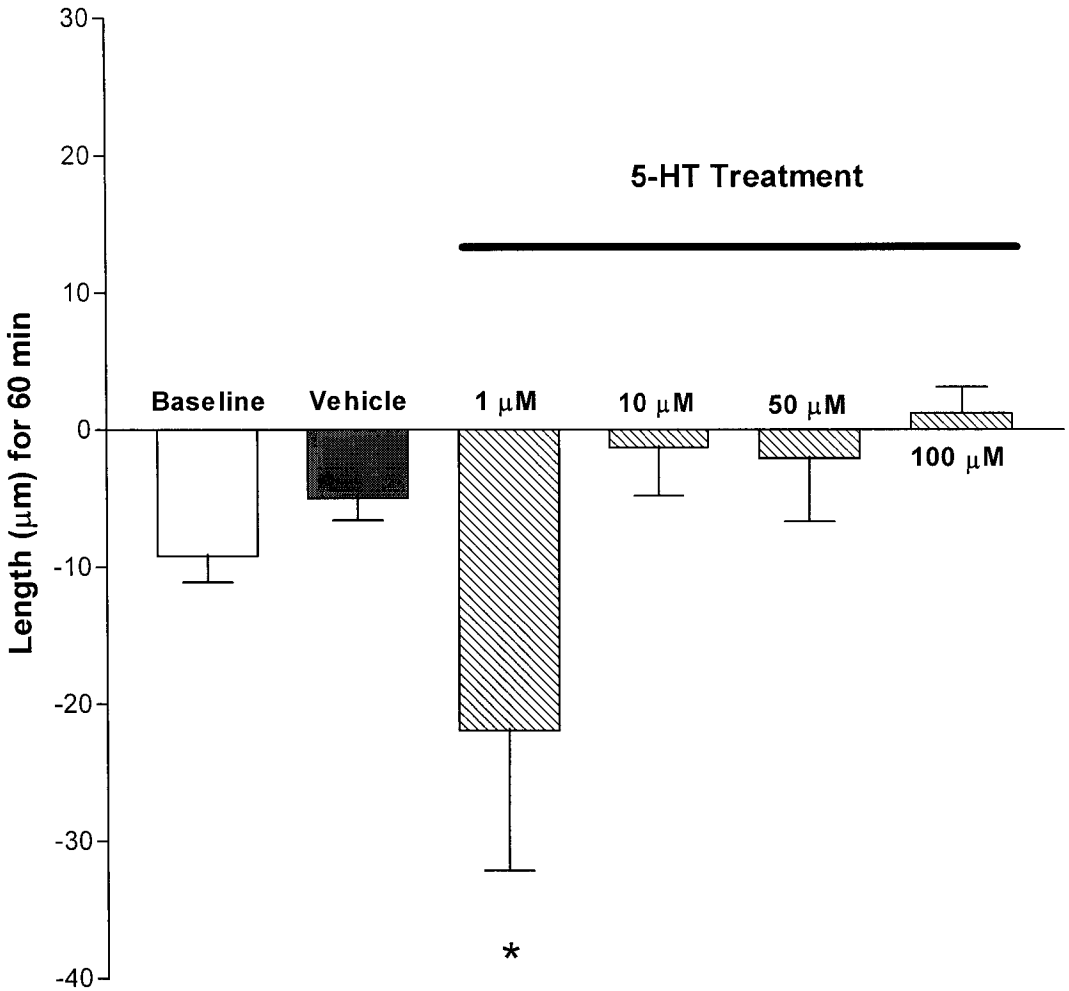


Figure 24. Effects of 5-HT on the total elongation rates of collapsing neurites (≤ 0 μm per hour) from 24 ~ 72 hour cultures (n=156, *p < 0.05, one-way ANOVA with Dunnett's multiple comparison test).

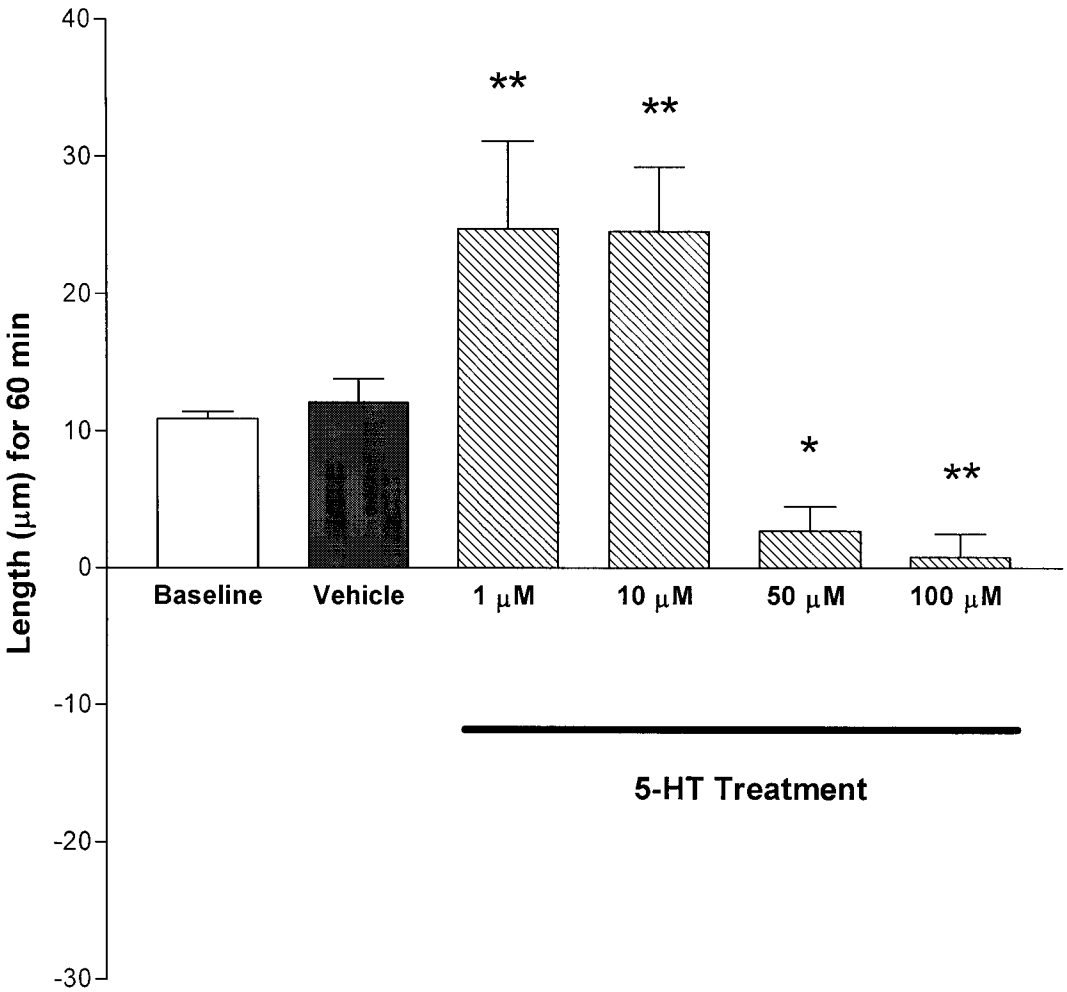


Figure 25. Effects of 5-HT on the elongation rates of slow-growing neurites from 24 hour cultures. 5-HT showed a dose dependent biphasic effect on neurite elongation rate (n=72, *$p < 0.05$, one-way ANOVA with Dunnett's multiple comparison test, **$p < 0.01$, one-way ANOVA with Dunnett's multiple comparison test).

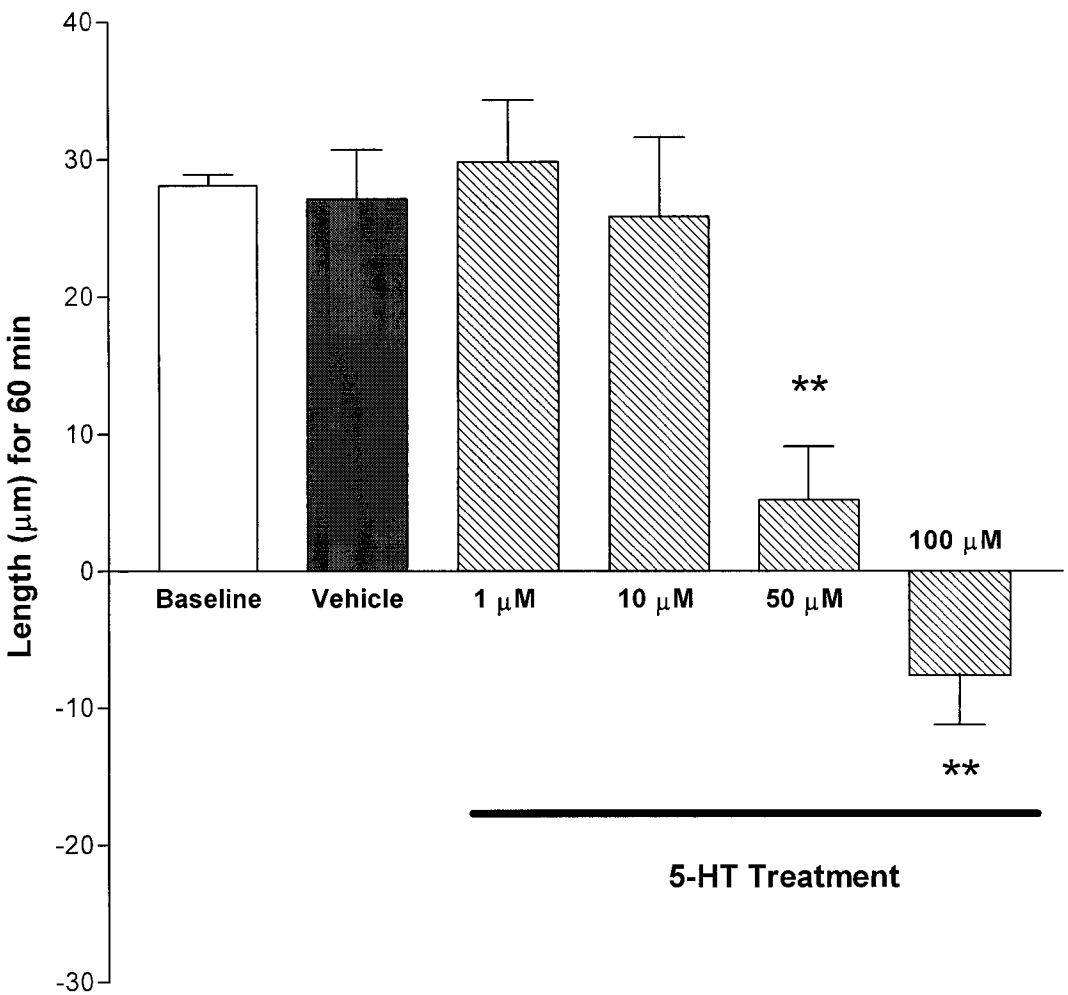


Figure 26. Effects of 5-HT on the elongation rates of fast-growing neurites from 24 hour cultures (n=46, **p < 0.01, one-way ANOVA with Dunnett's multiple comparison test).

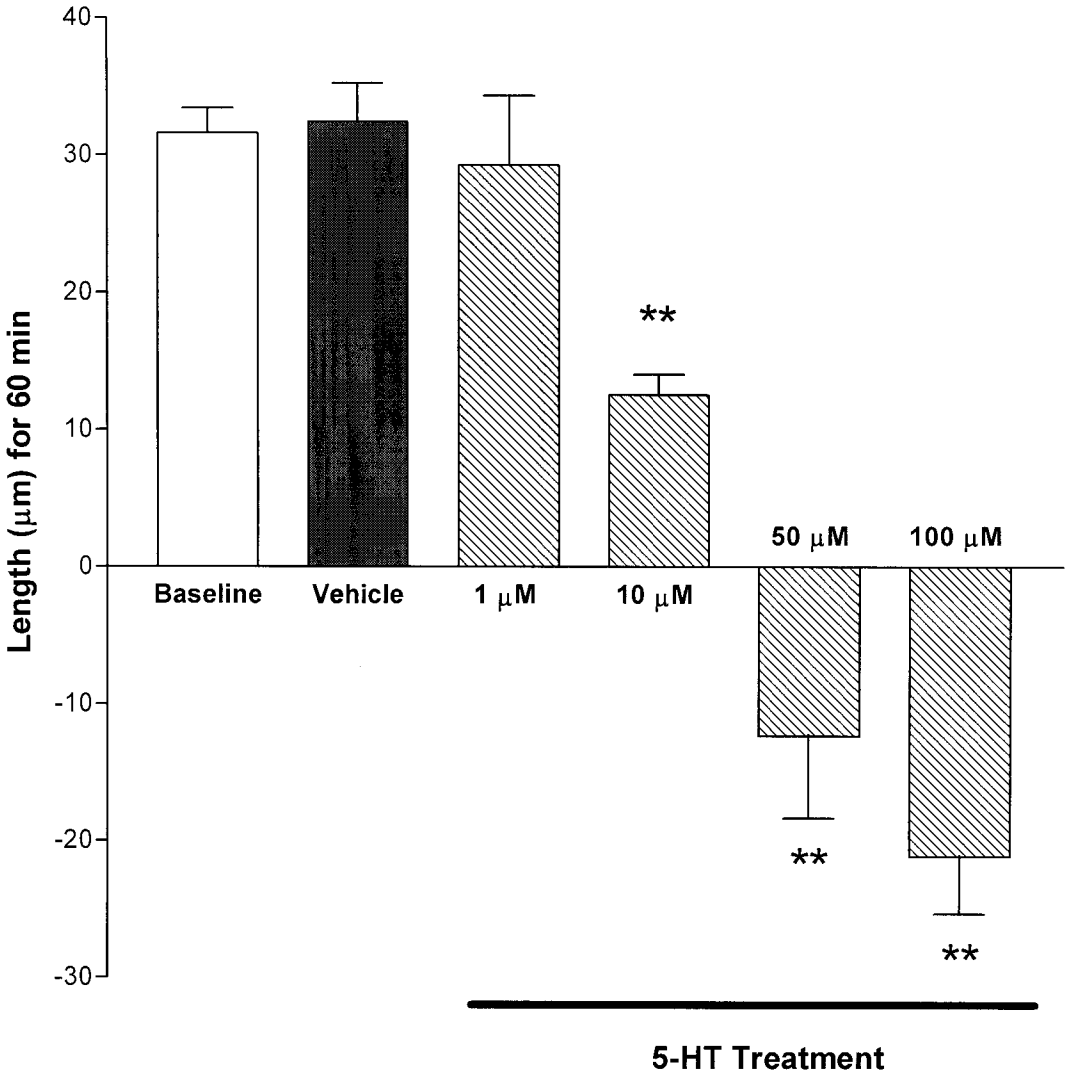


Figure 27. Effects of 5-HT on the elongation rates of fast-growing neurites from 48 hour cultures (n=48, **p < 0.01, one-way ANOVA with Dunnett's multiple comparison test).

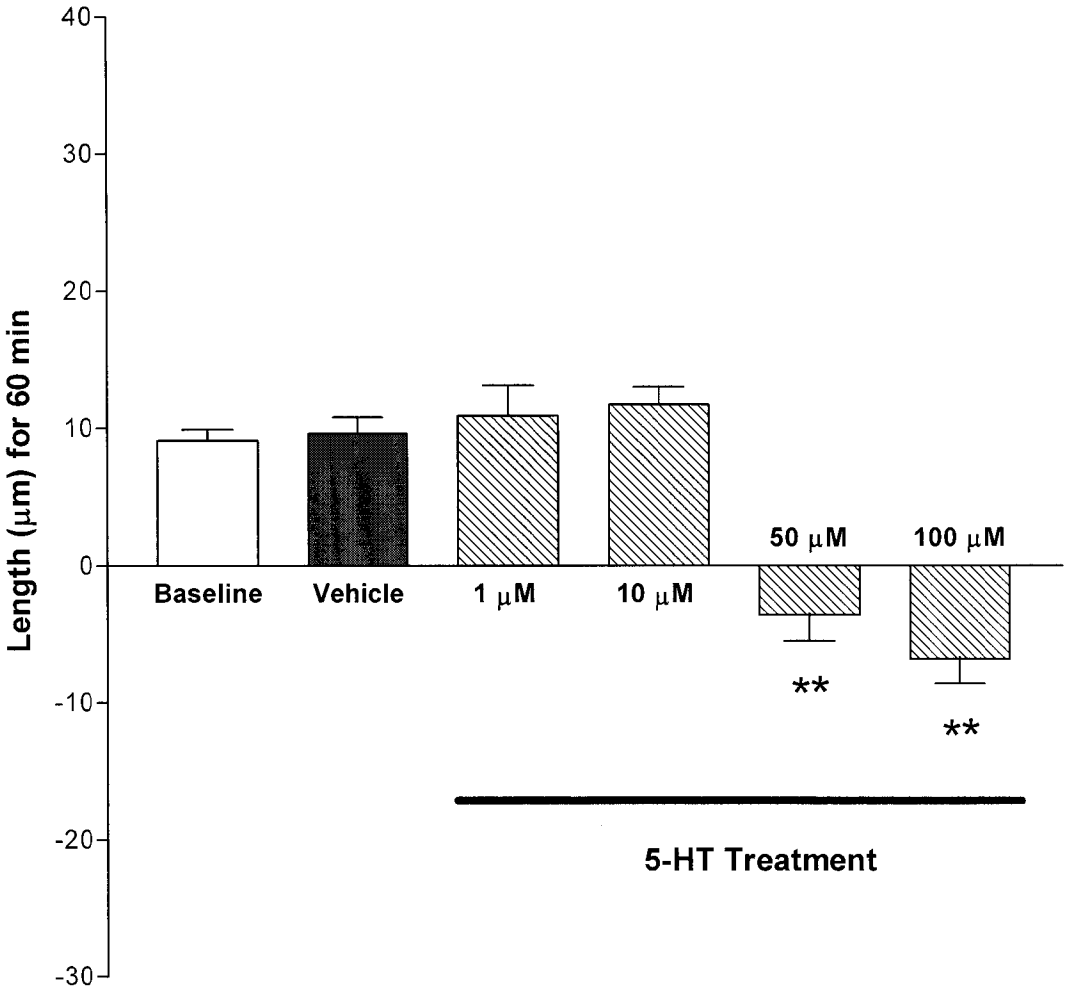


Figure 28. Effects of 5-HT on the elongation rates of slow-growing neurites from 72 hour cultures (n=58, **p < 0.01, one-way ANOVA with Dunnett's multiple comparison test).

Figure 29. Differential effects of 5-HT (10 μM) on the intracellular calcium levels in different parts of serotonergic neuron C1. A. Increased Ca^{++} levels in the cell body of neuron C1 (n=4). B. Calcium levels in the smaller growth cones (~ 20 μm) of neuron C1 (n=4). There was little Ca^{++} level changes observed. C. Increased calcium levels in the larger growth cones (~ 40 μm) of neuron C1 (n=5). Similar increase of Ca^{++} level observed in the cell body was observed in the peripheral domain of the growth cones. The neurite with a larger growth cone might be involved in a Ca^{++} signal propagation into the central domain of a growth cone (inset in C). Inset images were shown in pseudo-colors to indicate the intracellular calcium level changes. The F ratio (340/380) provides a relative measure of cytoplasmic free calcium concentration.

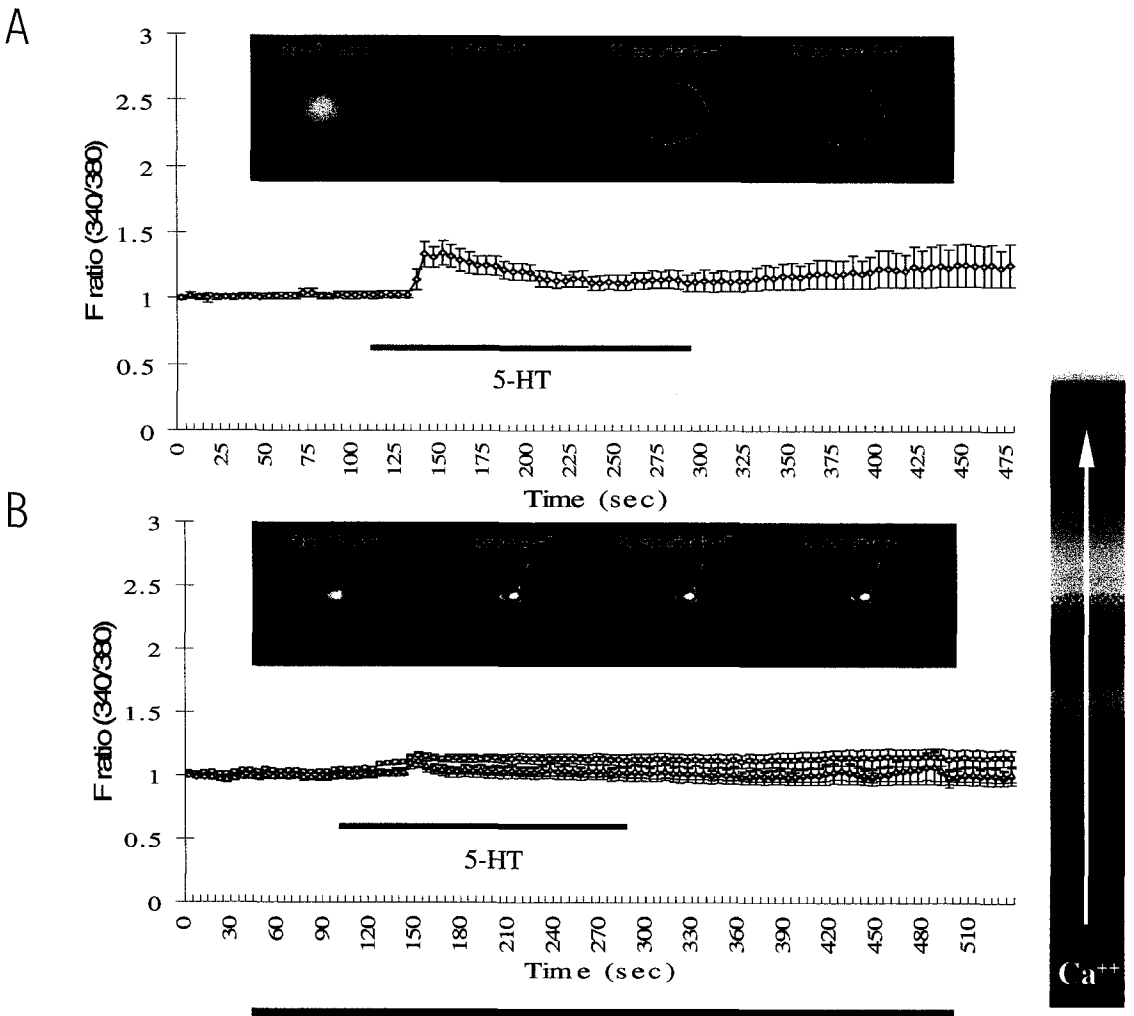
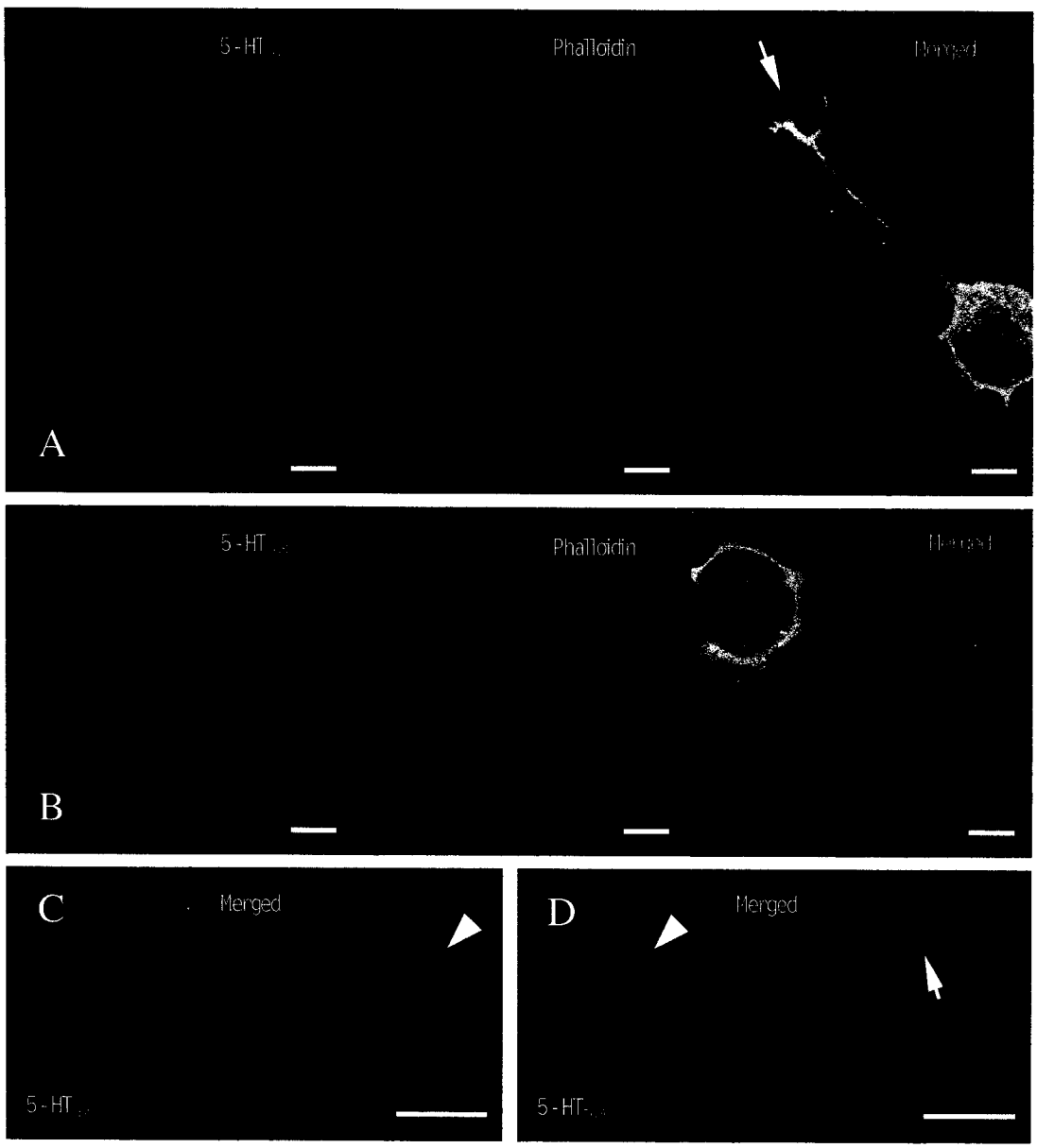


Figure 30. Localization of newly cloned *Helisoma* 5-HT receptors in serotonergic neuron C1. A. Expression of 5-HT_{1hel} receptor (left, immunoreactivity of anti-5-HT_{1hel} receptors; middle, immunoreactivity of phalloidin-TRITC; right, merged). Phalloidin-labeling was applied to show accurate neuronal morphology. Expression of 5-HT_{1hel} receptors were found in some fast-growing neurites with smaller growth cones as well as the cell body. B. Expression of 5-HT_{7hel} receptors observed only in the cell body area (left, immunoreactivity of anti-5-HT_{7hel} receptors; middle, immunoreactivity of phalloidin-TRITC; right, merged). C. 5-HT_{1hel} receptor was not expressed in slow-growing neurites with larger growth cones (merged). D. 5-HT_{7hel} receptors were never expressed in any of neurites at different elongation rates (merged). Arrow heads indicate a slow-growing neurite and arrows indicate a fast-growing neurite. Pre-absorption controls and keyhole limpet hemocyanin (KLH) controls did not affect both anti-receptors' immunoreactivity. All scale bars indicate 40 μm.



DISCUSSION

The morphology of neurons is profoundly influenced by various factors in the nervous system that can modulate neurite outgrowth. In the present study on an identified neuron from *Helisoma trivolvis*, the extension of neurites requires the presence of outgrowth-promoting factors, which are released from the snail's brain into the defined culture medium (DM). The results demonstrated in this thesis provide the first descriptive account of neurite outgrowth in the serotonergic neuron C1 using a single isolated cell culture system. Tonic inhibitory effects of 5-HT on the onset of neurite elongation were revealed. Moreover, differential regulatory roles of 5-HT in regulating the neurite outgrowth of serotonergic neuron C1 were also examined in the present study. Some previous studies used *in vivo* embryonic neuronal models, or dissociated neuronal cultures, to investigate the effects of 5-HT on neurite outgrowth (Whitaker-Azmitia and Azmitia, 1986; Haydon et al., 1987; Budnik et al., 1989; Goldberg and Kater, 1989; Liu and Lauder, 1991; Diefenbach et al, 1995). However, the present study is a pilot work to investigate the regulation of neurite outgrowth at the level of a single serotonergic neuron. The results outlined above will be discussed in relation to growth factors, neuronal death and initial states of neurite outgrowth in the first section and to neurite extension, growth cone morphology, calcium signaling and possibility of autoregulation in the second section.

1) Tonic inhibitory effects of 5-HT on controlling the phase transition of neurite outgrowth from initiation to elongation

The hypothesized role of 5-HT in controlling phase-transition of neurite outgrowth is demonstrated by several major findings from this cell culture study of the serotonergic neuron C1. First, onset of neurite elongation of neuron C1 was inhibited in cultures with the normal 72 hour CM, but not with the modified CM. Second, trace amounts of 5-HT were found in the normal 72 hour CM during brain-conditioning, in contrast to the present modified CM. Third, *p*CPA decreased 5-HT level in the normal 72 hour CM, partly by attenuating tryptophan hydroxylase expression in brains used for conditioning. Fourth, experimental reduction of 5-HT from the normal 72 hour CM rescued the earlier phase transition from initiation to elongation. Fifth, low doses of exogenous 5-HT added into the optimal CM caused a similar late onset of neurite elongation typical of standard 72 hour CM, while higher doses of 5-HT induced cytotoxic effects. Taken together, I conclude that 5-HT induces tonic inhibitory effects on neurite outgrowth by revealing the late onset of neurite elongation.

Optimal culture conditions for neurite outgrowth of serotonergic neuron C1

Optimal culture conditions for regenerative neurons normally require a combination of various substrate-bound and dissolved factors, some of which are obtained in the brain-conditioning process. Initiation of neurite outgrowth does not

always need exogenous neuronal growth factors, whereas neurite elongation requires extrinsic growth-promoting factors (Acklin and Nicholls, 1990). Some extrinsic factors such as ECM molecules and neurotrophic factors bind to their receptors and activate intracellular signaling cascades, resulting in reorganization of the cytoskeleton, neurite formation, and neurite extension. Guiding factors such as semaphorins, ephrins, netrins and Slits are also required to support neurite outgrowth. Some growth inhibitory factors can halt or repulse neurite advance in unfavorable or pathological conditions. For example, central neurons in vertebrates fail to regenerate their axons after injury because of outgrowth-inhibitory guiding factors in myelin, around damaged neurons and at the glial scar (McKerracher and Winton, 2002; David and Lacroix, 2003).

Generally speaking, neurotransmitters such as monoamines are known to have inhibitory effects on neurite outgrowth. It has been suggested that very high levels of 5-HT (up to 40 μg/g) are present in the nervous system of molluscs (for review, see Parent, 1981). The actual 5-HT content in *Helisoma* snail brain was previously analyzed by HPLC (128.9 ng/brain; Gadotti et al., 1986), and found to be in agreement with the concentration determined in the present study (130 ng/brain). In a previous study on vertebrate neurons, a trace amount of 5-HT (0.07 ± 10 ng/μl, equal to about 0.4 ~ 50 μM) was detected in the vertebrate culture medium (neural basal medium; Lieske et al., 1999), although it was not revealed how neural basal (NB) medium was prepared. Moreover, the amount of endogenous 5-HT released into the define medium (DM) by the cerebro-buccal ganglionic ring of *Aplysia* was determined to be 0.4 – 1.2 pmole per hour by

using a radioenzymatic microassay of [H^3] melatonin formed from 5-HT (Gerschenfeld et al., 1978). In the present study, I also report that normal 72 hour CM contains a trace amount of 5-HT (about 0.8 μM), whereas a lower amount of dopamine is also observed in the first 24 ~ 48 hour of conditioning. These monoamines are released into the medium from snail brains, which are used in the conditioning process. Consequently, the trace amount of these monoamines in the conditioned medium may induce specific tonic inhibitory effects on neurite outgrowth of the serotonergic neuron C1 through specific receptors. Interestingly, buccal neuron B19 does not undergo a similar delay in neurite initiation even though its neurite outgrowth can be inhibited by 5-HT (Haydon et al., 1987), perhaps because of its different profile of serotonin receptors.

During the dissection and trypsinization procedures prior to the conditioning incubation, brain tissues are subjected to harsh conditions, which may cause the release of tonic inhibitory factors into the medium. Moreover, many peripheral nerve trunks are transected during the dissection, providing release sites for inhibitory and growth-promoting factors into the medium. In a previous study, monoamines from food-deprived juvenile snails were released into the conditioned water and were found to have tonic inhibitory influences on *Helisoma* larval development under a 24 hour food-deprived condition (Voronezhskaya et al., 2004). Thus, my protocol for brain-conditioning involves a new step that replaces the first 24 hour CM with fresh defined medium. This is a strategy to remove inhibitors such as monoamines during the conditioning period and depends on the hypothesis that most of the monoamine release occurs in

the first 24 hours of the conditioning procedure. In my new protocol, monoamines such as 5-HT and dopamine were actually absent in the modified media. The subsequent 96 hour incubation period with the fresh defined medium may allow more growth-promoting conditioning factors to be released rather than inhibitory factors, while remaining monoamines have ample opportunity to be degraded.

The brains used for conditioning are recycled twice, but the amount of conditioning factors released into the 3rd CM may be reduced compared with the 1st and the 2nd CM. Thus, the 3rd CM produced a lower percentage of neurons with elongating neurites compared with the 1st and the 2nd modified CM, but similar to the normal 72 hour CM (see Figure 9. A). A reduction of growth-promoting factors in the 3rd CM is likely the cause of the poorer performance of the 3rd CM, rather than an increase in inhibitory factors characteristic of the 72 hour CM.

Lower doses of 5-HT treatments and phase transition of neurite outgrowth

The effects of 5-HT on neurite outgrowth are very neuron-specific (Haydon et al., 1984 & 1987; Murrain et al., 1990; Koert et al., 2001; Park et al., 2003). From experiments on identified molluscan neurons, 5-HT has primarily been shown to play negative roles on neurite outgrowth. For example, 5-HT treatments have been reported to cause inhibition of neurite extension (Haydon et al., 1984 & 1987), growth cone collapse (Koert et al., 2001), a reduced number of filopodia and decreased actin assembly at the growth cone leading edge (Zhou and

Cohan, 2001), inhibition of growth cone motility and synaptogenesis (Haydon et al., 1984 & 1987), and decreased branching (Diefenbach et al., 1995). In contrast, neurite outgrowth of thalamic neurons in rodents and some embryonic *Helisoma* neurons is facilitated by lower concentrations of 5-HT (Lieske et al., 1999; Lotto et al., 1999; Goldberg et al., 1991). All these morphological studies are based on observations during the elongation or guidance phases of neurite outgrowth, after neurite initiation has occurred.

Because the onset of neurite elongation in neuron C1 had spontaneously occurred under the optimal culture condition such as m-CM2 (see Figure 9. A), a late onset of neurite elongation seems to be attributed to the existence of inhibitory factors in the standard CM (s-CM). The present study suggests that lower doses (1 μM and 10 μM) of 5-HT treatments into the modified optimal CM (m-CM2) can slow down neurite initiation and inhibit spontaneous neurite elongation happening right after the completion of neurite initiation including neuritogenesis. However, these concentrations of 5-HT did not cause any significant effect on the percentage of elongating neurons (see Figure 13. A), which is increased by brain-conditioning factors (Berdan and Ridgway, 1992; Williams and Cohan, 1994). 5-HT may have interactive roles in regulating the elongation-inducing effects of brain-conditioning factors. Thus, the onset of neurite elongation from the initiation phase may be directly affected by tonic inhibitory 5-HT in CM. A previous study on freshly isolated soma has shown that 5-HT has a blocking effect on neuritogenesis in 24 hour cultured CGCs (Koert et al., 2001). However, this previous study did not examine the percentage of

elongating neurons beyond 24 hours, and thus could not actually report the late onset of neurite elongation. As the neuritogenesis is usually the last step of initiation of neurite outgrowth, their dose (10 μM) of 5-HT can be considered to slow down the process of initiation phase. Interestingly, this is the same concentration of 5-HT as used in the present study to observe the slow initiation. In the present study there appeared to be several segregated smaller lamellipodia with a greater number of protruding filopodia, in the presence of 10 μM 5-HT compared with other concentrations. However, in most cases (~ 90 %) the completion of neuritogenesis did not occur within first 24 hours. The parallels between the two studies may not be surprising because CGC is an identified serotonergic giant neuron found in cerebral ganglia of *Lymnaea stagnalis*, and is thought to be homologous to neuron C1 of *Helisoma trivolvis*.

The inhibitory effects of lower doses of 5-HT on the onset of neurite elongation can be explained as follows. Lower doses of 5-HT (1 and 10 μM) may initially inhibit the onset of neurite elongation thereby blocking the neuritogenesis. Over time, the concentration of 5-HT in culture may fall to a sub-threshold level due to some monoamine-inactivating steps such as oxidation, reuptake and glutamylation (Sloley and Goldberg, 1991), resulting in the disinhibition of neuritogenesis. This would result in the late onset of neurite elongation.

This explanation is only supported by the fact that a decreased 5-HT concentration to a sub-threshold level does not have an inhibitory effect on the onset of neurite elongation. However, it cannot be ruled out that a small amount

of 5-HT still remains in culture after several days, inhibiting the onset of neurite elongation as much as an initial dose. Actually, both 1 μM and 10 μM 5-HT treatments showed the late onset of neurite elongation on the same 3rd day in culture (see Figure 13. C). The exogenously applied 10 μM 5-HT seemed not to be fully catabolized even after the 3rd day in culture. Further, the concentration of uncatabolized 5-HT remaining in the medium may be higher than 1 μM. However, the leftover from the initial 10 μM 5-HT did not show the late onset of neurite elongation. Thus, these findings imply that the 5-HT-inhibited onset of neurite elongation may not be affected in a dose dependent manner and 5-HT may delay the onset.

In a more specific biological sense, inhibition is used for a restraining action that causes the reduction in the rate of overall process, while term of delay means putting off a reaction until a later time than expected (Hodgson et al., 1988). Accordingly, inhibitory effects will be removed after an active restraining factor becomes inactive. However, delaying effects will be independent of the inactivation of an initial restraining factor. This means that delaying effects will appear later even in the presence of an active restraining factor. While it is likely that the primary action of 5-HT is to inhibit the onset of neurite elongation, such that elongation only occurs after 5-HT drops below active levels, it remains possible that 5-HT acts to delay the onset instead. In this scenario, the onset of neurite outgrowth occurs later than normal, but does not require 5-HT to drop below active levels. To clarify whether the 5-HT in CM inhibits or delays the onset of neurite elongation, 5-HT clamping experiments (Figure 31) are

suggested for future study. In these suggested clamping exeperiments, addition and clamping of about 1μM 5-HT into the m-CM2 should inhibit the onset of neurite elongation. If neurite elongation still begins around 3-5 days in culture under the 5-HT clamping, then the effect of 5-HT is to delay, rather than prevent, the onset of neurite elongation.

Higher doses of 5-HT treatments and cytotoxicity

I directly tested the effects of exogenous 5-HT on cell bodies that were isolated without axon stumps. In the present study, lower concentrations of exogenous 5-HT inhibited the onset of neurite elongation. However, higher doses (50 μM and 100 μM) of 5-HT had cytotoxic effects rather than inhibitory effects on neurite outgrowth. The significantly fewer neurons with elongating neurites could be caused by the cytotoxic effects of higher doses of 5-HT directly to the soma, because the lower doses of 5-HT did not induce any significant change on percentage of elongating neurons. Neuron C1s treated with 50 μM 5-HT did not show any morphological indication of neurite initiation. Under these conditions, most neuron C1s appeared unhealthy because they exhibited cell-swelling, membrane-blebbing, and a mega-nucleus. These cytotoxic morphological changes were also prominent in neuron C1s treated with 100 μM of 5-HT, which ultimately induced neuronal death.

Cells die through one of two main cell death mechanisms, necrosis or apoptosis. Necrotic cells swell and lyse if they are too severely damaged to

maintain their physiological functions. Mitochondrial swelling, disintegration of organelle membranes and membrane-blebbing are also basic morphological features of necrosis (Levin et al., 1999; Clarke, 1999; Jellinger, 2001). In contrast, apoptotic cells are typically shrunken with condensed or fragmented nuclei although membrane blebbing may also be observed as for necrosis (Jellinger, 2001). In the present studies with higher doses of 5-HT, serotonergic neuron C1s likely underwent necrosis. The observed morphological features of cell-swelling, diffused nucleus and membrane blebbing fit the morphological criteria for necrotic cell death (Levin et al., 1999). Freshly isolated neuron C1s might be injured during cell culture procedures when their nerve trunks were cut. High concentrations of 5-HT might exacerbate this injury and induce necrotic cell death, by not allowing the damaged membrane to fully recover. Thus, it is necessary for regenerating neurites to undergo a minimum recovery time before regenerative neurite outgrowth can occur (Zhou and Cohan, 2001; Kim et al., 2003). This argument may support the fact that high doses of 5-HT such as 50 and 100 μM have often been used in neurite elongation studies, where a minimum recovery time was given for neurons to extend their neurites.

There are a few studies that demonstrated the controversial roles of 5-HT in inducing neuronal cell death (Zilka-Falb et al., 1997; Tajima et al., 2004), or in protecting from neurotoxicity (Kamei et al., 1991; Garssadi et al., 1994; Cummings and Walker, 1996). Recently, 5-HT-induced neuronal death has been reported in rat cerebrocortical cells, whereby the dose-dependent cytotoxicity was entirely due to necrosis (Tajima et al., 2004). This neurotoxicity occurred

substantially with 100 μM of 5-HT, which is the same concentration used for the 5-HT neurotoxicity of the present study. Morphological features from their studies included blebbing and mitochondrial membrane disintegration, which have been known to be associated with excessive free cytosolic calcium and extracellular glutamate (Sapolsky, 2001; Nicholls, 2004). Glutamate is the most common excitatory neurotransmitter implicated in neuronal excitotoxicity that is linked to acute neuronal insults and chronic neurodegenerative diseases. Thus, it cannot be ruled out that the present 5-HT-induced necrotic cell death may be due to an interactive signaling pathway related to glutamate excitotoxicity. For example, high dose of 5-HT may cause membrane depolarization and induce excessive stimulation of glutamate receptor/ion channel complexes, which trigger high calcium influx (Choi, 1987) and a cascade of intracellular necrotic events. Because the present study used a single neuronal culture system to rule out intercellular interactions, there were no glial cells in culture. Thus, applied 5-HT could not have a chance to modulate the glial conversion of glutamate to glutamine (Meller et al., 2002). Cell-swelling is known to be attributed to rapid influxes of Na^+, Cl^- and H_2O through kainatic acid (KA) receptors (McDonald and Johnston, 1990). Thus, 5-HT may interact with KA receptors, or more likely its own receptor/ion channels, such as $5\text{-}HT_3$ receptors, that mediate rapid influxes of Na^+, Cl^- and H_2O to induce cell-swelling. Catecholamine neurotransmitters and their metabolites have also been reported to show putative neurotoxic effects (Burke et al., 2004). However, the catecholamine neurotoxicity was not tested here, and thus, cannot be discussed in the present study. So far, the underlying

mechanisms for 5-HT-induced neuronal death are unknown. In addition, the cell death may be caused by overlapping complex events, involving both apoptotic and necrotic pathways. Further experiments examining DNA fragmentation, calcium signaling and caspase activation will give a more complete interpretation.

pCPA and tryptophan hydroxylase

Tryptophan hydroxylase is the rate-limiting enzyme for 5-HT biosynthesis (Lovenberg et al., 1967; Jéquier et al., 1967 & 1969; Gál and Whitacre, 1982). Therefore, experimental attempts to regulate enzyme activity or expression can lead to modifications of 5-HT levels in the brain. *p*CPA is one of the most specific chemicals used to reduce the level of 5-HT by inhibiting tryptophan hydroxylase (Gál and Whitacre, 1982). The physiological and molecular mechanisms of tryptophan hydroxylase inhibition by *p*CPA treatment are still unclear. In a previous study, *p*CPA was suggested to be incorporated, at or near the active substrate-binding site of tryptophan hydroxylase (Gál et al., 1970; Fratta et al., 1973; Gál and Whitacre, 1982). 5-HT biosynthesis in *C. elegans* requires the *tph-1* gene, which encodes a tryptophan hydroxylase (Sze et al., 2002). In a previous HPLC study, *p*CPA successfully reduced the embryonic 5-HT contents, without significantly altering embryonic dopamine levels (Dieffenbach et al., 1995). In that study, *p*CPA's effectiveness was confirmed by the transient abolition of 5-HT immunoreactivity in the embryonic serotonergic neuron ENC1.

The present study applied *p*CPA through two different routes; bath-application and intramuscular-injection. In bath-application, 0.02 % *p*CPA was added directly into the defined medium at the beginning of the brain-conditioning period for the s-CM. In intramuscular-injection, 0.1 mg *p*CPA was injected intramuscularly into feet of *Helisoma* 6 hours before conditioning media. After completion of a 72-hour conditioning period, both treatments of *p*CPA attenuated the 5-HT content of 72 hour CM (s-CM) to insignificant levels (less than 0.2 μM) for inhibiting neurite initiation. These levels were close to those observed in the modified 96 hour CM. Attenuation of 5-HT levels by both treatments was in a time-dependent manner. Diefenbach and colleagues (1995) also demonstrated that the 5-HT content of *Helisoma* embryos was significantly reduced within first 24 hours after 1 hour bath-treatments of the same dose of *p*CPA (0.02 %) as used in the present study.

In the present study, the intramuscular injection of *p*CPA revealed a maximal decrease of 5-HT content in the medium 72 hours after an initial injection, while bath-treatment of *p*CPA revealed a maximal reduction of 5-HT within the first 24 hours. Moreover, maximally decreased expression of tryptophan hydroxylase has been demonstrated 48 hours after a single intraperitoneal injection of *p*CPA in previous studies (Richard et al., 1990; Weissmann et al., 1990). This time-discrepancy in the variations of 5-HT levels may be attributed to different numbers of serotonergic neurons affected through different *p*CPA-delivery routes. Both the nature and the degree of drug effect may be influenced by the route of administration, due to effects at the portals of entry

or to effects on pharmacokinetic processes. For example, toxins injected by the intravenous route would be expected to result in the highest degree of toxicity. When administered by other routes, the approximate descending order of toxicity is inhalation > intraperitoneal > subcutaneous > intramuscular > intradermal > oral > topical (Klaassen, 1986; Gossel and Bricker, 1990). Bath-treatment is likely to have similar drug efficiency to intravenous injection or inhalation. Thus, effects of *p*CPA on tryptophan hydroxylase via the three different routes are comparable (bath-treated, 24 hr > intraperitoneal, 48 hr > intramuscular, 72 hr).

Furthermore, bath-treated *p*CPA may still remain in the medium during the conditioning periods, although there is a possibility for *p*CPA to be broken down. Therefore, in the present study, availability of the bath-treated *p*CPA was likely even higher than intramuscular-injected *p*CPA. The half-life of tryptophan hydroxylase is about 1.5 days in physiological conditions (Richard et al., 1990). Metabolites of *p*CPA such as *p*-chlorophenylacetic acid do not appear to affect the activity of tryptophan hydroxylase (Gál and Whitacre, 1982).

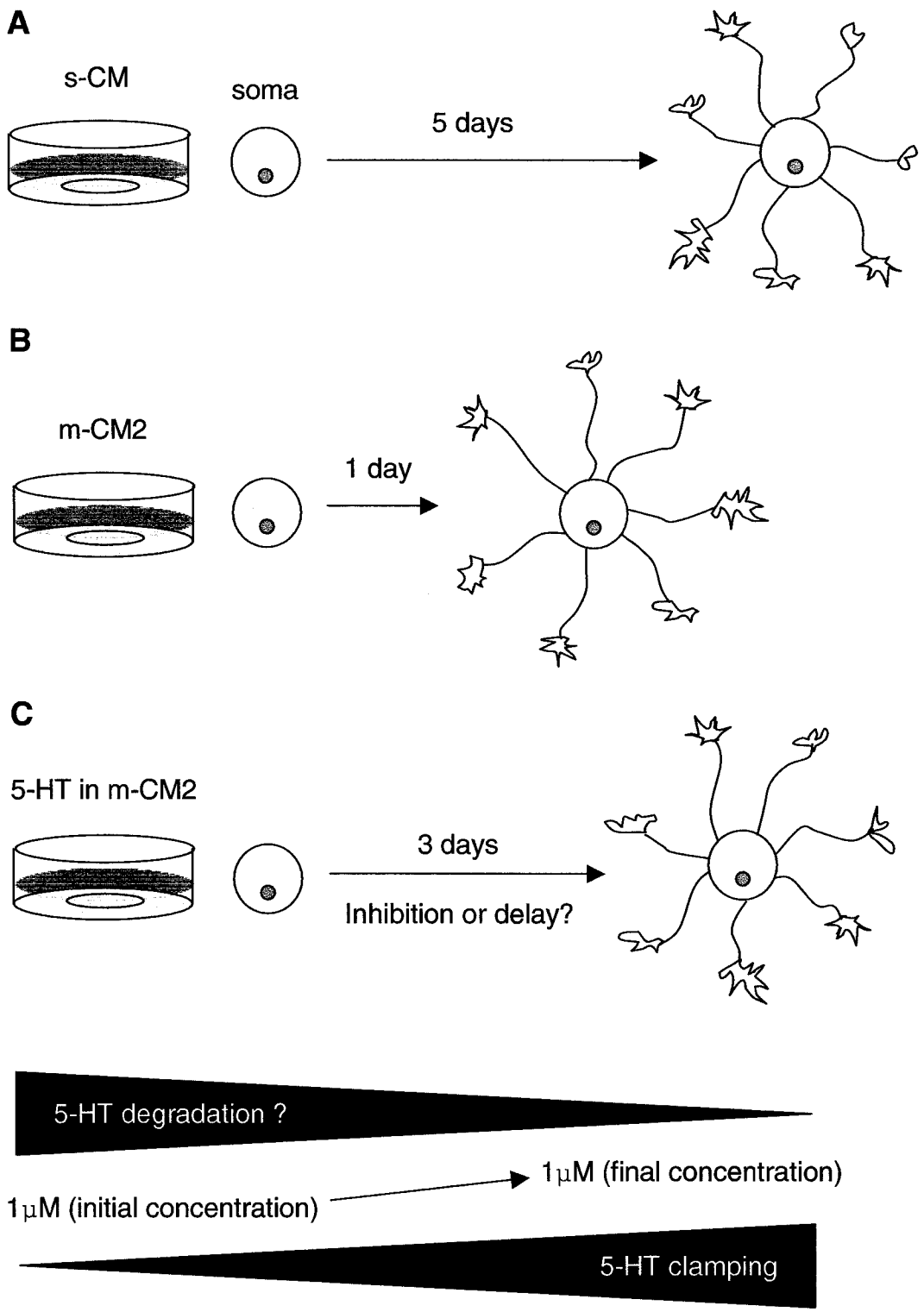
Tryptophan hydroxylase was previously found in cell bodies and axon terminals of the raphe neurons (Weissmann et al., 1987; Raison et al., 1995), whereas such localization studies were not performed in snail brains. *p*CPA induced a dramatic decrease in the number of tryptophan hydroxylase-immunoreactive cells (Raison et al., 1996) supporting the action that it affects the expression of tryptophan hydroxylase. Besides these immunocytochemical studies, attenuated expression of tryptophan hydroxylase by *p*CPA (300 mg/kg, ip) was quantified through an immunoblot of rat raphe neurons (Richard et al.,

1990). Western blotting analysis in the present study showed that both types of *p*CPA treatments decrease the protein expression of tryptophan hydroxylase in snail brains. Bath-treated *p*CPA appears more effective in reducing the enzyme expression than intramuscular-injection, consistent with the present HPLC study on 5-HT contents in the s-CM (see above). Therefore, reduced 5-HT synthesis by *p*CPA in the present study, might be induced by tryptophan hydroxylase down-regulation. Interestingly, bath-applied 5,7-DHT, which has been commonly used to deplete 5-HT, did not attenuate the expression of tryptophan hydroxylase, whereas both *p*CPA and 5,7-DHT increased the expression of tubulin protein in brains. That is not surprising because both agents can reduce 5-HT levels in snail brains, which is commonly known to be an inhibitory factor in neurite outgrowth (Haydon et al., 1987; Cohan et al., 1987; Goldberg, 1998). Tubulin has the same molecular weight (55 kDa) as tryptophan hydroxylase, and thus was detected with anti β-tubulin after stripping anti-tryptophan hydroxylase from the PVDF membranes. Since the formation of new β-tubulin has been suggested to be a specific marker for neurite outgrowth (Edde et al., 1983; Sekimoto et al., 1995; Dybowski et al., 1999; Petridis et al., 2004), the increased tubulin expression in the present study suggests a specific inhibitory effect of *p*CPA on tryptophan hydroxylase activity in order to reinitiate the neurite outgrowth suppressed by tonic inhibitory 5-HT in the s-CM.

Taken together, cultured neuron C1 initially forms a relatively huge lamellipodium surrounding its soma and projects many filopodia under its own optimum culture

condition such as our modified 2nd CM that lacked 5-HT. The lamellipodium protruded into several smaller lamellipodia, rearranged cytoskeleton proteins and formed initial neurites to prepare the neuron for neurite elongation. 5-HT is released into CM during the conditioning process to sub-cytotoxic levels. It causes a delay in the onset of neurite elongation, probably by inhibiting outgrowth until the concentration of 5-HT falls below active levels. The modified CM procedure developed in this thesis results in a lower level of 5-HT in the present CM. Thus, the improvement of the culture system allows for an earlier onset of neurite elongation, especially in the serotonergic neuron C1 that has never been used before for neurite outgrowth studies. The late onset of neurite outgrowth can be recovered by *p*CPA treatments, reducing the expression of tryptophan hydroxylase in brains and decreasing 5-HT levels in CM. In conclusion, I suggest tonic inhibitory roles of 5-HT in the regulation of phase transition during neurite outgrowth of the serotonergic neuron C1.

Figure 31. The onset of neurite elongation in neuron C1. There are two possible models (inhibition or delay) to explain effects of 5-HT on the onset of neurite elongation. A. Standard conditioned medium (s-CM) induced the onset of neurite elongation five days after cell plating. B. The second modified conditioned medium (m-CM2) induced the onset of neurite elongation within 24 hours after plating. C. 5-HT (1 μM) added to m-CM2 also caused late onset of neurite elongation at three days in culture. If the degradation of 5-HT by oxidation lowers the [5-HT] concentration and releases the neuron from inhibition, clamping the [5-HT] at levels found in s-CM on day 1 should prevent the onset of neurite elongation. If neurite elongation still began around 3-5 days in culture even when the [5-HT] was clamped, then the effect of 5-HT is to delay, rather than prevent, the onset of neurite elongation.



2) Differential regulatory effects of 5-HT on neurite elongation

Central neurons in the vertebrate brain are known not to regenerate (Schwab et al., 2005) although peripheral neurons are often capable of regeneration (Hall, 2005). The ability of molluscan central neurons to regenerate relies on intrinsic and extrinsic factors (Murphy et al., 1985). These neurons form growth cones and elongate neurites *in vitro* during regeneration. Important modulatory roles of 5-HT, an extrinsic diffusible cue for neurite elongation, were demonstrated in this study using an identified cerebral serotonergic neuron C1 under its optimal culture condition (m-CM2). First of all, the serotonergic phenotypic expression of the neuron C1 in cerebral ganglia was confirmed using immunohistochemical techniques, and the neuron C1 in cultures was examined for the confirmation of 5-HT phenotype using immunochemical labelling. Immunoblotting for tryptophan hydroxylase helped neuron C1 to be identified as a 5-HT synthesizing neuron. Under the optimal outgrowth condition, neuron C1 had many neurites at different elongation rates including slow-growing, fast-growing and collapsing neurites. Slow-growing neurites grew at a rate of 1 ~ 20 μm per 60 minutes, whereas fast-growing neurites grew at a rate of >20 μm per 60 minutes. Collapsing neurites had negative values in total outgrowth for 60 minutes. 5-HT affected neurite elongation rates in the serotonergic neuron C1 and changed the appearance of associated growth cones. In addition, two types of neurites with different growth cone widths responded differentially to 5-HT, changing their intracellular calcium levels in a different manner. The phenotypic

expression of two newly cloned *Helisoma* 5-HT receptors such as 5-HT_1hel and 5-HT_7hel was found to differ depending upon the neurite elongation rate. For instance, the soma and only some of the fast-growing neurites expressed 5-HT_1hel receptor, while 5-HT_7hel receptor was only localized to cell bodies. Taken together, 5-HT is likely to modulate neurite elongation rates of neuron C1 by transforming the growth cone structure and by affecting intracellular calcium levels through different subtypes of *Helisoma* 5-HT receptors.

Phenotypic comparison of neuron C1 in situ and in culture of Helisoma cerebral ganglion.

In a previous study of Granzow and Rowell (1981), a bilaterally symmetrical pair of serotonergic neurons at the anterior edge of the dorsal surface of cerebral ganglia was described in the freshwater pond snail, *Helisoma*. These cells are yellowish, often having a crescent band in contrast with paler surrounding cells. Thus, these cells are readily recognizable. Homologous cerebral neurons have also been identified in other gastropods including *Aplysia californica, Helix aspersa, Lymnaea stagnalis, Limax marginatus,* and *Tritonia diomedea* (Kerkut et al., 1967; Salanski et al., 1968; Osborne and Cottrell, 1971; Pentreath et al., 1973; Weinreich et al., 1973; Berry and Pentreath, 1976). Four common features of all these neurons have been suggested; 5-HT content, large size, a complex projection with axons extending down the cerebrobuccal connectives into buccal nerve trunks and the ability to influence neurons in the buccal ganglia. All four of

these features have been confirmed for the monopolar neuron C1 of *Helisoma* in previous studies (Granzow and Rowell, 1981; Murphy et al., 1985; Gadotti et al., 1986). The present study has again confirmed those features using *in situ* and/or *in vitro* immunostaining techniques for 5-HT, and extended this analysis by revealing tryptophan hydroxylase expression in neuron C1 through an immunoblotting technique. Tryptophan hydroxylase is a rate-limiting enzyme for 5-HT biosynthesis, and its presence in neuron C1 indicates that 5-HT is synthesized in this neuron, rather than produced in neighboring cells, released and transported into neuron C1 (Goldberg and Kater, 1989).

There have been at least 5 serotonergic neuronal clusters (I-V) identified in each cerebral ganglion (Goldberg and Kater, 1989). Neuron C1 and several surrounding satellite cells express phenotypic immunoreactivity of 5-HT in cluster I. The present study confirms these previous findings (Goldberg and Kater, 1989) on the distribution of cerebral serotonergic neurons and provides greater detail on the 5-HT immunoreactive cells around neuron C1. Although the resolution produced by the wholemount 5-HT immunofluorescent staining can be varied due to the thickness of the ganglionic sheath and the degree of antibody penetration, neuron C1 has always been identified as the largest serotonergic neuron (diameter of its soma; 80 ~ 100 μm) in the cerebral ganglion. However, its size does not much exceed that of other non-serotonergic neurons in the cerebral ganglion, and thus should not be referred to as a giant cell, as done in other gastropods such as *Aplysia* and *Helix*. Compared with 5-HT immunofluorescent staining of the cerebral ganglia, intracellular injection of Lucifer Yellow, a

fluorescent dye, into neuron C1 reveals different and complimentary representations of the morphology of neuron C1 (Murphy et al., 1985). Lucifer Yellow injection into neuron C1 is optimal for staining the soma, major axon branches and dendritic arborization in the neuropile of the ipsilateral cerebral ganglion. However, immunocytochemical staining can reveal terminal axon branches on distant targets and show an extensive plexus of fine fibers in the sheaths of ganglia and nerve trunks.

Cultured neuron C1 has also been found to express 5-HT-like immunoreactivity. This finding suggests that the axon-severed neuron C1 can conserve its original serotonergic phenotype during the cell culture procedure. All parts of neuron C1 including neurites and growth cones displayed 5-HT-like immunoreactivity, whereas some other neurons examined in control experiments of the present study did not show 5-HT-like immunoreactivity in any part of the neuron. These findings are in contrast to a previous co-culture study, where even non-serotonergic neurons as well as neuron C1s showed 5-HT-like immunoreactivity in their soma (Murphy et al., 1985). However, several neurons such as neuron C1 and B5 were co-cultured on the same dish in that study while the present study used a single cell culture system. Thus, 5-HT released from the serotonergic neuron could be taken up by the other neurons in the same culture. Neuron density might also induce 5-HT-like phenotypic expression in other non-serotonergic neurons. Likewise, a previous cell culture study on sympathetic neurons supports differential neurotransmitter phenotypic expression depending on neuronal density (Adler and Black, 1985). Moreover, dissociating and culturing

procedures changed neurotransmitter phenotype plasticity in rat dorsal root ganglionic neurons (Schoenen et al., 1989). In light of these examples of phenotypic plasticity, it was critical to confirm that the 5-HT phenotype is preserved in neuron C1s that are subjected to the optimal culture condition (m-CM2) for the neurite outgrowth studies.

Three different rates of neurite elongation found in cultured serotonergic neuron C1s.

Neurons require addition of new plasma membrane material to extend their projections. Local incorporation of a fluorescent lipid analog into the plasma membrane of fast-growing *Xenopus* neurites revealed an anterograde bulk membrane flow that correlated with neurite elongation (Popov et al., 1993). The rate of membrane flow depended on the position of the labeled membrane segment along the neurite, increasing with distance from the soma. This previous study suggests that neurite elongation involves the direct addition of new plasma membrane along the neurite and at the cell body, but not at the growth cone. Thus, the rate of addition of new membrane to the neurite may determine the rate of neurite elongation. It has also been known that neurite elongation is associated with rates of microtubule advance and F-actin turnover (Summers and Kirschner, 1979; Lin and Forscher, 1995). The present results revealed that elongating neurites in neuron C1 have various elongation rates, even in the same neuron. Neuron C1 exhibited a bimodal distribution for rates of neurite elongation

(see Figure 18), and its neurites were separated into two populations. Based on a trend regression analysis for frequency distribution over elongation rates, 20 μm length outgrowth per 60 minutes was a boundary to determine these two different elongation-rate populations of neurites. Thus, I designated that slow-growing neurites grow at a rate of 1 ~ 20 μm per 60 minutes while fast-growing neurites grow faster than 20 μm per 60 minutes. At the beginning of the present study, these two groups of neurites were hypothesized to have significantly different responses to the application of 5-HT. Identification of collapsing neurites was clear since they have negative values in total outgrowth for 60 minutes.

All three different groups of neurites were found in 24 ~ 72 hour cultures showing an interesting age-dependent pattern. A larger population of slow-growing neurites was found in 24 hour cultures and the number of fast-growing neurites was much higher than that of collapsing neurites. In 48 hour cultures, fast-growing neurites were found in higher occurrence than slow-growing neurites. However, this higher occurrence of fast-growing neurites was not observed in 72 hour cultures. Instead, the number of collapsing neurites increased depending on the number of days in culture. Thus, initial neurites of neuron C1 might grow slowly and have large growth cones, and then gradually grow more rapidly with smaller growth cones, followed by collapse. A similar differential age-dependent pattern of neurite outgrowth has also been shown between young and mature brainstem-spinal neurons (Borisoff et al., 2000) and between co-cultured young and mature hippocampal and entorhinal neurons (Li et al., 1995). Younger brainstem-spinal neurons show robust neurite elongation, whereas most mature

brainstem-spinal neurons fail to extend neurites or require a longer time for the onset of neurite elongation. The diminished outgrowth capacity of mature neurons in the above previous studies is in line with the reduced elongation-rate in 72 hour cultures in the present study. In addition, the differential age-dependent outgrowth between axons and dendrites was reported in the long-term culture study using hippocampal neurons (Mattson and Kater, 1988). Axons continued to grow at a relatively constant rate while dendritic outgrowth slowed during the second week of culture and ceased by the end of third week.

The present age-dependent pattern of elongation rates may be due to the presence or absence of different neuronal growth factors in the brain-conditioned medium at different culture stages. To test this, an HPLC analysis should be performed, along with experiments testing the effects of antibody-preabsorption or addition of known neuronal growth factors. It is suggested that different ECM molecules can induce differential rates of neurite elongation (Wehrle-Haller and Chiquet, 1993). Laminin elicits neurites that not only grow fast but are also thin, as is characteristic of axons (Rivas et al., 1992). Laminin is also shown to affect microtubules as well as actin networks (Tang and Goldberg, 2000). Moreover, acute addition of laminin to rat sympathetic neurons induces immediate microtubule advance and its bundling within the initial widely spread growth cone, followed by the outgrowth of thin, rapidly growing nascent axons (Tang and Goldberg, 2000). In contrast to the fast outgrowth caused by laminin and fibronectin, tenascin expressed within the developing peripheral nervous system induces a slower rate of neurite outgrowth after a lag phase. Strength of

adhesion to the ECM substrates is also positively correlated with the amount and complexity of neurite outgrowth in developing neurons (Letourneau, 1975; Chamak and Prochiantz, 1989). The strength of cell adhesion to the ECM molecules, can be enhanced by increasing the level of NCAMs (Bozyczko and Horwitz, 1986; Tomaselli et al., 1988; Wu et al., 1996). Therefore, the existence of three different classes of elongation rates in neuron C1 neurites under the optimum culture condition may be understood with changes in the adhesion strength following changes in levels of intrinsic NCAMs. However, there is no evidence, so far, that NCAMs are differentially expressed in neuron C1 neurites undergoing different elongation rates. This is an area to be explored in the near future. A recent study suggests that the high level of cytoplasmic dynein light chain Tctex-1 at hippocampal growth cones drives fast neurite extension by modulating actin dynamics and Rac1 activity (Chuang et al., 2005). This is another case that explains involvement of intrinsic factors in the regulation of outgrowth rate. In the present study, some putative growth cues in CM may trigger rapid and local synthesis, degradation and endocytosis of proteins, providing a fast and flexible way for growth cones to respond to cues in their microenvironment and to alter their responsiveness. Thus the localized synthesis, and down-regulation of receptor proteins by mechanisms such as membrane trafficking and internalization may contribute to understanding various neurite elongation rates and may contribute to understanding morphological changes of growth cones when neurites age.

Functional relationships between growth cone width and rate of neurite elongation.

Various neurite elongation rates were previously suggested from identified *Helisoma* neurons (Boddy, 2002), and different morphology of growth cones was often observed in outgrowing neuronal cultures. Interestingly, there is an *in vivo* study that tried to correlate growth cone morphology with elongation rate (Brittis et al., 1995). In this study, growth cone size of retinal ganglionic axons becomes bigger when outgrowth slows down. However, this study did not strictly measure neurite elongation rate, but growth cone extension rate toward a target. Therefore, a more practical correlation needs to be established for further study. Present results first suggest that growth cone widths may be relevant to neurite elongation rates, showing some significant differences in the growth cone width between slow-growing neurites and fast-growing neurites. Slow-growing neurites (11 ~ 20 μm per hour) displayed growth cone widths around 30 ~ 40 μm, while fast-growing neurites (36 ~ 40 μm per hour) had growth cone widths around 20 μm. Thus, growth cones of 30 ~ 40 μm in width were excellent predictors of a slow rate of neurite elongation. These slow-growing neurites were observed primarily in 24-hour cultures. In contrast, growth cones of 20 μm in width were excellent predictors of a fast rate of neurite elongation. These fast-growing neurites are observed primarily in 48-hour cultures. These findings will make it possible to compare cellular and molecular properties from slow- and fast-growing neurites without the requirement of neurite outgrowth measurements.

Moreover, these results are consistent with a previous report that smaller growth cones may be associated with fast-growing neurites containing closely bundled microtubules (Burden-Gulley and Lemmon, 1996).

Differential effects of 5-HT on neurite elongation through the activation of its putative receptors.

5-HT is known to selectively inhibit neurite outgrowth in many different types of neurons (Haydon et al., 1984 & 1987; Janakait et al., 1988; McCobb et al., 1988; Sikich et al., 1990; Lauder, 1990; Lauder et al., 1992; Diefenbach et al., 1995; Urbina et al., 1996; Koert et al., 2001). In contrast, facilitatory effects of 5-HT on neurite outgrowth are also suggested in thalamic neurons (Lotto et al., 1999; Lieske et al., 1999). Thus, biphasic fine-tuning effects including both inhibition and facilitation may be putative roles of 5-HT in modulating neurite outgrowth during neuronal development and regeneration. There are a couple of previous studies showing the biphasic effects of 5-HT in mass-dissociated neurons, including vertebrate embryonic monoaminergic neurons (Liu and Lauder, 1991) and silk moth antennal lobe neurons (Kim et al., 2003). However, all of those studies included many different types of cells in their cultures. Thus, their biphasic effects may have occurred because of a differential effect of 5-HT on many different cell types. Goldberg and colleagues (1991) also have suggested biphasic effects of 5-HT on neurite outgrowth using molluscan embryonic or adult mass-dissociated cultures. In their study, 5-HT arrested

neurite elongation in a significant percentage of elongating neurites in a dose-dependent manner, whereas 5-HT caused the reinitiation of neurite elongation in a significant percentage of non-elongating neurites. Moreover, this study proposed the possibility that different neurites from the same adult neuron might display differential sensitivity in their outgrowth response to 5-HT. The present study supports the idea by using an identified single neuron culture, showing that 5-HT has differential effects on neurite outgrowth in the same cell, depending on the rate of neurite elongation. There is another example of differential effects of neurotransmitters on neurite outgrowth from the present laboratory. In that study, an atypical neurotransmitter, nitric oxide (NO), enhanced elongation in slow-growing neurites of buccal ganglionic neuron B5, whereas NO inhibited elongation of fast-growing neurites in the same neuron (Boddy, 2002).

In slow-growing neurites of the present study, 5-HT had biphasic effects on the regulation of neurite elongation. Lower doses of 5-HT (1 and 10 μM) facilitated neurite elongation while higher doses of 5-HT (50 and 100 μM) inhibited elongation and/or collapsed growth cones. Therefore, the present study provides the first evidence to show biphasic effects of 5-HT on the neurite outgrowth of a single identified serotonergic neuron. Thus, neurites of neuron C1 can respond differentially to varying 5-HT concentrations. In contrast to neurons in mass-dissociated cultures, the present single cultured neuron C1 could not possibly be affected by cellular interactions. Accordingly, these biphasic effects on the control of neurite elongation rate are attributed to direct 5-HT action on its receptors in neuron C1. The direct action of 5-HT may stimulate one or two

different type(s) of 5-HT receptor, changing $[Ca^{++}]_i$ levels either in a dose-dependent manner or in an affinity-dependent manner.

In the present study, the biphasic effects were only observed in slow-growing neurites, but not in fast-growing neurites. Thus, the same lower doses (1 and 10 μM) of 5-HT could either facilitate elongation of slow-growing neurites or inhibit elongation of fast growing-neurites. This finding is consistent with the idea that different types of 5-HT receptors are involved in mediating these various effects. Dose-dependent 5-HT regulation of $[Ca^{++}]_i$ levels may contribute to the biphasic modulation of neurite elongation rates as well (Figure 32). For example, slow-growing neurites in the present study might initially have a lower $[Ca^{++}]_i$, which is below the elongation-optimal level but within the elongation-permissive range. Exposure of neuron C1 to 5-HT might increase the $[Ca^{++}]_i$ in a dose-dependent manner via a putative 5-HT receptor. Hence, lower doses of 5-HT might raise the $[Ca^{++}]_i$ of slow-growing neurites up to the optimal level to switch them into fast-growing neurites. However, higher doses of 5-HT might drive the $[Ca^{++}]_i$ beyond the level supporting optimal elongation, thus producing inhibition. All above assumptions reflect well how 5-HT receptor subtype(s) can be involved in the biphasic effects of 5-HT on elongation of slow-growing neurites (Figure 33). To test if one or two receptors are involved in mediating the biphasic effects, an efficient antagonism against dose-responses for the biphasic effects of 5-HT on neurite elongation has to be explored in future studies. Mianserin, an antagonist with a broad spectrum of 5-HT receptor affinity (van der Ven et al., 2006; Harvey, 2003; Anttila and Leinonen, 2001), is not likely to be a good choice for the

blockade of those biphasic effects on neurite elongation, based on my preliminary experiments.

The present finding that 1 μM of 5-HT effectively increased the elongation rate of slow-growing neurites suggests the involvement of a high affinity 5-HT receptor (Figure 33). In a previous study, a wide range of 5-HT concentrations (0.05 ~ 500 μM) tended to reinitiate neurite elongation in *Helisoma* stable embryonic neurons, in a dose-dependent manner (Goldberg et al., 1991). However, 5-HT doses lower than 50 μM did not show a significant reinitiation. Therefore, a putative high affinity 5-HT receptor involved in the present outgrowth-facilitatory effect seems to be different from the *Helisoma* embryonic 5-HT receptor that reinitiates neurite elongation. Although 5 μM of 5-HT was not tested in the present study, this dose was previously reported to facilitate elongation of some neurites of the buccal ganglionic neurons in *Helisoma* (Goldberg et al., 1991). This previous finding is in line with the present study, where lower doses of 5-HT facilitated elongation of slow-growing neurites of neuron C1 through a putative high affinity 5-HT receptor. Interestingly, a high affinity G protein-coupled 5-HT receptor (5-HT_{ap1}) was found in every ganglia of *Aplysia* (Angers, et al., 1998). Similar to the vertebrate 5-HT_1 receptor family, the 5-HT_{ap1} receptor is also negatively coupled to adenylate cyclase. It suggests that reduced cAMP levels may be involved in the facilitatory mechanism of neurite elongation in the present study. Neurotransmitter-receptor affinity is similar to the relationship between a lock and key. Thus, a putative high affinity 5-HT receptor proposed in the present study should have the best-fit binding site for 5-HT to

elicit the greatest elongation response from neuron C1. The receptor affinity for 5-HT can be determined by receptor-ligand binding assays in future approaches.

In fast-growing neurites, 5-HT showed only inhibitory effects on neurite elongation in a dose-dependent manner (Figure 34). These findings could be due to the presence of fast-growing neurites in the optimal intracellular Ca^{++} level for neurite elongation. Therefore, either increase or decrease of $[Ca^{++}]_i$ through putative 5-HT receptor(s) could slow down neurite elongation. If the $[Ca^{++}]_i$ levels rose higher than the elongation-permissive range, growth cones might collapse. 1 μM of 5-HT did not significantly affect the elongation of fast-growing neurites. This suggests that a different subtype of 5-HT receptor from the putative high affinity 5-HT receptor that promotes faster outgrowth in slow-growing neurites, may exist in fast-growing neurites. 10 μM of 5-HT induced fast-growing neurites to slow-down, whereas higher doses of 5-HT (50 and 100 μM) caused most neurites to collapse. Interestingly, a similar inhibitory effect caused by 10 μM 5-HT was found in the axonal regeneration of neuron C1 in the cerebro-buccal ganglia culture (Murrain et al., 1990). However, the neuron C1s in the ganglia cultures might have both *in situ* slow-growing and fast-growing neurites, which were inhibited by 10 μM 5-HT. This is different from the present effects of 10 μM 5-HT on slow-growing neurites. Thus, those different effects of 10 μM 5-HT on *in situ* and *in vitro* neurite outgrowth might be due to different culture conditions. I have not yet determined if the 5-HT receptors involved in inhibiting fast-growing and slow-growing neurites are the same. This determination should be performed by blocking the 5-HT inhibition in respective elongating neurites, after the

identification of antagonists, the development of antibodies or molecular knockdown experiments for the putative inhibitory 5-HT receptors. Moreover, it cannot be ruled out that two different types of inhibitory 5-HT receptors could be involved in inhibition or collapse of fast-growing neurites, respectively.

In collapsing neurites, all doses of 5-HT led to a cessation of neurite outgrowth, which recovered their outgrowth after the removal of 5-HT (data not shown). However, 1 μM of 5-HT often induced an irreversible collapse. 5-HT is known to induce growth cone collapse of *Helisoma* neurons (Zhou and Cohan, 2001) that is characterized by loss of leading edge F-actin and a decrease in the number of radially aligned F-actin bundles. A recent study has supported that collapsing factors can induce alterations in the growth cone cytoskeleton. A common change induced by collapsing factors in the cytoskeleton of the peripheral domain, the thin lamellipodial area of growth cones, is a decline in the number of radially aligned F-actin bundles that form the core of filopodia (Torreano et al., 2005). In their studies, 5-HT depolymerises and possibly debundles F-actin filaments that form the core of filopodia. Additionally, they also suggest a dense actin-ring around the central domain of growth cones, formed by other collapsing factors (Zhou and Cohan, 2001; Torreano et al., 2005). The actin-ring inhibits microtubule extension into the P-domain that is a necessary event for neurite elongation.

On the other hand, the present study first suggests that 5-HT might influence the age-dependent occurrence pattern of different elongation-rate neurites (see Figure 19 & Figure 25-28). This finding implies that 5-HT may have

long term, programmed, fine-tuning effects *in vivo* on neurite outgrowth of neuron C1. A previous *in vivo* study suggested that serotonergic neuron C1 has a regulatory mechanism of neurite outgrowth (Murphy et al., 1985). *In vivo*, neuron C1 sprouts within 12 hours of axotomy, shows distinctive neuritic structures 2 to 3 days after axotomy, and regenerates within 8 days to recover its regulation of feeding motor output from buccal ganglionic neurons such as B19. However, it is not clear whether the regenerative neurite outgrowth is autoregulatory.

Putative sources of 5-HT that can affect neurite elongation of neuron C1

Serotonergic neuron C1 exists as a bilaterally symmetrical pair in the cerebral ganglia of *Helisoma trivolvis*. This neuron can display spontaneous action potentials *in situ* at a firing rate of two impulses per second. The pattern of firing in neuron C1 can be modified *in vivo* by a high level of inhibitory synaptic input (Granzow and Rowell, 1981). If intracerebral inhibitory serotonergic sources are one of the high level synaptic inputs, endogenous 5-HT possibly modulates the neurite outgrowth of neuron C1 *in vivo*. Three possible *in vivo* sources for 5-HT are the following. First, neuron C1 may have synaptic inputs from its surrounding serotonergic neurons, which belong to the serotonergic neuronal cluster I (see Figure 17). Second, neuron C1 may also have synaptic inputs from other long-distance neurons, which belong to the serotonergic neuronal cluster II – V (see Figure 17). These two cases are related to effects of 5-HT on neurite outgrowth through 5-HT homo-receptors. Last, serotonergic neuron C1 may self-

release its synthesized 5-HT to regulate its own neurite elongation, which is the case for 5-HT autoregulation in neurite elongation. In this case, 5-HT autoreceptors may mediate a regulatory mechanism in neurite elongation.

Possibility of 5-HT autoregulation in controlling neurite elongation rate

Regulatory roles of neurotransmitters in neurite outgrowth have been elucidated with many previous studies using molluscan central neurons (Haydon et al., 1987; Bulloch, 1987; McCobb et al., 1988; McCobb and Kater, 1988; Goldberg and Kater, 1989; Price and Goldberg, 1993; Diefenbach et al., 1995; Spencer et al., 1996 & 1998; Koert et al., 2001) and vertebrate developing neurons (Lankford et al., 1988; Mattson et al., 1988; Mattson and Kater, 1989; Lauder et al., 1989; Sikich et al., 1990; Riad et al., 1994; Yan et al., 1997; Hery et al., 1999). 5-HT is most commonly studied for its modulatory roles in neurite elongation of non-serotonergic neurons. However, serotonergic neurons can be hypothesized to release their own neurotransmitter, not only to regulate the neurite outgrowth of other neurons, but also to autoregulate their own neurite outgrowth. Thus, it is of interest to discuss how self-released 5-HT affects neurite outgrowth of the same serotonergic neuron through putative 5-HT autoregulatory receptors.

Developing raphé neurons contain 5-HT immediately after their differentiation and rapid extension of axons towards the forebrain (Lauder, 1990). This may be correlated to the assumption that self-released 5-HT may play an

inhibitory role in the autoregulation of neurite outgrowth. The 5-HT-mediated inhibitory autoregulation of neurite outgrowth was suggested before from several previous studies using vertebrate or invertebrate serotonergic neurons (Haydon et al., 1984; Whitaker-Azmitia and Azmitia, 1986; Budnik et al., 1989; Diefenbach et al., 1995; Koert et al., 2001). However, some of those studies applied exogenous 5-HT instead of examining endogenous 5-HT effects on neurite outgrowth, and the others did not show if 5-HT released from a serotonergic neuron directly affected the neurite elongation of the same neuron. Thus, it is difficult to conclude that autoregulation of neurite outgrowth has previously been shown in serotonergic neurons. In the present study, a single serotonergic neuron C1 was cultured to offer a possibility of future study for autoregulatory effects of 5-HT on neurite outgrowth. Under this single cell culture condition, neurite outgrowth of neuron C1 was only influenced by exogenously applied 5-HT. However, this regulation was not yet induced by self-released endogenous 5-HT. To overcome all above technical difficulties for an examination of autoregulatory neurite outgrowth, a single serotonergic neuron culture should be prepared and examined under control of its endogenous 5-HT level. *p*CPA and 5,7-DHT have been known to be good candidates to downregulate the endogenous 5-HT level in *Helisoma* (Diefenbach et al., 1995). Moreover, *p*CPA was an effective agent in the present study to reduce 5-HT levels during brain-conditioning periods (see Figure 14). Therefore, direct application of *p*CPA to the single neuron C1 culture will be favorable for future study of 5-HT autoregulation in neurite outgrowth. Molecular manipulation to silence the gene expression of putative 5-HT receptors

or the rate-limiting tryptophan hydroxylase enzyme using RNA interference (RNAi) will be an excellent alternative. RNAi refers to the introduction of homologous double stranded RNA (dsRNA) to specifically target a gene's product, resulting in null or hypomorphic phenotypes (Fire et al., 1998).

What aspects of neurite outgrowth should be measured for future 5-HT autoregulation study? It is not clear so far, since the reduction of endogenous 5-HT induced by an early *p*CPA treatment can change an elongation status before determining the elongation rates of sample neurites. The present study will suggest one possibility in following. It was already discussed that the age-dependent pattern of elongation rates in the present study (see Figure 19) may be due to different contents of neuronal outgrowth factors in different stages of cultures. In other words, populations of individual collapsing, slow-growing and fast-growing neurites might be dependent on the breakdown of present conditioning factors. However, it cannot be ruled out that an endogenous factor from the cultured neuron C1 might autoregulate the elongation rates of various neurites. If 5-HT were a candidate for the endogenous factor, it could be synthesized and locally released to autoregulate elongation rates for the age-dependent pattern. Since the present application of 5-HT to the culture already disturbed the age-dependent pattern of elongation rates (see Figure 25, 26, 27 and 28), involvement of 5-HT in autoregulation can be examined in the near future, comparing the age-dependent pattern of elongation rates after the inhibition of endogenous 5-HT synthesis.

Some molluscan giant serotonergic neurons homologous to *Helisoma* neuron C1 (Weiss and Kupfermann, 1976) can re-uptake 5-HT released from other neurons in the cerebral ganglia (Osborne et al., 1975; Gerschenfeld et al., 1978; Burrell and Stefano, 1981). This fact raises the possibility that those identified giant serotonergic neurons can contain 5-HT, but cannot synthesize 5-HT. Thus, it is important to verify the biosynthesis of 5-HT in neuron C1 in order to offer a possible model for autoregulation of 5-HT in neurite outgrowth. Here, the presence of tryptophan hydroxylase, the 5-HT synthesis rate-limiting enzyme in neuron C1, was verified through immunoblotting. This enzyme was detected as a 55 kDa band, consistent with the fact that the antibody was prepared using the 55 kDa active enzyme (Joh et al., 1975). Although release of endogenous 5-HT from neuron C1 has not directly been tested in the present study, there are previous correlated studies supporting the release of endogenous 5-HT from cerebral giant serotonergic neurons (Koert et al., 2001; Ghirardi et al., 2004). Using 5-HT-sensitive sniffer cells, release of 5-HT from neurites and growth cones of the *Lymnaea* cerebral giant cell (CGC) and the *Helix* neuron C1 was demonstrated. The 5-HT release may be enhanced with stimulation of cAMP-PKA signaling system increasing calcium influx (Funte and Haydon, 1993; Ghirardi et al., 2004). For more supportive information for the 5-HT relase, the amperometric analysis using a carbon fiber electrode can be applied to a future study for electrochemical detection of locally released oxidizable 5-HT.

Taken together, the present study using the single neuron C1 culture may offer a good candidate model system to test the possibility of 5-HT autoregulation in neurite outgrowth.

Involvement of intracellular calcium levels in the regulation of neurite outgrowth

Maintenance of calcium at appropriate levels in growth cones is important to determine the status of neuronal elongation (Kater and Mills, 1991). There are effective transport mechanisms for allowing Ca^{++} to enter growth cones (Connor et al., 1990; Ghosh and Greenberg, 1995). The calcium homeostasis may be disturbed by extrinsic factors. In the present study, it was initially hypothesized that 5-HT induces differential changes in the intracellular calcium concentration, $[Ca^{++}]_i$ from different elongation-rate neurites. To answer this question, direct measurements of $[Ca^{++}]_i$, from slow- or fast-growing neurites are necessary. However, it is difficult to measure the $[Ca^{++}]_i$ directly from them in cultured neuron C1s, since fura-2 AM loading for 45 minutes had a negative impact on the elongation-rate measuring process. The present study reported that the width of a growth cone could be significantly associated with a certain elongation rate (see Figure 21). Therefore, I used this correlation to measure $[Ca^{++}]_i$ in growth cones whose widths I specifically knew were correlated with a particular neurite elongation rate. For instance, growth cones of widths 30 - 40 μm were always correlated with slow-growing neurites and were used to measure $[Ca^{++}]_i$ in slow-growing neurites. Additionally, growth cones of width ~15 - 20 μm were always

correlated with fast-growing neurites and were used to measure $[Ca^{++}]_i$ in fast-growing neurites. In the present study, 10 μM of 5-HT was applied to test effects of 5-HT on $[Ca^{++}]_i$ during the regulation of neurite elongation. With this dose, elongation of slow-growing neurites was promoted, and elongation of fast-growing neurites was inhibited. Thus, the hypothesized differential effects of 5-HT on $[Ca^{++}]_i$ levels may be observed in this experiment.

Transient changes in $[Ca^{++}]_i$ have long been known to be involved in the regulation of neurite outgrowth and act as key regulators of growth cone guidance in response to extrinsic growth factors *in vitro* and during *in vivo* development (Lankford and Letourneau, 1989; Connor et al., 1990; Holliday and Spitzer, 1990; Kater et al., 1990; Kater and Mills, 1991; Goldberg et al., 1992; Gu and Spitzer, 1995; Gomez and Spitzer, 1999; Hong et al., 2000; Gomez and Spitzer, 2000). Elevations of $[Ca^{++}]_i$ may require Ca^{++} influx across the plasma membrane through Ca^{++} channels. Many neurons express many different types of voltage-gated Ca^{++} channels and neurotransmitter-gated Ca^{++} channels (Bixby and Spitzer, 1984; O'Dowd et al., 1988; Connor et al., 1990; Gleason and Spitzer, 1998). It is not clear why many different Ca^{++} channels are expressed in neuronal structures. However, the channel activity modulated by membrane potential changes or phosphocycling through kinase/phosphatase reactions may be associated with subtle changes in $[Ca^{++}]_i$. In addition to Ca^{++} influx, release of Ca^{++} from intracellular stores through IP_3-sensitive and/or ryanodine-sensitive Ca^{++} channels can also regulate neurite outgrowth. In the *in vivo* previous studies, negative regulation of IP_3-sensitive channels actually inhibited neurite elongation

(Takei et al., 1998; Gomez and Spitzer, 2000). This implies that a rise of $[Ca^{++}]_i$ through IP_3 channels could be related to facilitation of neurite elongation.

In the cytoplasm where $[Ca^{++}]_i$ increases, calcium-binding calmodulin activates a wide variety of enzymes including Ca^{++}-calmodulin-dependent protein kinases, adenylate cyclase, cyclic nucleotide phosphodiesterase, a protein phosphatase called calcineurin, and nitric oxide synthase (Courtney and Nicholls, 1992; Rasmussen and Means, 1989). Moreover, the outgrowth-promoting GAP-43 is present in growth cones and has been identified as a substrate of PKC and calcineurin (Apel and storm, 1992). Thus, GAP-43 phosphorylated by PKC may promote neurite outgrowth by increasing the amount of F-actin, while dephosphorylation of GAP-43 by calcineurin may slow neurite outgrowth by reducing the amount of F-actin (He et al., 1997).

It has also been suggested that neurite elongation may occur over an outgrowth-permissive range of $[Ca^{++}]_i$ (Kater and Mills, 1991). Maximal outgrowth may occur specifically within a narrow optimal outgrowth zone of the permissive $[Ca^{++}]_i$ range. Based on the calcium set-point hypothesis for the regulation of neurite elongation, different elongation rates of neurites in the present study may be associated with various $[Ca^{++}]_i$ levels, although calcium-independent mechanisms can also mediate transitions between slow- and fast-growing states. Accordingly, slow-growing neurites may be in an outgrowth-permissive range of $[Ca^{++}]_i$, but outside of a narrow optimal outgrowth zone of $[Ca^{++}]_i$, thereby showing slower rates of elongation. In contrast, fast-growing neurites are assumed to be within a narrow optimal outgrowth zone of $[Ca^{++}]_i$ (Figure 32).

The present study revealed that $[Ca^{++}]_i$ increased in the somata and in the larger growth cones of neuron C1 after 5-HT treatment. In contrast, $[Ca^{++}]_i$ in the smaller growth cones of neuron C1 did not change after 5-HT treatment. These findings may suggest that elevations of $[Ca^{++}]_i$ in the somata and in the larger growth cones can be associated with a regulatory mechanism of neurite elongation. The present study previously suggested that the width (~ 40 μm) of larger growth cones used is a predictor of some slow-growing neurites, while the width (~ 20 μm) of smaller growth cones is a predictor of some fast-growing neurites (see Figure 21). Moreover, in the present study (see Figure 22), elongation of slow-growing neurites was promoted by 10 μM of 5-HT, which is the same concentration used in these calcium experiments. Thus, elevations of $[Ca^{++}]_i$ in the larger growth cones may be associated with a facilitatory effect of 5-HT on elongation of slow-growing neurites. In this case, slow-growing neurites transformed to fast-growing neurites. Similar differences in Ca^{++} regulation and sensitivity to $[Ca^{++}]_i$ of different types of neurites were shown in the previous study, where the outgrowth of phasic motor axons were more sensitively inhibited to $[Ca^{++}]_i$ changes than tonic motor axons (Arcaro and Lnenicka, 1997). The Ca^{++}-dependent effects on neurite outgrowth were reduced by the Ca^{++} channel blockers such as Mg^{++}, in contrast to the greater inhibition produced by Ca^{++} ionophore such as A23187.

5-HT (concentration; not described in the study) was shown to depolymerize F-actin of the *Helisoma* B19 growth cone (Torreano et al., 2005), probably involving an increase in $[Ca^{++}]_i$ (Murrain et al., 1990; Torreano et al., 2005). A rise

in $[Ca^{++}]_i$ could activate ADF/cofilin by dephosphorylation through Ca^{++}/calmodulin activated protein phosphatase such as calcineurin (Meberg et al., 1998). Activation of ADF/cofilin facilitated neurite elongation by increasing the actin tread-milling rate, as indicated by increased F-actin turnover (Meberg and Bamburg, 2000). In addition, GAP-43 phosphorylated by Ca^{++}-activated-PKC may promote neurite outgrowth and increase the amount of F-actin. These previous findings are parallel with the present facilitatory effect of 10 μM 5-HT on neurite elongation, although there is a contrary study suggesting that 5-HT depolymerization of F-actin is related to the growth cone collapse (Torreano et al., 2005).

On the other hand, no changes in $[Ca^{++}]_i$ of the smaller growth cones may indicate that fast-growing neurites have already been within a narrow optimal outgrowth zone of $[Ca^{++}]_i$ for the maximal elongation rate, and that this dose (10 μM) of 5-HT may not be strong enough to disturb the calcium homeostasis. In the present study of 5-HT effects on neurite elongation, lower dose (10 μM) of 5-HT inhibited elongation of fast-growing neurites whereas higher doses of 5-HT (50 and 100 μM) induced growth cones of fast-growing neurites to collapse. Thus, it is implied that a higher dose of 5-HT (at least over 10 μM) is required to collapse the growth cone of fast-growing neurites and the inhibitory effect of 5-HT (10 μM) in fast-growing neurites is Ca^{++}-independent. However, it is unclear from the present study whether the Ca^{++}-independent elongation-inhibitory mechanism induced by 10 μM 5-HT is different from the collapsing mechanism caused by higher doses of 5-HT (50 and 100 μM). There are several studies showing that

collapsing factors such as NI-35, collapsin and 5-HT may increase $[Ca^{++}]_i$ through Ca^{++} influx and/or Ca^{++} stores (Bandtlow et al., 1993; Gomez and Spitzer, 2000; Zhou and Cohan, 2001). Therefore, higher $[Ca^{++}]_i$ levels in the growth cones of fast-growing neurites probably caused by higher doses of 5-HT (50 and 100 μM) may be involved in collapsing mechanisms rather than in the inhibition of elongation. Actin bundles in *Helisoma* growth cones are common cytoskeletal targets of collapsing factors, which may use calcium-signaling pathways (Zhou and Cohan, 2001). Based on the previous study, 5-HT is suggested to increase cAMP levels and cause calcium influx. The increased $[Ca^{++}]_i$ may induce growth cone collapse via actin bundle loss by inhibiting calcium-regulated actin-bundling proteins such as α-actinin and frimbrin (Figure 35). In addition, actin-binding β-thymosin was recently identified in neurites of outgrowing neurons in *Lymnaea* neuron cultures (van Kesteren et al., 2006). Thus, this protein can be a candidate target of calcium-activated enzymes.

Neurite retraction is often accompanied with the growth cone collapse. The retraction of microtubules is known to be associated with elevated phosphorylation of myosin light chain (Hirose et al., 1998). However, involvement of calcium in the mechanism of retraction is unknown. Additionally, 5-HT induced the formation of a dense actin-ring around the central domain of growth cones. The formation of the actin-ring occurred subsequent to the loss of actin filament bundles and inhibited microtubule extension into the peripheral domain of growth cones (Torreano et al., 2005). The microtubule extension probably related to myosin phosphorylation is critical to neurite elongation.

The present study revealed an interesting difference in the intracellular calcium responses to 5-HT between peripheral and central domains of the larger growth cones. The central domain of the larger growth cone had a rapid elevation of $[Ca^{++}]_i$ along the neurite shaft, whereas the peripheral domain showed smaller change in $[Ca^{++}]_i$. Since elevations of $[Ca^{++}]_i$ in the larger growth cones are related to a facilitatory effect of 5-HT on elongation of slow-growing neurites, the prominent elevation of $[Ca^{++}]_i$ in the central domain of a larger growth cone could be a main mechanism responsible for 5-HT's facilitation of elongation in slow-growing neurites. Central domains of the larger growth cones are suggested to have more functional Ca^{++} entry sites into the growth cone of slow-growing neurites than peripheral domains. A previous imaging study showed a similar localization of voltage-gated Ca^{++} entry sites in neuronal growth cones from adult *Helisoma* (Connor et al., 1990). From that study, the regions of highest Ca^{++} are suggested to be influx sites, since the ion is free to diffuse within the cytosol. In the resting state, Ca^{++} levels (~ 150 nM) are relatively uniform among adult *Helisoma* neurons (Murrain et al., 1990; Connor et al., 1990). The resting Ca^{++} levels in neurons generally vary by about 35 nM in different parts of individual cells, and no one area is significantly different from another. However, the resting $[Ca^{++}]_i$ increases up to around 300 nM after addition of 5-HT (50 μM) or an electrical stimulation of the *Helisoma* neurons, showing clear differences in $[Ca^{++}]_i$ within the terminal growth cone and back along the neurite. This is consistent with the present suggestion that Ca^{++} entry sites may be unevenly distributed between central domains and peripheral domains of the larger growth

cones. Moreover, the distribution of Ca^{++} entry sites changes between freshly isolated cell bodies without neurites and with neurites, thereby suggesting the redistribution of Ca^{++} entry sites from the soma to growing neurites of the neuron during neurite outgrowth (Connor et al., 1990). It is also interesting from the previous study that dormant old neurites have more Ca^{++} entry sites in the peripheral domain of their growth cones than newly elongating neurites. This may be related to growth cone collapse of dormant neurites.

From previous studies (Gomez and Spitzer, 2000), two different types of Ca^{++} transients have been suggested; Ca^{++} spikes and Ca^{++} waves. The spikes involve Ca^{++}-dependent action potentials and Ca^{++}-induced calcium release. They propagate rapidly throughout neurons, stimulating elevation of $[Ca^{++}]_i$. The waves are localized to growth cones and spread passively by diffusion, decaying in amplitude with distance from the growth cone. In the present study, similar Ca^{++} diffusion was observed from an adjacent shaft (about 100 μm away from the transition zone) of the slow-growing neurite into the central domain of a growth cone. The origin Ca^{++} entry site on the adjacent shaft was determined based on the Ca^{++} diffusion rate (30 - 40 μm/min). Therefore, the elevation of $[Ca^{++}]_i$ observed in the central domain in the present experiments may be mainly caused by Ca^{++} waves through diffusion of the calcium signal into the central domain of a growth cone. This may suggest that facilitatory effects of 5-HT on the elongation of slow-growing neurites could be attributed to the Ca^{++} entry and diffusion from the proximal neurite shaft, but not from the somata.

Involvement of newly cloned Helisoma 5-HT receptors in the regulation of neurite outgrowth

Helisoma 5-HT receptors such as 5-HT_{1hel} and 5-HT_{7hel} are newly cloned receptors (Mapara, 2001). Their corresponding antibodies such as peptide 5-HT_{1hel} (residues 409 – 423, YSRTREKLELKRERK) and peptide 5-HT_{7hel} (residues 246 – 261, YFKIWRVSSKIAKAEA) were used to localize intracellular phenotypic expression of those 5-HT receptors. The present study applied phalloidin-labeling to show the neuronal morphology in detail. Various controlled experiments including the pre-immune serum and KLH preabsorption controls were carried out in order to rule out non-specific binding opportunities.

I have shown that 5-HT_{1hel} receptors are found in some fast-growing neurites with smaller growth cones (~ 10 - 20 μm) as well as the cell body. In contrast, 5-HT_{1hel} receptors were not expressed in slow-growing neurites with larger growth cones (~ 30 - 40 μm). Thus, expression of 5-HT_{1hel} receptors in some fast-growing neurites is likely related to the differential effects of 5-HT on neurite outgrowth, which is dependent on various elongation rates observed in the present study. Since 10 μM of 5-HT did not affect resting $[Ca^{++}]_i$ of fast-growing neurites with small growth cones, the previously proposed Ca^{++}-independent inhibition of 5-HT on fast-growing neurites may be associated with these 5-HT_{1hel} receptors only found on fast-growing neurites. Further, 5-HT_{7hel} receptors were found only in the cell body, and not in any neurites. It is also interesting to note that mammalian 5-HT_{1A} autoreceptors are commonly found in

serotonergic cell body areas such as raphe nuclei (Sotelo et al., 2000; Zhang et al., 1990). The expression of both 5-HT_{1hel} and 5-HT_{7hel} receptors in the soma suggests that hypothesized autoregulatory roles of 5-HT in neurite outgrowth may operate through molluscan putative 5-HT receptors pharmacologically similar to mammalian 5-HT autoreceptors found in soma or dendrites. These mammalian somatodendritic autoreceptors are known to regulate the availability of 5-HT released from the vertebrate serotonergic neurons using negative feedback mechanisms such as the reduction of firing rate or inhibition of 5-HT synthesis (Cerrito and Raiteri, 1980; Rogawski and Aghajanian, 1981; Westerink et al., 1990; Stamford et al., 2000; Ahn et al., 2005).

The pharmacological profile of the putative 5-HT outgrowth-regulatory receptors on neuron C1 in *Helisoma* is unknown. Pharmacological characterization of a novel 5-HT receptor mediating the inhibition of neurite outgrowth was suggested in *Helisoma* neuron B19 (Price and Goldberg, 1993). This 5-HT receptor has a relatively high affinity for 5-HT, and is positively coupled to the elevation of cAMP. This intracellular cAMP increase directly activates a class of cyclic nucleotide-gated sodium channels, leading to sodium influx, membrane depolarization, and activation of voltage-gated calcium channels (Price and Goldberg, 1993). Furthermore, the resulting elevation of $[Ca^{++}]_i$ is suggested to inhibit neurite elongation and growth cone motility of neuron B19 through a calcium/calmodulin-dependent pathway (Cohan et al., 1987; Polak et al., 1991; Davenport et al., 1996; Torreano and Cohan, 1997; Rehder and Cheng, 1998). However, it has not been determined whether this novel 5-HT receptor

also plays a regulatory role in neurite outgrowth of serotonergic neuron C1s. To address the possibility that this novel 5-HT receptor is involved in neurite elongation control, a sodium-dependent inward current can be first measured through sodium substitution experiments over 5-HT treatments. If the same novel 5-HT receptors are involved in the regulation of neuron C1 elongation, the use of the cAMP analog (8-bromo-cAMP), the adenylate cyclase activator (forskolin), or the phosphodiesterase inhibitor (IBMX: isobutyl methylxanthine) to increase intracellular cAMP levels can result in the same sodium-dependent inward current. Although studies in molluscs have provided abundant information on the numerous physiological actions of 5-HT, more studies are required to clarify the pharmacology and diversity of the 5-HT receptor system in molluscs.

5-HT_{1hel} and 5-HT_{7hel} receptors share characteristics of the seven transmembrane G-protein coupled receptors (Parries et al, 2003). In addition, these cloned receptors are closer to the mammalian 5-HT receptors which couple to adenylate cyclase than those stimulating phospholipase C. It has been suggested that mammalian 5-HT receptor subtypes associated with cyclic nucleotides include the 5-HT_1, 5-HT_4, 5-HT_6 and 5-HT_7 receptor families. It is postulated that the inhibitory action on mammalian neurons may be mediated through 5-HT_1 receptor subtypes, while the excitatory actions of 5-HT may be mediated through 5-HT_2 receptor subtypes (Peroutka and Snyder, 1981 & 1982). The 5-HT_1 receptor family in vertebrates not only couples to G-proteins, but also inhibits adenylate cyclase reducing cAMP levels (Middlemiss and Tricklebank, 1992; Boess and Martin, 1994; Peroutka, 1994; Martin and Humphrey, 1994). It

is interesting that hyperpolarization through 5-HT_{1A} receptor may be induced by opening potassium channels (Cooper et al., 1996). 5-HT_{1A} receptors play a critical role in mediating inhibition of cell firing in the midbrain raphé nuclei (Sprouse and Aghajanian, 1986; Stamford et al., 2000) where neurite outgrowth is inhibited by 5-HT. However, there is a controversial study testing effects of a well-known 5-HT_{1A} receptor agonist on adenylate cyclase activity (Urbina et al., 1996). 8-OH-DPAT has been reported to induce an increase in the activity of adenylate cyclase in goldfish retina in contrast to inducing a decrease in the activity of adenylate cyclase in the hippocampus of rat. Moreover, the increased cAMP level by 8-OH-DPAT caused the inhibition of neurite outgrowth, and WAY 100135, a selective 5-HT_{1A} receptor antagonist, significantly blocked the inhibition of neurite outgrowth (Lima et al., 1994; Urbina et al., 1996). These different results are likely due to the non-selective binding of 8-OH-DPAT between 5-HT_{1A} and 5-HT_7 receptor subtypes. The possible regulation of neurite outgrowth through 5-HT_{1hel} or 5-HT_{7hel} receptors may be similar to the regulatory mechanisms through mammalian 5-HT_{1A} receptor negatively coupled to adenylate cyclase or mammalian 5-HT_7 receptor positively coupled to adenylate cyclase. Thus, they may mediate the regulation of neurite elongation in serotonergic neuron C1s through regulation of cAMP levels. Further approaches using pharmacological agents to induce antagonism of those receptors or RNAi to silence the gene expression of those receptors will elucidate the involvement of those newly cloned 5-HT receptors in the regulation of neurite outgrowth in serotonergic neurons.

Taken together, neurite outgrowth of an identified serotonergic neuron C1 may be regulated by activating putative 5-HT receptors on the somata and/or neurites. These regulatory mechanisms may be dependent on concentrations of 5-HT, thereby showing differential effects of 5-HT on neurite outgrowth at different elongation rates. Endogenous 5-HT possibly involved in the regulation of neurite outgrowth of serotonergic neuron C1s can be released from other serotonergic neurons in the cerebral ganglia and/or self-released from neuron C1. Therefore, lower levels of 5-HT that diffuse from endogenous-releasing sites to the growth cones of slow-growing neurites may facilitate neurite elongation of the neuron C1 to lead its neurites to a target area as quickly as possible, whereas temporal and spatial higher levels of 5-HT around endogenous-releasing sites may inhibit or collapse slow-growing neurites of the neuron C1 not to reach a false target. In addition, 5-HT may inhibit or collapse fast-growing neurites of the neuron C1, which passed their targets or approached false targets.

Figure 32. A model for 5-HT dose-dependent modulation of $[Ca^{++}]_i$ levels in the growth cone of serotonergic neuron C1. Neurite elongation rates in slow-growing and fast-growing neurites could be regulated depending on the $[Ca^{++}]_i$ levels (This figure was modified from the calcium set-point hypothetic diagram of Kater and Mills, 1991).

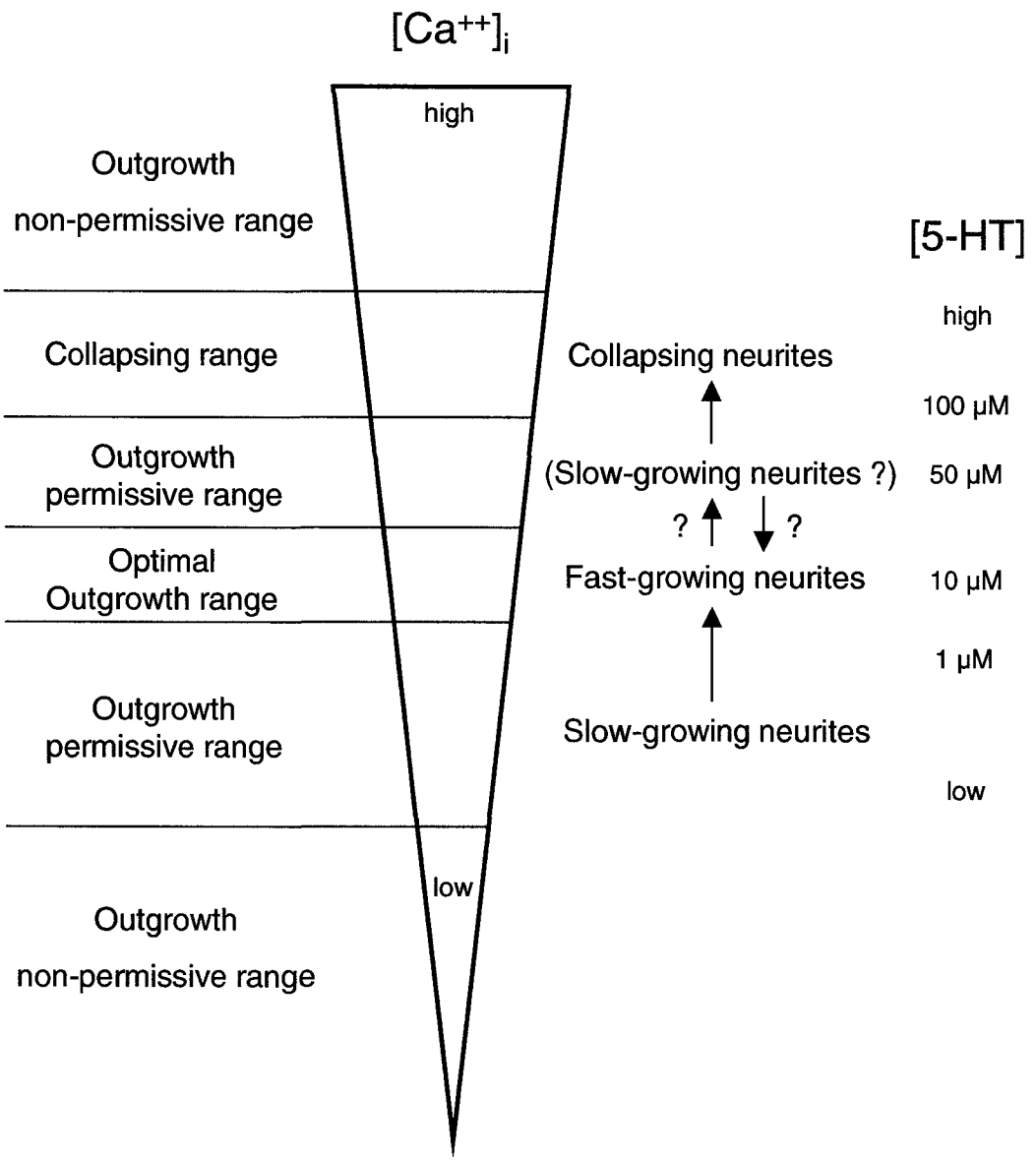


Figure 33. A model for biphasic effects of 5-HT in the regulation of slow-growing neurites. In the present study, both 5-HT_{1hel} and 5-HT_{7hel} receptor expression was only found in soma, but not in neurites. 5-HT_{1hel} may be a high affinity G protein-coupled 5-HT receptor which is assumed to negatively couple to adenylate cyclase (AC). 5-HT_{7hel} may be a G protein-coupled 5-HT receptor which is assumed to positively couple to adenylate cyclase. 5-HT binds to a 5-HT_{7hel} receptor, which associates with a GTP-binding (G_s) protein. The activated Gs protein interacts with adenylate cyclase causing accumulation of cAMP. The cAMP then binds to nucleotide-gated sodium channels, causing their activation and an inward sodium current. The depolarization resulting from the sodium influx activates voltage-gated Ca^{++} channels. More voltage-dependent Ca^{++} channels may open in a 5-HT dose-dependent manner, causing the higher rise of $[Ca^{++}]_i$. In lower doses of 5-HT, a small rise of $[Ca^{++}]_i$ may be related to facilitation of neurite elongation. The Ca^{++} release from intracellular stores induces much higher $[Ca^{++}]_i$ levels which may drive slow-growing neurites to a cessation stage. In higher doses of 5-HT, a big rise of $[Ca^{++}]_i$ may also produce the inhibition of neurite elongation or growth cone collapse. On the other hand, 5-HT_{1hel} receptor associates with an inhibitory G protein (G_i), which reduces the intracellular cAMP level. The reduced cAMP level interferes with the opening of sodium channel, reducing $[Ca^{++}]_i$ levels to facilitate the elongation of slow-growing neurites.

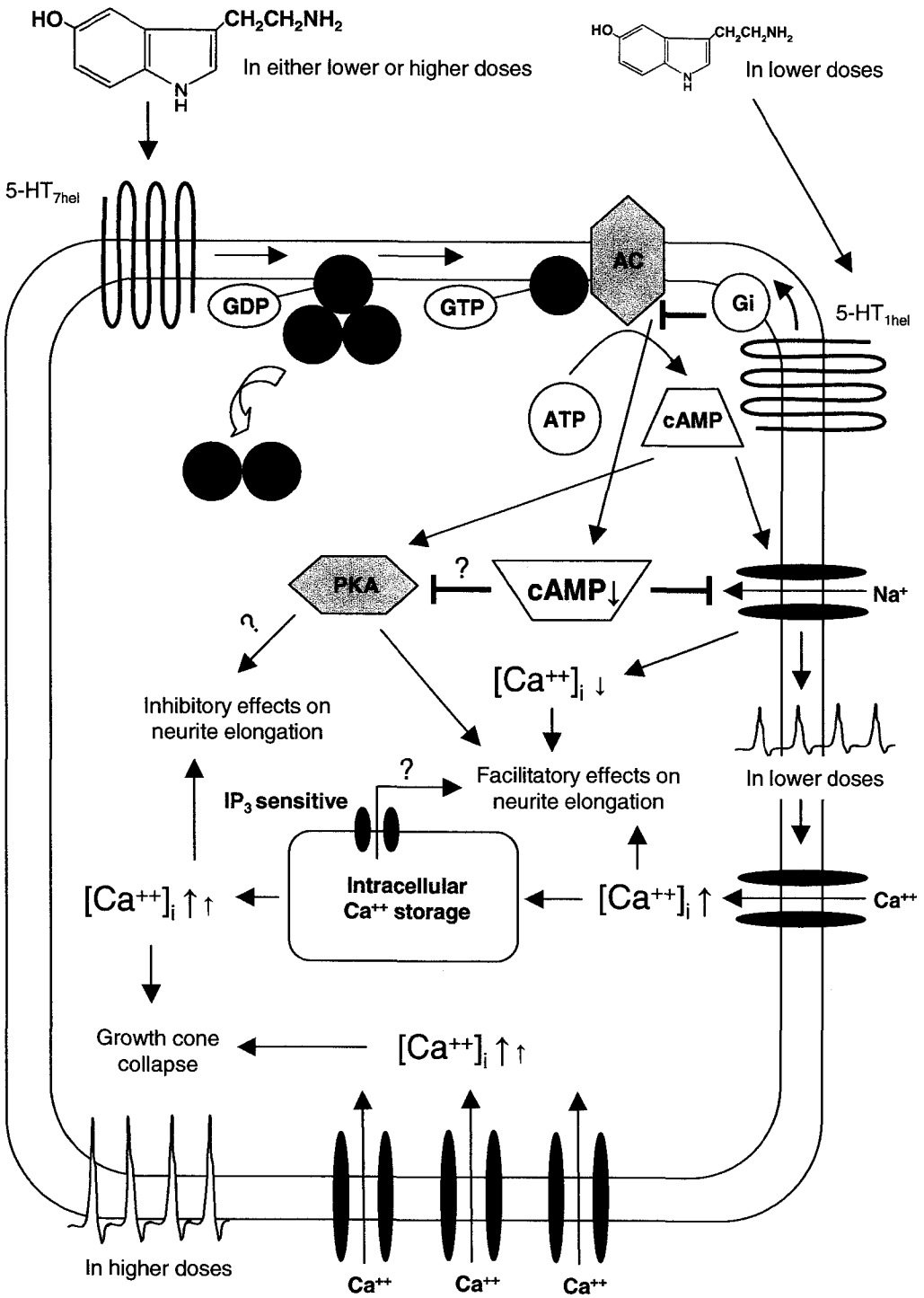


Figure 34. A model for dose-dependent effects of 5-HT in the regulation of fast-growing neurites. In the present study, both 5-HT_{1hel} and 5-HT_{7hel} receptor expression was found in soma, and 5-HT_{1hel} receptors were expressed in neurites. Fast-growing neurites may already have the optimal $[Ca^{++}]_i$ for rapid elongation. Therefore, either increase or decrease in $[Ca^{++}]_i$ levels through those 5-HT receptors may always induce inhibitory effects of 5-HT on the elongation of fast-growing neurites. Moreover, a Ca^{++}-independent PKA-associated signaling pathway may be involved in the 5-HT inhibition of neurite elongation. The activation of 5-HT_{1hel} receptor may reduce the intracellular cAMP level and lower the activation of PKA, causing inhibition of the neurite elongation rate.

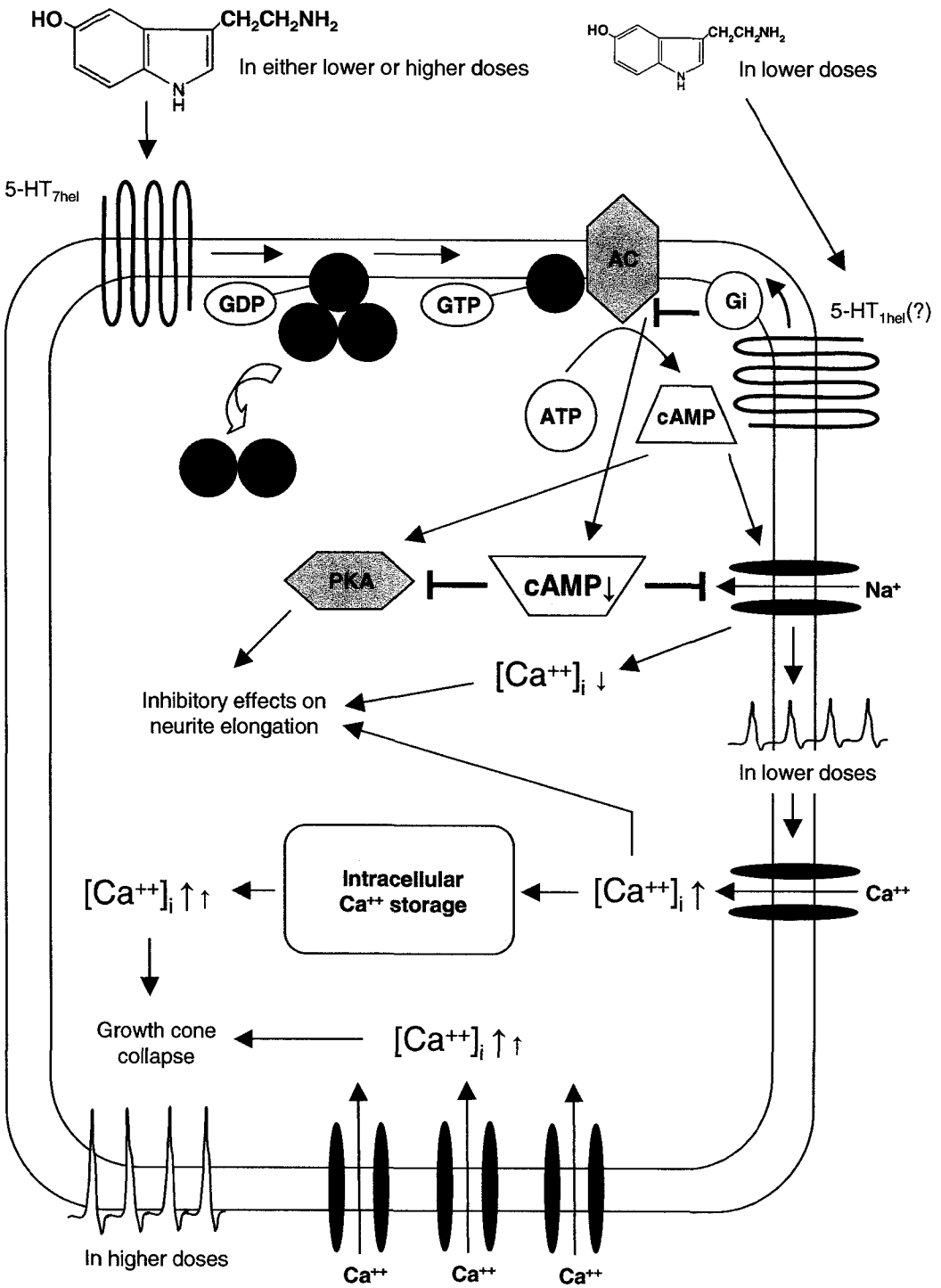
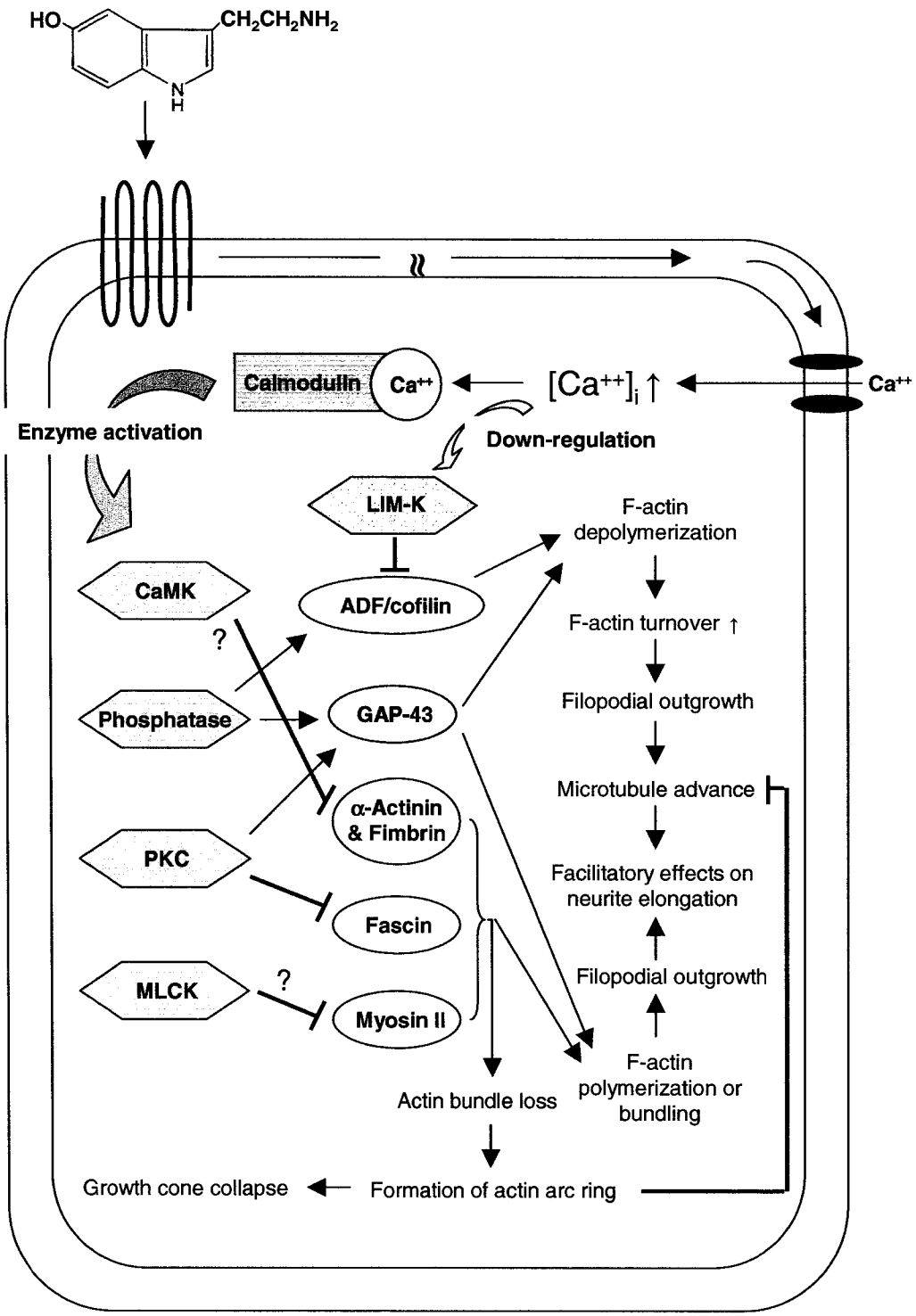


Figure 35. A model for roles of calcium in the regulation of neurite elongation. 5-HT increases $[Ca^{++}]_i$ levels and increases the amount of Ca^{++}-bound calmodulin. Ca^{++}/Calmodulin activates various enzymes, which can be related to the regulation of neurite outgrowth. The enzyme activation triggers down-streaming signaling molecules such as ADF/cofilin, GAP-43 and actin bundling proteins (α-actinin, fimbrin, fascin & myosin II), which regulates neurite outgrowth. CaMK, Ca^{++}-calmodulin-dependent protein kinase; MLCK, myosin light chain kinase; PDE, cyclic nucleotide phosphodiesterase; Phosphatase, a protein phosphatase called calcineurin; PKC, protein kinase C; LIM-K, LIM kinase.



CONCLUSION

An identified serotonergic neuron C1 of the cerebral ganglion of the snail *Helisoma,* when isolated from their ganglionic environment and plated in cell culture, grows new neurites that are tipped with motile growth cones. A small amount of the neurotransmitter 5-HT in standard culture conditions surrounding the plated neurons causes tonic inhibitory effects on the onset of neurite elongation, and a high dose of 5-HT leads to necrotic neuronal death. Furthermore, addition of 5-HT to modified culture conditions producing an active neurite elongation causes differential regulatory effects on elongating neurites at different rates.

All of the above dose-dependent differential effects of 5-HT including facilitation, inhibition and collapse can be suggested to be used *in vivo* to extend neurites of serotonergic neuron C1 to their target area leading to proper branching, synapse formation and physiological activity of specific target cells . Therefore, suggestions from the present study strongly support that 5-HT has fine-tuning effects on neurite outgrowth. In addition, these fine-tuning roles of 5-HT in the regulation of neurite outgrowth may involve autoregulatory mechanisms to control the neuronal regeneration of serotonergic systems and recover serotonergic physiological functions such as feeding activity.

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