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Neurotrophic Signaling Endosomes:
Clathrin-Coated Vesicles Propagate the NGF Signal Through the Ras/MAPK Pathway

by

Charles L Howe

DISSERTATION

Submitted in partial satisfaction of the requirements for the degree of

DOCTOR OF PHILOSOPHY

in

Neuroscience

in the

GRADUATE DIVISIONS

of the

UNIVERSITY OF CALIFORNIA SAN FRANCISCO

and

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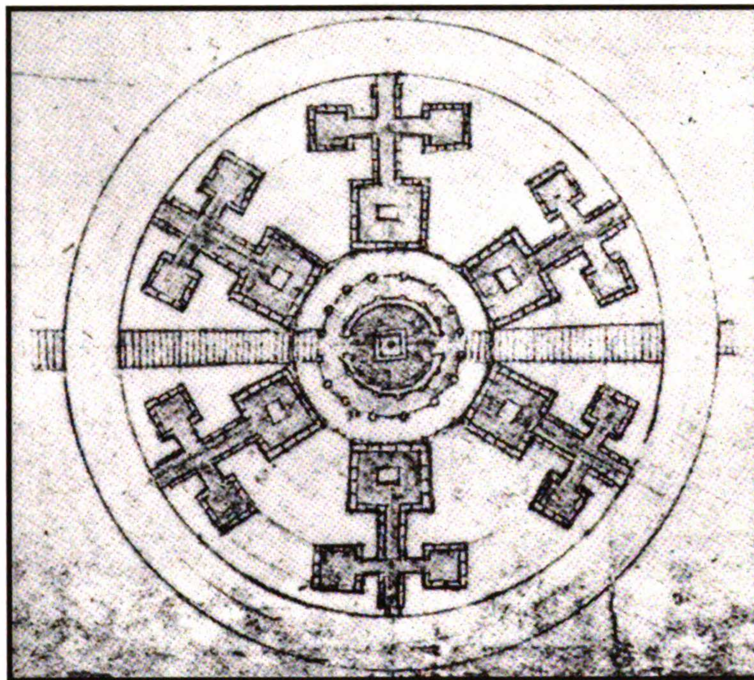

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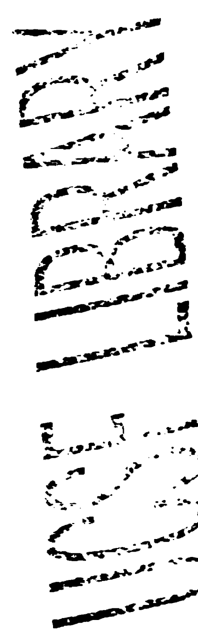
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Unending pain and loss I have known
in an immortal chain
of a million mortal lives,
but in each, you are there,
a light in the darkness,
glittering like a jewel
on the black velvet of the night sky,
my loves for you strung out behind me
like a string of shimmering pearls.

For H, February 2000

Her eyes are always open
She never lets me sleep.
Her dreams, so brightly lit,
Make the sun vanish,
Make me laugh, weep, laugh, and
Speak without having anything to say.

Paul Eluard 1924

Preface

First and foremost among the people who have supported me in this endeavor is my wife Heidi. I owe her everything – without her this work would have been an impossibility. From the moment she convinced me not to turn the U-haul around outside of the Amana Colonies in Iowa, to the moment that she understood I was coming home late tonight to finish typing this document, she has provided me with strength, conviction, and courage that I never would have found on my own. I love her intelligence, her creative spark, her mettle and her tenderness, her resolve, and her powerful spirit. She has sacrificed much that I might accomplish this work.

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I also thank my parents for the love and sacrifice they have provided throughout my life. I owe them more than can ever be repayed. My mother, Salli, has perhaps the strongest character of anyone I know, and I am grateful for whatever bit of that character rubbed off on me while I was growing up. Likewise, my father, Chuck, has the most raw intelligence of anyone I know – I hope that someday it will be said that I was as smart as my old man.

When I called Heidi's parents 7 years ago to ask for permission to marry their daughter, I never anticipated that I would acquire an entire extended family in the deal. But I did, and

that acquisition has been one of the biggest rewards in my life. Without the support of Mary and Ray I am sure that none of this would have been possible. I am beholden to them for all of their love and for making me feel like a part of the family. Ta gra agam ort. Rath Dé ort.

I thank Bill for all of his guidance, support, and friendship. Since the day I walked into his office and told him I was going to rotate through his lab and then ingenuously told him exactly what project I was going to work on, he has let the reins hang loose and let me find my own way. At the same time, however, he has always been there to put me back on the path when I strayed - and I have strayed frequently. If I am a scientist, then it is only as a result of his tempering. I think that I would not have fluorished in any environment other than the one Bill provided, and I thank him for letting me learn how to think about science in a manner that anneals my talents to the skills necessary to succeed.

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The good friends I have made in the last 6 years are one of the best rewards of this endeavor. Without Janice Valletta, Alex Krüttgen, Manish Butte, and Michael Silver my life would be impoverished. I thank Janice for making Heidi and I feel at home in the alien landscape of California. I thank Alex for his energy and enthusiasm. I thank Manish

for being someone I can talk to and count on. And I thank Michael for being himself – you're still my hero Jim.

I have also had the pleasure of working with a number of interesting people, some of whom I hope I can count as friends. Pavel Belichenko, Jon Cooper, and Jean-Dominique Delcroix are three that I would single out as people who I hope will remain in my life long after I have moved on in my career.

I also wish to acknowledge the support and friendship of fallen comrades.

This work is also dedicated to past mentors. The most prominent among them is Helena Jones. Her acumen and erudition inspire me to this day.

Finally, I sincerely thank the members of my committe, especially the chairman, Bob Messing. His support and energy have been of infinite help to me during my exile from UCSF. Likewise, Rob Edwards has always offered his enthusiasm and intelligence, all the way back to my orals exam, and I thank him for being a constant in the whirlwind of changes that have happened since I started graduate school. I thank Mark von Zastrow for all of his input, and for helping me out of one of the weird situations that seemed to

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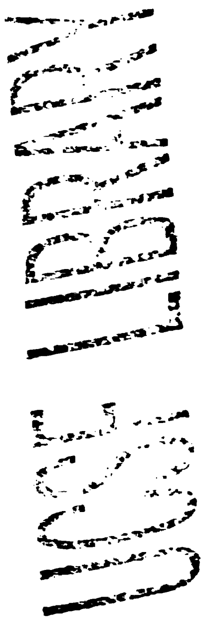
plague my graduate career. Last, but certainly not least, I thank Eric Shooter for his participation. I am honored that he is a member of the committee that granted me a PhD, and I respect the history that links him to me via Bill.

In terms of the work presented in this thesis, I would like to acknowledge the help of Eric Beattie and Janice Valletta. Chapter 2 is the result of work done in collaboration with Eric, while elements of Chapter 3 could not have been done without the indispensable help of Janice.



Chapter 2 has been previously published in the Journal of Neuroscience (J Neurosci 20:7325-7333, 2000), and is used with permission. As co-first authors, Eric Beattie and I contributed equally to all aspects of this work. I acknowledge the input of the other authors, Andrew Wilde and Frances Brodsky, and, of course, William Mobley.

Chapter 3 represents work submitted for publication at the time of this writing. As first author, I made major contributions to all aspects of the work, including performing all of the experiments described. Janice Valletta contributed her remarkable talents to several of the experiments described at the end of the chapter, and I thank her for her help.

Chapter 5 is a book chapter previously published in the *Neurobiology of the Neurotrophins*, edited by Italo Mocchetti. It is reprinted with the permission of the publisher, F. P. Graham Publishing Co. William Mobley contributed fundamentally to the final structure and organization of the chapter.

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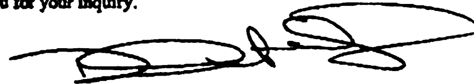

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Abstract

NEUROTROPHIC SIGNALING ENDOSOMES: Clathrin-Coated Vesicles Propagate the NGF Signal Through the Ras/MAPK Pathway. Charles L Howe

The target-derived neurotrophic factor, nerve growth factor (NGF), signals through its receptor tyrosine kinase TrkA to promote the survival, differentiation, and maintenance of neurons. How the NGF signal generated in axon terminals is conveyed to the cell body is unknown. The 'signaling endosome hypothesis' postulates that NGF:TrkA complexes are internalized at axon terminals and retrogradely transported to the cell body, where they initiate local signal transduction cascades that exert pleiotropic effects on cell survival and differentiation. Many receptor tyrosine kinases are internalized and downregulated from the plasma membrane via clathrin-mediated endocytosis. I hypothesized that TrkA might also be internalized via a clathrin-mediated mechanism, and the vesicles formed as a result of this internalization might function as signaling endosomes. I found that signaling through TrkA led to the redistribution of clathrin within cells, and the recruitment of clathrin to membranes, including the plasma membrane. Moreover, I found that NGF signaling induced the formation of a complex containing activated TrkA, the clathrin adaptor protein AP2, and the clathrin heavy chain. These findings led me to develop a protocol for isolating a highly purified clathrin-coated

vesicle fraction. Utilizing this cellular fractionation protocol, I found that NGF treatment induced the formation of clathrin-coated vesicles that contained NGF bound to activated TrkA. Importantly, Shc, and Ras were recruited to clathrin-coated vesicles in NGF treated cells. Unexpectedly, I found that the MAP kinases Erk 1 and 2 were present in clathrin-coated vesicles, and that following NGF treatment, Erk1 and 2 were activated within these vesicles. This activity was judged by the phosphorylation state of Erk 1 and 2, and by the ability of NGF-induced clathrin-coated vesicles to signal in vitro to activate Elk, a transcription factor that is a downstream physiological target of Erk. Though others have previously provided evidence in support of signaling from internalized membranes, the findings reported here are the first to document the existence of endosomes that signal, and suggest that TrkA activation induces the formation of signaling endosomes derived from clathrin-coated membranes.

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One has to multiply thoughts to the point where there

aren't enough policemen to control them.

Stanislaw Jerzy Lec

Chapter 1

Introduction to Neurotrophic Signaling Endosomes

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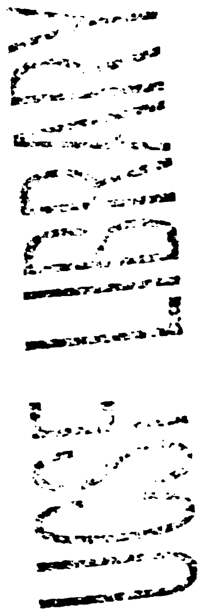
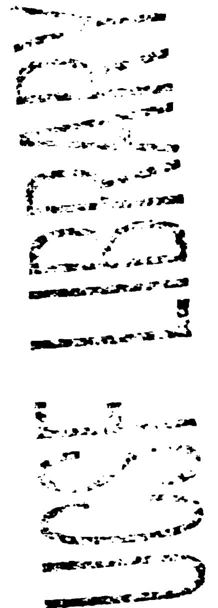
1. Introduction

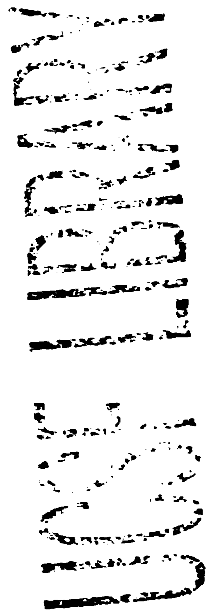
Neurons are linked together in complex circuits in which each neuron acts as an information processing node within a larger network. Efficient function of this network requires that signals be transmitted reliably from one neuron to the next. This information transfer takes place at the synapse, a highly specialized structure that occurs at the apposition of the presynaptic axon terminal and the postsynaptic dendritic or somal membrane. Information flow from the presynaptic terminal to the postsynaptic element is mediated by neurotransmitters, chemical messengers that bind to specific receptors on the postsynaptic plasma membrane in order to transduce pleiotropic intracellular events in the postsynaptic neuron. These events range from electrical excitation, via ionic flux, to morphological plasticity, via kinase-mediated signal transduction cascades. However, information flow is not unidirectional. Reciprocal flow of information occurs from the postsynaptic cell to the presynaptic terminal, instructing the terminal as to the degree of activity in the postsynaptic cell, as well as to the fitness and propriety of the connection. The information flowing in this retrograde direction may be mediated by several factors, including secreted protein factors such as neurotrophins and growth factors, membrane-anchored factors such as adhesion proteins, and soluble membrane-permeant factors such as lipid-derivatives and gases. All of these agents elicit signals within the presynaptic element. Many of these signals are local, in that they provide information that is used to control events within the presynaptic terminal. However, some signals, particularly those



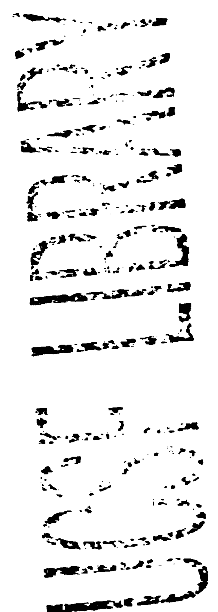
generated in response to secreted protein factors, are meant as long-distance bulletins. These signals are intended to transmit information from the postsynaptic element to the cell body of the presynaptic terminal receiving the secreted protein factor. For example, the postsynaptic element, as a target for the presynaptic terminal, may instruct the presynaptic neuron cell body to maintain or retract its connection. The postsynaptic element may also instruct the presynaptic neuron to elaborate or strengthen existing connections. Some retrograde signals may even instruct the presynaptic cell body to commit suicide or alter phenotype. These signals, necessarily signals-at-a-distance, may be communicated from the presynaptic terminal to the cell body via several mechanisms. A signal generated at the plasma membrane of the axon terminal may simply diffuse through the cytoplasm, eventually reaching the cell body and registering an effect. Such a mechanism is certainly viable for short distance communication. However, the distances over which some axonal signals must traverse are, in terms of the kinetics of diffusion, essentially infinite. Communication over such distance requires an active process. One such active process is a regenerating wave of signal, elicited at the terminal, but propagated by a wave that is periodically amplified by local signal transducers along the length of the axon. This type of signal transmission is akin to radio communications that utilize transponders to boost signal strength for long-distance transmission. However, perhaps the most efficient mechanism for signaling-at-a-distance involves the packaging of a secreted factor signal into a discrete, coherent, membrane-



bounded organelle that is moved along the length of the axon via a cytoskeleton-based transport machine. This type of signal is akin to writing a letter and sending it by mail to a specifically addressed recipient. Unlike the diffusion- and regenerating wave-based mechanisms, this quantal method of signaling from the postsynaptic element to the presynaptic neuron cell body requires far fewer resources to accomplish the communication. Furthermore, it is resistant to noise and cross-talk, as essentially each quantal element of the downstream signal can reconstruct the initial stimulus signal upon arrival at the cell body. In contrast, wave-based signals are open to interference by other signals generated either at the same presynaptic terminal or at other sites within the presynaptic neuron, including the cell body. Moreover, wave-based communications lack specificity – anyone tuned in to the appropriate frequency can listen to any radio communication. On the other hand, quantal signals can be addressed to highly specific cellular locations. Hence, quantal signals, in the form of membrane-bounded organelles containing factors secreted by the postsynaptic element bound to receptors removed from the presynaptic plasma membrane, can serve to communicate highly specific information using limited resources in a noise-resistant manner. Such a signal appears to be one mechanism by which neuronal targets instruct innervating neurons about the level of available trophic support and the fitness of nascent connections formed during development. As discussed below, the neurotrophic factor hypothesis requires that such communication occur in order to shape the connectivity of the nervous system and



control plasticity in response to environmental stimuli. A corollary of this hypothesis is that communication is mediated by quantal signal packets containing postsynaptically-derived neurotrophin bound to neurotrophin receptors derived from the presynaptic axon terminal. This signaling endosome hypothesis posits that these packets are retrogradely transported through the axon to the cell body, carrying the target-derived communication in a discrete and efficient parcel. Upon arrival in the cell body, this quantal signaling packet elicits local signal transduction cascades that modify transcriptional and translational events necessary to control cell survival, differentiation, and maintenance.

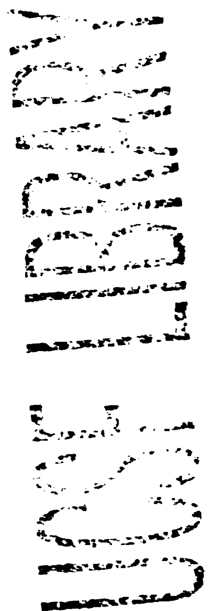


2. Neurotrophins and Neurotrophic Factors

2.1 The Neurotrophic Factor Hypothesis and Pre-History of Nerve Growth Factor

The critical role of the target in the survival, differentiation, and phenotypic maintenance of innervating neurons during development has been appreciated since the early 1900's. Classic experiments using either target ablation via limb bud extirpation or target expansion via the surgical addition of supernumerary limbs showed that the number of peripheral sensory and sympathetic neurons, as well as the number of central motor neurons, is regulated by the size of the target field they innervate (Bueker 1948; Detwiler 1920; Hamburger 1934; Hamburger 1939; Hamburger and Levi-Montalcini 1949; Shorey 1909). This concept forms the central element of the "Neurotrophic Factor Hypothesis" (Figure 1.1). It states that innervating neurons compete for a limited supply of a target-

derived trophic factor in order to match innervation density to target size and to ensure innervation propriety (Purves 1986). The first experimental evidence of the neurotrophic factor hypothesis dates to the work of Marian Shorey in 1909 and 1911. She discovered that extirpation of limb bud target fields in chick embryos led to a consequent loss of neurons in the corresponding ganglia (Shorey 1909). She went on to hypothesize that the differentiation and development of neurons is controlled by factors produced by target tissues, stating that "the sources of stimulation will always be found to be the metabolic products of other tissues" (Shorey 1911). The focus was shifted from control of differentiation to control of neuronal survival by the experiments of Viktor Hamburger, who hypothesized that the severe hypoplasia of neural populations resulting from limb bud extirpation was the result of death of differentiated neurons, rather than the failure of neuronal precursors to differentiate (Hamburger 1934). An exogenous source of a "metabolic product" capable of controlling the survival of neurons was discovered in 1948 by Elmer Bueker. He provided evidence that grafting fragments of mouse sarcoma 180 into the body wall of a 3-day old chick embryo resulted in ectopic innervation of the neoplastic tissue by sensory fibers emanating from the adjacent dorsal root ganglia, and in the consequent enlargement of the ganglia (Bueker 1948). However, the scope and importance of this source of survival-promoting factor was not appreciated until Rita Levi-Montalcini, under the guidance of Viktor Hamburger, discovered that such neoplastic tissue was able to elicit a massive response from sympathetic ganglia. This



response was evidenced by ganglionic volume increases to six-fold the size of control ganglia, and by the ectopic innervation of blood vessels, sex glands, thyroid, parathyroid, and spleen (Levi-Montalcini 1952; Levi-Montalcini and Hamburger 1951). These data led to the hypothesis that the neoplastic cells were releasing a soluble and diffusible factor that controlled the survival and differentiation of sympathetic neurons. This hypothesis was further confirmed when Levi-Montalcini grew explanted sensory and sympathetic ganglia in proximity to, but not in contact with, mouse sarcomas 180 or 37 (another sarcoma of identical origin to 180). Within 24 hours of co-culturing these tissues, a robust halo of nerve fibers was found emanating from the ganglia, with maximal fiber density on the side facing the sarcoma (Levi-Montalcini and others 1954). This discovery led Stanley Cohen, working with Levi-Montalcini and Hamburger, to isolate from the tumors a nucleo-protein fraction containing the nerve growth-promoting activity (Cohen and others 1954). In an effort to remove the nucleic acids from this fraction, Cohen employed the phosphodiesterase enzyme found in snake venom, and essentially stumbled upon one of the most potent sources of the nerve growth-promoting factor. During an experiment to test whether the nucleic acids or the proteins within the nucleo-protein fraction were responsible for promoting the fiber outgrowth from ganglia, a small amount of snake venom was added to the cultures. Cohen's hypothesis was that the snake venom would degrade the nucleic acids, and if they were responsible for the fiber growth, no halo would be found. Surprisingly, addition of the snake venom resulted

in a dramatic increase in the density of fibers produced by the explanted ganglia. Furthermore, addition of snake venom alone to explanted ganglia provoked a dense halo of nerve fibers. These results led to the subsequent biochemical purification of a small protein capable of inducing fiber outgrowth from explanted ganglia, and capable of provoking hyperplasia of sensory and sympathetic ganglia in chick embryos following injection of the protein into the yolk every day for several days (Cohen 1959; Cohen and Levi-Montalcini 1956; Cohen and others 1954; Levi-Montalcini and Cohen 1956). Finally, Cohen discovered that the same protein was present in mouse submandibular salivary glands (Cohen 1960), and on the basis of further purifications, a single protein named Nerve Growth Factor (NGF) was isolated.

NGF is the prototypical member of a family of growth factors collectively named the neurotrophins, comprised of brain-derived neurotrophic factor (BDNF), neurotrophin-3 (NT-3), NT-4/5, NT-6, and NT-7 (Lewin and Barde 1996). The neurotrophins have since been joined by a growing list of growth factors that mediate neuronal survival and development, including glial-derived neurotrophic factor (GDNF) and members of the cytokine family such as leukemia inhibitory factor (LIF) and ciliary neurotrophic factor (CNTF) (DeChiara and others 1995; Durbec and others 1996; Moore and others 1996). However, NGF remains the best studied nerve growth-promoting agent, and the first factor to fulfill the requirements of the neurotrophic factor hypothesis. This includes the

following evidence: 1) administration of NGF prevents both naturally occurring and experimentally induced cell death in specific neuronal populations; 2) administration of anti-NGF antibodies leads to the death of virtually all sensory and sympathetic neurons; 3) NGF is bound to specific receptors on axon terminals, internalized, and retrogradely transported to the cell body, where it elicits a host of transcriptional events; 4) NGF protein and mRNA is present within the targets of sensory and sympathetic neurons, while NGF protein but not mRNA is found within the cell bodies of these neurons; and 5) transgenic ablation of either NGF or its receptors results in the loss of specific sensory and sympathetic neuronal populations (Reichardt and Farinas 1999). Together, these findings serve both to verify the validity of the neurotrophic factor hypothesis, and to showcase NGF as the prototypic neurotrophic factor.

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2.2 The Neurotrophins

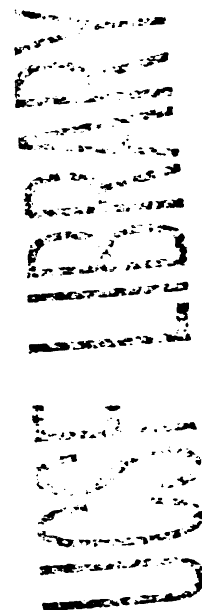
2.2.1 Nerve Growth Factor

The family of neurotrophic factors includes members of the neurotrophins, the fibroblast growth factors, the transforming growth factors, and the cytokines. Each of these factors exhibit specific tissue expression patterns and act to promote the survival of distinct but overlapping neuronal populations (Table 1). Of the neurotrophins, NGF, BDNF, and NT-3 genes have been identified in all higher vertebrates, including the teleost fishes, and NGF, BDNF, NT-3, and NT-4/5 have been identified in all tetrapods, except for birds,

which appear to lack the gene for NT-4/5 (Hallbook 1999; Hallbook and others 1991). NGF, BDNF, NT-3, and two novel neurotrophins named NT-6 and NT-7 are found in bony fish (Gotz and others 1994; Lai and others 1998; Nilsson and others 1998), while two other highly divergent neurotrophins are found in the jawless fish *Lampetra fluviatilis* and *Myxine glutinosa* (Hallbook and others 1998; Kumar and Hedges 1998). Evidence that substitution of only seven amino acids in the NT-3 sequence is sufficient to confer the biological activity of NGF, BDNF, and NT-3 to the mutant protein suggests that NGF and BDNF may have evolved from NT-3 (Urfer and others 1994). However, other phylogenetic evidence indicates that NGF and NT-3 may have formed from the gene duplication of one intermediate ancestral gene, while BDNF and NT-4/5 formed from the gene duplication of another intermediate ancestor (Hallbook 1999).

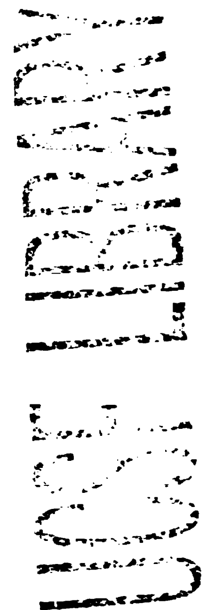
As described above, NGF was discovered as the nerve-growth promoting factor of mouse sarcomas 180 and 37, of snake venom, and of the mouse submandibular salivary gland. The NGF isolated from mouse submandibular gland occurs in two distinct forms. The first, named 7S NGF due to its sedimentation coefficient, is a high molecular weight complex of three protein subunits named α-, β-, and γ-NGF (Varon and others 1967a; Varon and others 1967b). The second form, referred to as 2.5S NGF (Bocchini and Angeletti 1969), exhibits a large degree of terminal proteolytic modification, but is effectively indistinguishable from the β-subunit of 7S NGF. It is this subunit, either as

derived from 7S NGF or as 2.5S NGF, that contains all of the relevant neurotrophic activity of NGF. The mature form of β-NGF (henceforth, just NGF) is a sequence of 118 amino acids proteolytically derived from a 307 amino acid precursor protein via cleavage of 187 N-terminal residues and 2 C-terminal residues (Scott and others 1983). The sequences derived from human, guinea pig, rat, bovine, chicken, cobra, viper, *Xenopus*, and salmon NGF indicate that there is a high degree of conservation within specific regions of NGF that are likely to be important for receptor interaction and maintenance of three-dimensional structure (Ebendal and others 1986; Goedert 1986; Hallbook and others 1991; Meier and others 1986; Schwarz and others 1989; Selby and others 1987a; Selby and others 1987b; Ullrich and others 1983a; Ullrich and others 1983b; Whittemore and others 1988; Wion and others 1986). This structure is comprised of seven β strands oriented in three anti-parallel pairs, with elongated loops at either end that contribute to binding specificity (McDonald and Blundell 1991; McDonald and others 1991; Wlodawer and others 1975). In particular, the N-terminal six residues of NGF contribute significantly to both binding specificity and binding affinity (Shih and others 1994). NGF, like the other neurotrophins and many other growth factors, belongs to the cystine knot superfamily of proteins, and this cystine knot region is highly conserved among the individual neurotrophins (Butte and others 1998). Finally, NGF and the other neurotrophins exist exclusively as dimers that share an interface containing an extensive buried hydrophobic surface. It has been suggested that dimerization is necessary to



stabilize the neurotrophin structure by burying these hydrophobic regions within the core of the dimer (Sun and Davies 1995).

While NGF expression is very high in the mouse submandibular gland, its expression in physiological targets of neuronal innervation is much more limited. Quantification of NGF mRNA levels in target regions using an NGF cDNA hybridization assay revealed that the density of sympathetic innervation was directly correlated to the level of NGF mRNA, and that expression was highest in tissues such as the heart and the iris, which receive dense sympathetic innervation (Heumann and others 1984; Shelton and Reichardt 1984). Analysis of NGF protein levels by ultrasensitive immunoassay provided similar results (Korsching and Thoenen 1983; Thoenen and others 1983). Utilizing these same techniques, NGF was detected in the central nervous system as well, with specific expression found in several neuronal populations, including hippocampal neurons (Ayer-LeLievre and others 1988; Korsching and others 1985; Shelton and Reichardt 1986; Thoenen and others 1987; Whittemore and Seiger 1987). The induction of choline acetyltransferase by NGF in basal forebrain cholinergic neurons, and the retrograde transport of radiolabeled NGF from the hippocampus and cortex to the basal forebrain, indicates that NGF is a neurotrophic factor for specific central neuronal populations (Gnahn and others 1983; Seiler and Schwab 1984). This topic is taken up at length in



chapter 5 of this manuscript, which deals with the control of plasticity within the central nervous system by NGF-mediated modulation of cholinergic function.

2.2.2 Brain-Derived Neurotrophic Factor

The initial attempts to isolate neurotrophic factors distinct from NGF led to the discovery of an activity present in media conditioned by a glioma cell line that was able to induce the morphological differentiation of neuroblastoma cells (Monard and others 1973; Monard and others 1975). This "glioma factor" was found to induce neurite outgrowth from explants of chick embryo dorsal root ganglia (DRG), and this activity was not blocked by antibodies against NGF (Barde and others 1978). Furthermore, the glioma conditioned media was more effective than NGF at promoting survival of cultured DRG neurons (Barde and others 1980). Attempts to justify a physiological relevance for this glioma-derived factor led to the isolation of a similar activity from chick and rat brain extracts, and from homogenates of postnatal chick spinal cord and embryonic chick heart, liver, and kidney (Barde and others 1980; Lindsay and Peters 1984a; Lindsay and Peters 1984b; Lindsay and Tarbit 1979). Identification of an activity in rat brain homogenates that was unique from NGF led to the eventual purification of BDNF from pig brains by Yves-Alain Barde in 1982 (Barde and others 1987; Barde and others 1982; Barde and others 1983; Barde and others 1985; Barde and Thoenen 1984; Barde and Thoenen 1985; Edgar and Barde 1983). BDNF was purified as a 12.3 kDa protein that shared many

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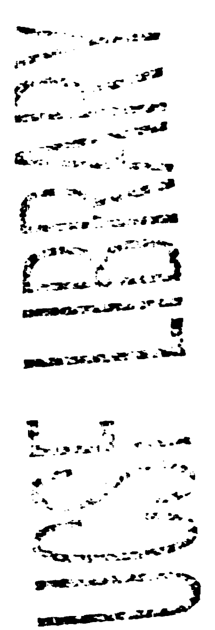
physiochemical and biological properties of NGF, and that exhibited specific activity for neurite outgrowth from DRG neurons (Hofer and Barde 1988). BDNF was subsequently cloned and found to contain highly conserved regions of sequence homology with NGF (Leibrock and others 1989). The first activity defined for BDNF that was clearly distinct from NGF was the support of survival and neurite outgrowth from neurons of the nodose ganglia isolated from chick embryos (Lindsay and others 1985a; Lindsay and Rohrer 1985; Lindsay and others 1985b). Such differential sensitivity to NGF versus BDNF was later extended to the general premise that sensory neurons of neural crest derivation, such as DRG, dorsomedial trigeminal ganglia, and jugular ganglia neurons, are responsive to NGF and BDNF, while neural placode-derived neurons, such as nodose ganglia and petrosal ganglia neurons, are insensitive to NGF but responsive to BDNF (Table 1) (Davies and others 1986a; Davies and others 1986b; Lindsay and others 1985a; Lindsay and Rohrer 1985; Lindsay and others 1985b). Furthermore, BDNF was found, not surprisingly, to support the survival and neurite outgrowth of several central neuronal populations, including retinal ganglion cells (Johnson and others 1986b; Rodriguez-Tebar and others 1989), spinal motor neurons, basal forebrain cholinergic neurons, cerebellar granule cells, and cortical and hippocampal neurons (Ernfors and others 1994; Jones and others 1994; Reichardt and Farinas 1999). Finally, the role of BDNF in plasticity of cortical and hippocampal connections has established itself as perhaps the most exciting function of neurotrophins within the central nervous system. Competition among axonal



terminals for a limited supply of target-derived BDNF has been implicated in the experimental and developmental plasticity of the visual cortex (Berardi and Maffei 1999; Berardi and others 2000; Cabelli and others 1995), and in the plasticity of learning and memory within the hippocampus (Carmignoto and others 1993; Kang and others 1996; Kang and Schuman 1995a; Kang and Schuman 1996; Kang and Schuman 2000; Kang and others 1997; Kang and Schuman 1995b; Korte and others 1995; Korte and others 1998; Lessmann and others 1994; Li and others 1998a; Li and others 1998b). Chapter 5 of this manuscript contains further review of this subject.

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2.2.3 Neurotrophin-3 and Neurotrophin-4/5

Following the isolation, cloning, and sequencing of BDNF, the search for other growth factors related to these two neurotrophins accelerated. Based upon sequence homologies between NGF and BDNF, several groups designed degenerate oligonucleotide primers for PCR amplification of genomic DNA sequences that might encode other neurotrophin family members. Using this strategy, NT-3 was discovered in 1990 (Ernfors and others 1990a; Ernfors and others 1990b; Jones and Reichardt 1990; Kaisho and others 1990; Maisonpierre and others 1990; Rosenthal and others 1990), and NT-4/5 was isolated in 1991 (Berkemeier and others 1992; Berkemeier and others 1991; Hallbook and others 1991). NT-3 mRNA is expressed in numerous neuronal and non-neuronal tissues throughout the body, and exhibits a complex pattern of temporal expression that changes

from high embryonic expression to low overall adult expression levels, though significant expression remains within specific neurons of the hippocampus, cerebellum, and cortex (Maisonpierre and others 1990; Phillips and others 1990), and within tissues such as heart, liver, kidney, spleen, and muscle (Phillips and others 1990; Rosenthal and others 1990). Several populations of neurons in both the peripheral and central nervous systems are responsive to NT-3, including neurons of the DRG, nodose ganglion sensory neurons, motor neurons, basal forebrain cholinergic neurons, mesencephalic GABAergic neurons, dopaminergic neurons of the substantia nigra, hippocampal neurons, striatal neurons, neurons of the trigeminal mesencephalic nucleus and the locus ceruleus, and retinal ganglion cells (Table 1) (Yuen and Mobley 1996). NT-3 is a potential candidate for the control of diabetic sensory neuropathy. NT-3 supports the survival and neurite outgrowth of developing Ia proprioceptive afferent sensory neurons that project to muscle (Hohn and others 1990; Hory-Lee and others 1993), and administration of NT-3 to developing animals rescued sensory neurons from a normal program of cell death (Oppenheim and others 1993). These findings suggest that a failure in NT-3 signaling may play a role in the development of diabetic neuropathy, and suggest that exogenous trophic support of these neurons may be a viable therapeutic intervention. NT-3, like BDNF, has also been implicated in the developmental and learning- and memory-related plasticity of hippocampal and cortical neurons (Berninger and others 1993; Kim and others 1994; Lohof and others 1993). Similarly, NT-4/5 is expressed in several neuronal and non-

neuronal tissues throughout development and in the adult animal, where it exhibits overlapping but also distinct actions from BDNF (Reichardt and Farinas 1999; Yuen and Mobley 1996). Likewise, NT-4/5 appears to exhibit overlapping function with BDNF within the context of cortical and hippocampal plasticity (Schuman 1999).

2.3 Other Neurotrophic Factors

2.3.1 Glial-Derived Neurotrophic Factor

Glial cell line derived neurotrophic factor (GDNF) was initially identified as a glioma-secreted factor capable of eliciting survival of cultured embryonic ventral midbrain dopaminergic neurons (Lin and others 1993). Following its initial characterization, it was found to support the survival of several neuronal populations, including spinal motor neurons and sympathetic, parasympathetic, proprioceptive, enteroceptive, and small and large cutaneous sensory neurons (Buj-Bello and others 1995; Ebendal and others 1995; Henderson and others 1994; Kotzbauer and others 1996; Trupp and others 1995; Yan and others 1995). GDNF became a family in 1996, when a protein sharing 44% sequence homology, named neurturin, was discovered as a survival factor for a subset of sympathetic neurons, nodose ganglia sensory neurons, and neurons of the DRG (Kotzbauer and others 1996). Subsequently, two other members of the GDNF family were isolated: persephin (Milbrandt and others 1998) and artemin (Baloh and others 1998). Each of the members of the GDNF family exhibit specific patterns of expression,

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and support distinct but overlapping populations of neurons (Baloh and others 2000). Evidence of the roles played by these proteins establishes that the GDNF-like neurotrophic factors form a family of related factors that parallel the relationship between the NGF-like neurotrophins. Characterizing each of these factors as Neurturin-1 (GDNF), Neurturin-2 (neurturin), Neurturin-3 (persephin), and Neurturin-4 (artemin), would permit discussion of the neurotrophin versus neurturin families of neurotrophic factors, and would emphasize the basic similarities in the organization of these families.

2.3.2 Ciliary Neurotrophic Factor

As with the use of explanted sympathetic ganglia in the identification and isolation of NGF, development of cultures of chick ciliary ganglia provided a sensitive system for the characterization of novel neurotrophic factors. Work in the mid-60's proved that the chick ciliary ganglion is a population of purely cholinergic parasympathetic motor neurons that innervate the intraocular muscles of the eye (Hess 1965; Landmesser and Pilar 1972; Marwitt and others 1971). On the basis of this knowledge, several groups established co-cultures of explanted chick ciliary ganglia and chick hindlimb skeletal muscle, and began to investigate the factors released by the muscle cells which supported survival and neurite outgrowth of the ciliary neurons (Betz 1976; Hooisma and others 1975). This assay, modified such that the chick ciliary neurons were cultured in media conditioned by dissociated chick heart cells led to the isolation of a factor which

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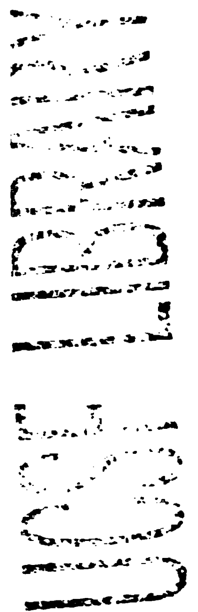
supported survival and neurite outgrowth in 15% of the cultured ganglia cells (Helfand and others 1976). Following up these experiments, and returning to the use of skeletal muscle cells either in co-culture with dissociated ciliary neurons or as producers of conditioned media, a factor was discovered that supported the survival and neurite outgrowth of essentially all ciliary neurons in culture (Nishi and Berg 1977; Nishi and Berg 1979). However, as these factors were identified using target fields other than the in vivo physiological target, it was realized that they likely represented factors with widespread neurotrophic effects, rather than effects specific for ciliary ganglia neurons. Hence, an assay system was developed utilizing dissociated ciliary neurons and serial dilutions of extracts from various tissues, including the intraocular muscles of the eye. It was found that chick eye extract contained significantly more ciliary-directed trophic activity than any other tissue (Manthorpe and Varon 1985). Biochemical purification of this trophic activity led to the isolation of a factor initially called CIPE, for choroid, iris, ciliary body, and attached pigment epithelium, from which the factor was isolated. It was defined as a ciliary neurotrophic factor, or CNTF (Manthorpe and others 1980). The factor was identified as a protein of roughly 40 kDa that exhibited very strong potency for neurite outgrowth and survival of chick ciliary ganglia neurons (Barbin and others 1984; Manthorpe and others 1982). However, the most rapid advances in purification and cloning of CNTF came when it was discovered that the rat sciatic nerve was a rich and readily available source of the factor (Lam and others 1991; Lin and others 1990; Lin



and others 1989; Manthorpe and others 1986; Stockli and others 1989). In addition to developing ciliary ganglionic neurons, CNTF has been found to serve as a trophic factor for several other neuronal populations, including sympathetic neurons (Barbin and others 1984; Ernsberger and others 1989), DRG neurons (Barbin and others 1984; Eckenstein and others 1990; Manthorpe and others 1981a; Manthorpe and others 1981b; Skaper and others 1986), spinal cord motor neurons (Arakawa and others 1990; Magal and others 1991; Oppenheim and others 1990; Wewetzer and others 1990), and striatal cholinergic neurons (Asada and others 1996; Hagg and others 1989). Recently, CNTF was shown to be protective of striatal neurons in several models of Huntington's disease (Emerich and others 1998; Emerich and others 1997a; Emerich and others 1997b), and was found to actually promote functional recovery of lost cognitive and motor skills attributable to striatal cell death and dysfunction (Mittoux and others 2000).

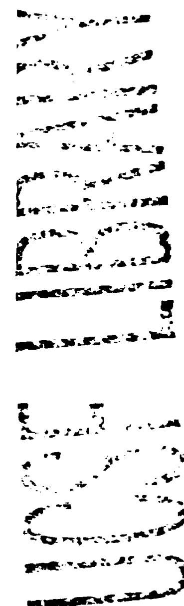
3. Neurotrophic Factor Receptors

3.1 TrkA

3.1.1 Evidence that TrkA Mediates an NGF Response

TrkA, a single transmembrane spanning, single polypeptide chain member of the receptor tyrosine kinase (RTK) superfamily, was initially discovered as an oncogenic fusion protein isolated from human colon carcinoma (Martin-Zanca and others 1986a; Martin-

Zanca and others 1986b). In its oncogenic form, the N-terminal 392 residues of normal TrkA were replaced by tropomyosin residues (hence tropomyosin receptor kinase, or Trk), leading to constituitive kinase activity and oncogenicity. Further genetic analysis revealed that in normal cells the proto-oncogene encoded a 140 kDa glycosylated protein containing an extracellular domain comprised of several immunoglobulin-like binding domains, a short, single transmembrane domain, and an intracellular domain encoding a tyrosine kinase (Figure 1.2) (Martin-Zanca and others 1989). It was also found that this protein exhibited a relatively restricted neuronal expression pattern (Martin-Zanca and others 1990). Following its initial discovery in 1986, the receptor remained an "orphan receptor" until 1991, when it was discovered that NGF evoked a rapid tyrosine phosphorylation of endogenous TrkA in PC12 cells and of exogenous TrkA in transfected fibroblasts (Kaplan and others 1991a; Kaplan and others 1991b; Klein and others 1991). Furthermore, NGF was found to bind to TrkA with high affinity ($K_d = 10^{-11}$ M; 0.1 to 0.01 nM NGF), and to elicit signaling cascades necessary for the biological responses of PC12 cells and neurons to NGF. For example, a subclone of PC12 cells that was non-responsive to NGF in terms of neurite outgrowth was shown to exhibit recovery of such responsiveness following transfection with human TrkA (Loeb and Greene 1993; Loeb and others 1991), and *Xenopus* oocytes transfected with TrkA exhibited germinal vesicle breakdown in response to NGF (Nebreda and others 1991). Further evidence for a role of TrkA in mediating biological responses to NGF came from studies of TrkA knockout



mice. It was found that many populations of NGF-responsive and NGF-dependent neurons were selectively winnowed or lost in animals missing one or both copies of the TrkA gene (Smeyne and others 1994). Finally, the neuronal localization of TrkA in the peripheral and central nervous systems correlates with NGF dependence and responsiveness, confirming that TrkA is the RTK that mediates biological responsiveness to NGF (Shelton and Reichardt 1984; Shelton and Reichardt 1986).

3.1.2 TrkA Expression in the Peripheral and Central Nervous Systems

TrkA is expressed in neurons of the trigeminal, dorsal root, and sympathetic ganglia within the peripheral nervous system (Martin-Zanca and others 1990), and within cholinergic neurons of the caudate-putamen and the basal forebrain in the central nervous system (Holtzman and others 1992). Mice homozygously transgenic for a disruption in the TrkA gene appear normal at birth, but show progressive hypotrophy and sensory dysfunction, noticeable by PD10. By PD20, half of the animals die. Surviving animals exhibit severe sensory defects, including non-responsiveness to painful stimuli and a failure to orient to vibrissal stimulation. Furthermore, the animals exhibit a severe defect in pupilary response indicative of a loss of sympathetic innervation. Indeed, the superior cervical ganglia of perinatal animals show both cell loss and actively degenerating neurons, and by PD10 the ganglia are extremely shrunken. Moreover, the dorsal root ganglia exhibit a loss of 70-90% of neurons, with small, NGF-dependent neurons

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preferentially affected. Likewise, the trigeminal ganglia of these mice are reduced by 70-90% in neuronal number, with the cell loss apparently distributed throughout the ganglia (Smeyne and others 1994). These findings are consistent with previous work showing that administration of anti-NGF antibodies to developing mice resulted in severe loss of neurons within specific peripheral ganglia (Johnson and others 1980; Ruit and others 1992), and suggest that these specific neuronal populations rely upon TrkA signaling for survival and maintenance.

As with the specific peripheral populations described above, a subpopulation of cells within the central nervous system known to express TrkA were affected by homozygous deletion of the TrkA gene. Evidence from in situ hybridization and immunohistochemistry shows that both caudate-putamen and basal forebrain cholinergic neurons express TrkA. Moreover, evidence from intraventricular injection of NGF into adult rats shows that these neuronal populations respond to NGF by upregulating choline acetyltransferase and TrkA mRNA (Holtzman and others 1992). These same populations were selectively reduced in number in mice expressing a homozygous deletion of TrkA, with an increase in TUNEL-positive staining in young animals suggesting an active process of cell death rather than a failure to differentiate. Furthermore, the cholinergic neurons which remained in the caudate-putamen and basal forebrain were atrophic (Fagan and others 1997). In addition, those neurons which were not lost exhibited

significantly decreased innervation of target regions such as the hippocampus and cortex, as marked by staining for acetylcholinesterase (Fagan and others 1997; Smeyne and others 1994) or p75NTR (Fagan and others 1997). In contrast to the peripheral populations sensitive to loss of TrkA expression, the cell loss within central nervous system populations was limited to 20-40% of cholinergic neurons, suggesting that only a subset of these neurons is dependent upon NGF for survival, and that the remaining cholinergic neurons require NGF for maintenance of phenotype and target innervation. The role of NGF in the function of basal forebrain cholinergic neurons is discussed more fully in chapter 5 of this manuscript.

3.1.3 TrkA Activation and Proximal Signaling

Upon binding of NGF to TrkA, the receptor is subjected to a series of events that characterize RTK signaling. This includes receptor dimerization, transphosphorylation of tyrosines in the activation loop leading to initiation of kinase activity, autophosphorylation of tyrosines outside of the activation loop, binding of specific signaling adaptors to these autophosphorylated sites, subsequent phosphorylation and activation of these adaptor proteins, and generation of a cascade of receptor-independent signaling pathways. Evidence for the requirement of receptor dimerization comes from studies in which transfected kinase-inactive mutants of TrkA act in a dominant-negative

fashion to suppress activation of endogenous TrkA (Jing and others 1992). Under conditions of TrkA overexpression, a high level of ligand-independent TrkA autophosphorylation is observed, apparently due to an increase in the probability of spontaneous TrkA:TrkA interactions (Hempstead and others 1992). Furthermore, gangliosides and ceramide can elicit ligand-independent TrkA activation, presumably by enhancing TrkA:TrkA interactions in the plane of the plasma membrane (MacPhee and Barker 1999; Mutoh and others 1995). Interestingly, ligand-dependent TrkA activation might also require the action of endogenous gangliosides, as inhibition of glucosylceramide synthase in PC12 cells inhibited TrkA activation in response to NGF, an effect that was reversed by exogenous supply of the ganglioside GM1 (Mutoh and others 1998).

Following NGF binding to human TrkA and receptor dimerization, tyrosine residues 670, 674, and 675 within the kinase activation loop are autophosphorylated (Figure 1.2) (Cunningham and others 1997). These residues, once phosphorylated, form specific charge-pair interactions with neighboring positively charged residues. These interactions stabilize a shift from a closed conformation of the kinase activation loop in which access to kinase substrates is blocked, to an open conformation in which the kinase activation loop can phosphorylate both intra- and intermolecular substrates (Cunningham and Greene 1998; Mitra 1991). Tyrosines 490 and 785 are two such intramolecular targets

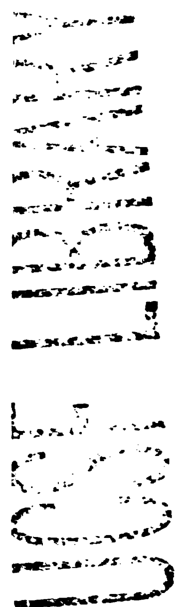
that are transphosphorylated after TrkA kinase loop activation (Figure 1.2) (Loeb and others 1994; Middlemas and others 1994; Stephens and others 1994). Tyrosine 785 is near the C-terminus of TrkA, within a consensus site for the binding of the SH2 domain of phospholipase C-γ (PLCγ) (Figure 1.2). This tyrosine is required for NGF-dependent recruitment of PLCγ to TrkA, and for the phosphorylation and activation of PLCγ (Vetter and others 1991). TrkA mutated at tyrosine 785 still mediates NGF-induced neurite outgrowth, but appears to be defective in induction of peripherin expression (Loeb and others 1994). Tyrosine 751, which is within a consensus domain for RTK binding of PI3 kinase (Obermeier and others 1993), also appears to play a role in PLCγ activation, as its mutation results in the loss of NGF-dependent PLCγ phosphorylation (though, oddly, not PI3 kinase activation) (Friedman and Greene 1999). Interestingly, tyrosine 785, in addition to PLCγ binding, also appears to play a role in the association of TrkA with the Csk homologous kinase (CHK), a protein which plays a role in NGF-induced neurite outgrowth (Yamashita and others 1999a). Injection of anti-CHK antibodies into PC12 cells blocked neurite outgrowth, though the fact that mutation of tyrosine 785 fails to do so suggests that CHK must interact with multiple or alternative residues within TrkA.

Another potential adaptor protein that interacts with tyrosine 785 is Cbl (Figure 1.2). NGF treatment leads to the tyrosine phosphorylation of Cbl (Galisteo and others 1995),

but evidence of a direct association between Cbl and TrkA is lacking. However, it was recently reported that mutation of tyrosine 785 in TrkB blocked BDNF-induced activation of both Cbl and PLCγ (McCarty and Feinstein 1999), suggesting that Cbl may in fact bind at this site. It is interesting to note that Cbl adapts many RTKs to the Src family of non-receptor tyrosine kinases, and that Src is tyrosine phosphorylated in response to NGF in PC12 cells through an as yet undefined mechanism (Sato and others 1998) (Howe unpublished observations). The role of Cbl in NGF and TrkA signaling is discussed further in chapter 4 of this manuscript.

Tyrosine 490, near the juxtamembrane domain of TrkA, is also involved in mediating TrkA signaling. A primary target of tyrosine 490 appears to be Shc, an adaptor protein that is critical to activation of the Ras signaling cascade (Figures 1.2 and 1.3) (Basu and others 1994; Obermeier and others 1994). While Shc was originally thought to interact with tyrosine 490 of TrkA via its SH2 domain, more recent work suggests that the interaction is in fact mediated via a phosphotyrosine binding domain (PTB) within Shc (Dikic and others 1995). Following phosphorylation of tyrosine 490 and subsequent binding and phosphorylation of Shc, the Grb2-Sos complex binds to phospho-Shc via an SH2 interaction (Rozakis-Adcock and others 1992), thereby bringing Sos into proximity to membrane-associated Ras and activating the MAP kinase signaling cascade. Sos, bound to Grb2 via two SH3 interactions (Cohen and others 1995), is a Ras GTP exchange

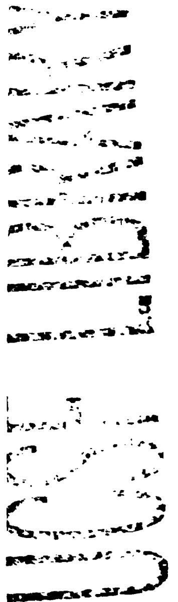
factor that promotes the transition from inactive Ras-GDP to active Ras-GTP (McCormick 1994). GTP-bound Ras then triggers a kinase cascade that results in the phosphorylation and activation of the MAP kinases, a family of proline-directed kinases that translocate into the nucleus and phosphorylate several transcription factor substrates (Hill and Treisman 1995). Interestingly, mutations in tyrosine 490 of TrkA do not abolish NGF induction of the MAP kinase signaling pathway. However, cells expressing TrkA with a double mutation at tyrosine 490 and tyrosine 785 do not exhibit MAP kinase activation or neurite outgrowth in response to NGF (Stephens and others 1994). This finding suggests that there is an as yet undiscovered complexity or redundancy to the interaction of adaptor proteins with tyrosines 490 and 785. One possible component in this additional complexity is the recent finding that Grb2 binds directly to activated TrkA at both tyrosine 785 and the kinase activation loop tyrosines (MacDonald and others 2000). This additional route to the Ras pathway may circumvent loss of either tyrosine 490 or tyrosine 785, but not loss of both tyrosines. Finally, overexpression of CHK, reported to bind tyrosine 785, as described above, leads to enhanced activation of the MAP kinase pathway, suggesting that this kinase may also mediate some of the complexity in the interaction between cascades downstream from either tyrosine 490 or tyrosine 785 (Yamashita and others 1999a).



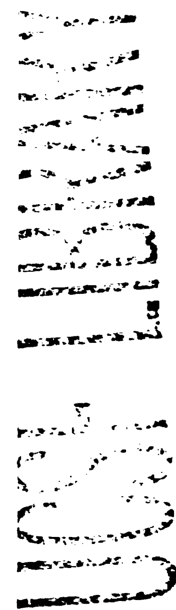
Tyrosine 490 also appears to mediate the interaction of TrkA with FRS-2, a novel membrane-anchored adaptor protein that is tyrosine phosphorylated in response to NGF (Figure 1.2) (Kouhara and others 1997; Ong and others 2000). Phosphorylated FRS-2 binds to the Grb2-Sos signaling unit, forming a multi-protein complex that includes Crk and the protein tyrosine phosphatase SHP-2 (Hadari and others 1998; Kouhara and others 1997; Meakin and others 1999). Formation of this complex is necessary for FRS-2 activation of the Ras pathway. FRS-2 appears to compete with Shc for binding to tyrosine 490 on TrkA, adding yet another layer of complexity to the signaling cascades elicited by NGF treatment (Meakin and others 1999).

Finally, tyrosine 490 may play a role in linking TrkA to the phosphotidylinositol 3-kinase (PI3 kinase) pathway via Grb2 and the Grb2-associated binder-1 (Gab1) protein. Gab1 was initially identified as a Grb2-associated protein in a human glial tumor expression library, and was also identified in a yeast 2-hybrid screen using the Met RTK as bait (Holgado-Madruga and others 1996; Weidner and others 1996). Gab1 is a member of a family of adaptor proteins that includes Gab2, IRS-1, IRS-2, and Dos, all of which exhibit sequence homology within their pleckstrin homology domains, and all of which link plasma membrane RTKs to intracellular signaling cascades (Bausenwein and others 2000; Gu and others 1998). Gab1 contains several SH2 and SH3 binding domains that recognize PI3 kinase and SHP-2, as well as Grb2, Nck, and Crk (Holgado-Madruga and

others 1996; Weidner and others 1996). Gab1 is tyrosine phosphorylated in response to signaling downstream from TrkA (Holgado-Madruga and others 1997), and it is also induced to associate with and activate the p85 subunit of PI3 kinase. Furthermore, overexpression of Gab1 reduced the concentration of NGF necessary for mediating cell survival in serum free conditions, while expression of a mutant Gab1 lacking the PI3 kinase binding sites enhanced apoptosis (Holgado-Madruga and others 1997). These data suggest that anti-apoptotic TrkA signaling to PI3 kinase and the Akt pathway is mediated by Gab1. This is supported by the finding that adenovirus-mediated expression of Gab1 in PC12 cells is sufficient to support enhanced survival, even in the absence of NGF signaling, and that this enhancement is correlated with increased PI3 kinase signaling (Korhonen and others 1999). However, Gab1 appears to utilize both the PI3 kinase pathway and the MAP kinase pathway to mediate its effect on cell survival, as pharmacological inhibition of both MAP kinase kinase (MEK) and PI3 kinase was required to fully suppress Gab1-mediated cell survival (Korhonen and others 1999). Finally, adenovirus-expressed Gab1 enhanced neurite outgrowth in response to NGF via a mechanism that was sensitive to either MEK inhibition or PI3 kinase inhibition (Korhonen and others 1999). These results suggest that Gab1 plays a role as an adaptor protein for both the PI3 kinase pathway and the MAP kinase pathway downstream from TrkA signaling. However, another member of the Gab family, Gab2, was recently identified as a substrate for tyrosine phosphorylation downstream of TrkA (Figure 1.2),



and Gab2 was found in complex with CrkL, C3G, and SHP-2 following NGF treatment of PC12 cells (Wu and others 2000). This finding suggests that Gab2 may adapt TrkA to the Rap1/B-Raf pathway by inducing NGF-dependent activation of C3G, a Rap GTP exchange factor. Activation of the Rap1 pathway leads to MEK activation in parallel with the Ras pathway, and it is possible that overexpressed Gab1 subsumed the role of endogenous Gab2 in mediation of neurite outgrowth.



The juxtamembrane region of TrkA, a unique region in the cytoplasmic domain of the receptor, has been implicated in carrying out several specific signaling functions downstream from NGF binding. This region contains the Shc NPQY binding site (Obermeier and others 1993), an adjacent IMENP site involved in differentiative signaling (Meakin and MacDonald 1998), and a KGF motif that is proximal to the transmembrane domain and is involved in NGF-induced neuritogenesis (Peng and others 1995). Transfection of PC12 cells with a chimeric EGF receptor containing the TrkA juxtamembrane domain led to EGF-induced neurite outgrowth and prolonged MAP kinase activation, suggesting that this domain is a critical element in differentiative signaling downstream from NGF binding (Yoon and others 1997). The juxtamembrane domain is also implicated in the association of activated TrkA with Abl, a non-receptor tyrosine kinase that is involved in the regulation of adhesion-dependent signaling and cytoskeletal remodeling that occurs during neuronal differentiation (Yano and others

2000). The basic pathway involves phosphorylation of the SH2 and SH3 domain-containing protein Crk (Reichman and others 1992), and consequent dissociation of a CrkII, paxillin, Abl complex (Escalante and others 2000), leading to activation of Abl. Activated Abl then feeds back into the system to regulate the phosphorylation of paxillin and Crk. Treatment of PC12 cells with NGF induces the tyrosine phosphorylation of paxillin, the formation of a Crk, paxillin, and Abl complex, and the association of Crk with TrkA (Matsuda and others 1994; Ribon and Saltiel 1996; Teng and others 1995; Torres and Bogenmann 1996). Formation of this complex is likely mediated via the association of Abl with the TrkA juxtamembrane domain (Yano and others 2000). It is interesting to note that tyrosine phosphorylation of Crk and paxillin are critical to the increased cell adhesion necessary for neurite outgrowth, and that Abl is involved in this pathway in Drosophila (Gertler and others 1989; Gertler and others 1993; Wills and others 1999). Further evidence that Crk plays a role in NGF-induced neurite outgrowth and differentiation comes from studies showing that v-Crk enhances NGF signaling through the Erk pathway (Teng and others 1996), and that dominant-negative Crk suppresses NGF signaling (Matsuda and others 1994). As mentioned earlier, Crk is found in complex with Grb2 and Sos within the Ras pathway, and it also associates with the Rap GTP exchange factor C3G (Matsuda and others 1994). This links Crk to the MAP kinase cascade in two ways. Interestingly, via its interaction with C3G, Crk may lead to the NGF-dependent sustained activation of Rap1. In this model, upon activation,

Rap1 binds to and activates B-Raf, thereby triggering the sustained activation of Erk that is a necessary component of neurite outgrowth (York and others 1998).

The Suc-associated Neurotrophic factor-induced Tyrosine-phosphorylated target protein, or SNT, is another signaling agent that interacts with the juxtamembrane domain of TrkA. SNT, which may or may not be identical to FRS-2 (Friedman and Greene 1999; Kouhara and others 1997), is considered a candidate for the factor that controls the decision between cell cycle progression and cell cycle arrest, a critical component of differentiative signaling. Its ability to bind the cyclin-dependent kinase substrate p13^{suc1}, and the fact that it is rapidly tyrosine phosphorylated in response to NGF, even in the absence of tyrosines 490 and 785 in TrkA (Rabin and others 1993), as well as the fact that cell cycle arrest also occurs in the presence of such mutations (Meakin and MacDonald 1998), suggests that SNT is indeed the mediator of this key decision. While the relationship between SNT and FRS-2 is still unresolved, recent evidence indicates that human FRS-2 does binds p13^{suc1} (and, interestingly, the SH3 domain of src) in a constituitive manner (Meakin and others 1999), strengthening the possibility that FRS-2 is an SNT.

Two other adaptor proteins which do not appear to interact with either tyrosine 490 or tyrosine 785 are rAPS and SH2-B, which were recently identified as TrkA substrates in

developing cortical and sympathetic neurons (Figure 1.2) (Qian and others 1998). Both rAPS and SH2-B were found in complex with Grb2, and either adaptor was able to mediate NGF induction of the Ras pathway. Furthermore, in nnr5 PC12 cells expressing extremely low levels of TrkA, co-transfection with either rAPS and a TrkA mutant lacking all tyrosines except those in the kinase activation loop, or with SH2-B and this TrkA mutant, led to robust neurite outgrowth (Qian and others 1998). Moreover, while the interaction between rAPS and Grb2 is at least partially dependent upon tyrosine phosphorylation of rAPS, Grb2 appears to bind to SH2-B constituitively via an SH3 interaction. Finally, antibodies to SH2-B inhibited NGF-dependent survival of cultured neonatal sympathetic neurons, and transfection with a dominant-interfering mutant of SH2-B completely blocked the elaboration of axons by cultured sympathetic neurons. This suggests that SH2-B and rAPS are critical elements in the TrkA signaling pathway necessary for both neurite outgrowth and survival, but that their interaction with TrkA may utilize a novel association mechanism.

3.1.4 TrkA Downstream Signaling Cascades

As indicated above, several signaling cascades downstream from TrkA activation have been identified. Foremost among these is the Ras pathway, which is implicated in pleiotropic responses to NGF (Figure 1.3). This pathway involves the recruitment of Shc, Grb2, and Sos to the plasma membrane, where the Ras GTP exchange factor Sos can gain

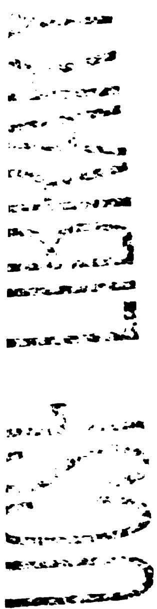
access to the membrane-associated small G-protein Ras (Segal and Greenberg 1996). Ras is targeted to the plasma membrane via farnesylation (Casey 1995), and resides at the plasma membrane in an inactive, GDP-bound state. Upon recruitment of Sos to the membrane, Ras is activated by exchange of GDP for GTP (McCormick 1994). GTP-bound Ras then recruits the serine-threonine kinase C-Raf to the plasma membrane (Marshall 1994; Van Aelst and others 1993; Wood and others 1992). In PC12 cells, Raf family members (Jaiswal and others 1994; Oshima and others 1991; Traverse and Cohen 1994) mediate the signal downstream from NGF by phosphorylating and thereby activating the dual-specificity MAP kinase kinase MEK1 (Figure 1.3) (Jaiswal and others 1994; Lange-Carter and Johnson 1994; Vaillancourt and others 1994). Raf catalyzes the phosphorylation of MEK1 at serine 217 and serine 221, activating it and inducing the further phosphorylation of two members of the MAP kinase family, extracellular signal-related kinases 1 and 2 (Erk 1/2) (Figure 1.3) (Crews and others 1992; Crews and Erikson 1992). Erk1/2 are phosphorylated on threonine 202 and tyrosine 204 by MEK1 (Payne and others 1991), leading to activation and translocation of Erk1/2 into the nucleus (Chen and others 1992). Erk1/2 are proline-directed serine-threonine kinases that phosphorylate the consensus sequence (P/L)-X-(T/S)*-P in several substrates (Mansour and others 1994; Marshall 1994), including Elk-1 (Miranti and others 1995). Phosphorylation of Elk-1 at serine 383 and serine 389 stimulates its interaction with the transcription factor serum response factor (SRF) and with the CAGGAT binding site within the c-fos gene

(Figure 1.3) (Gille and others 1995; Hill and others 1993; Mueller and Nordheim 1991; Treisman 1992). c-fos is an immediate early gene that is rapidly transcribed in response to many extracellular stimuli, including NGF (Ginty and others 1994; Greenberg and others 1986; Sheng and Greenberg 1990). A sequence called the c-fos SRE, or serum response element, is a 20 base pair region located roughly 310 base pairs 5' to the site of transcription initiation within the c-fos gene. It contains a core sequence called the CArG box, which is composed of the sequence $CC(A/T)_6GG$, and which serves as a binding site for the transcription factor SRF (Treisman 1992; Treisman 1994). The CAGGAT Elk-1 binding site within the c-fos promoter is directly adjacent to the CArG box, and SRF must bind to the CArG box prior to Elk-1 binding to the CAGGAT site within the SRE. Hence, SRF serves as a docking site for activated Elk-1, and SRF and Elk-1 must act together in PC12 cells to mediate induction of SRE-dependent c-fos transcription following NGF treatment (Hill and others 1993; Mueller and Nordheim 1991).

Additional transcription factors contribute to the regulation of c-fos transcription in response to NGF signaling. The cAMP regulatory element binding protein (CREB) is a transcription factor that binds to three sites within the c-fos promoter that differ from the SRE (Figure 1.3) (Berkowitz and others 1989). Mutation of these binding sites inhibits NGF induction of c-fos transcription without disrupting the SRE (Bonni and others 1995). Furthermore, NGF signaling leads to the phosphorylation of CREB at serine 133

via a Ras-dependent mechanism (Ginty and others 1994). This phosphorylation permits CREB interaction with SRF and Elk-1 (Bonni and others 1995; Ramirez and others 1997), possibly via the transcriptional coactivator protein CREB binding protein (CBP), which binds to phosphorylated serine 133 in CREB (Chrivia and others 1993), as well as to SRF (Ramirez and others 1997) and Elk-1 family members (Janknecht and others 1993). However, CREB may play an even more important role in transcriptional regulation of several NGF-specific delayed response genes, including the VGF gene. Mutation of the CREB binding site within the VGF gene significantly reduced NGF-induced VGF transcription (Hawley and others 1992). Interestingly, VGF transcription may require the cooperation of CREB with an as yet unidentified transcription factor product of an immediate early gene. CREB is persistantly phosphorylated at serine 133 for several hours after an initial NGF stimulus, and this may permit accumulated immediate early gene protein to interact with activated CREB . In contrast, EGF stimulation, which does not lead to VGF transcription, only transiently phosphorylates CREB, such that by the time sufficient immediate early gene product is present, activated CREB may no longer be available to cooperatively stimulate VGF transcription (Bonni and others 1995). This may be one mechanism by which NGF and EGF activate different transcriptional programs leading to either differentiation or proliferation (Marshall 1994).

The difference in temporal control of CREB phosphorylation induced by NGF or EGF is a specific example of a more general temporal difference elicited in the MAP kinase pathway by these two growth factors. In PC12 cells treated with NGF, there is a sustained activation of the MAP kinase pathway that persists for several hours. In contrast, EGF stimulation only transiently activates the MAP kinase pathway (Muroya and others 1992; Qui and Green 1992; Traverse and others 1992), suggesting that the temporal dynamics of Erk1/2 activation may account for a differentiative versus proliferative signaling outcome. One explanation for how two RTKs linked to very similar signaling pathways might induce such very different Erk activation kinetics requires a better understanding of the specific isoforms of certain adaptor proteins utilized in these cascades. For example, while both NGF and EGF appear to utilize the classic Shc/Grb2/Sos/Ras/C-Raf/MEK pathway to activate Erk, NGF also utilizes an accessory route to Erk activation that utilizes Gab2/CrkL/C3G/Rap1/B-Raf/MEK (Figure 1.4). This second pathway, unique to NGF signaling, promotes sustained activation of Erk1/2 (Wu and others 2000; York and others 1998), while the Ras-mediated pathway utilized by both receptors only results in a rapid and transient Erk activation. Hence, NGF activation of the Rap1 pathway may lead to Erk activation that is sustained long enough to induce either sufficient immediate early gene transcription, and translation of protein products that are able to interact with activated CREB, or that is sufficient to induce transcription of novel delayed response genes. However, this simple model is



tempered by the fact that expression of a mutant Rap that blocks sustained Erk activation in response to NGF does not block neurite outgrowth in PC12 cells (York and others 1998). On the other hand, complete inhibition of Erk activation either by pharmacological inhibition of MEK or transfection with a dominant-interfering MEK mutant does block NGF-induced neurite outgrowth (Cowley and others 1994; Pang and others 1995), and inhibition of Ras activity by microinjection of a Ras-neutralizing antibody also blocks differentiation (Hagag and others 1986). This indicates that Ras-dependent signaling is critical to NGF-induced differentiation, suggesting that some very early event triggered by a Ras- and C-Raf-mediated transient activation of the Erk pathway is necessary for priming the cell to respond differentiatively to the later and sustained activation of Erk by the Rap1 and B-Raf pathway.

A further level of control of NGF-induced immediate early gene transcription and translation comes from parallel activation of the Rsk pathway downstream from Ras (Figure 1.3). The Rsk serine-threonine kinase was originally isolated as a 90 kDa cell-cycle regulated kinase that phosphorylated the S6 protein of the 40S ribosomal subunit (Erikson and Maller 1991; Erikson and others 1991). This p90 kinase (Ribosomal S6 Kinase, hence Rsk) was itself found to be regulated by serine-threonine phosphorylation, and Erk1/2 were subsequently identified as the kinases responsible for this regulatory phosphorylation (Sturgill and others 1988; Zhao and others 1996). Rsk is a unique

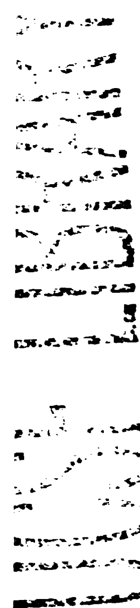
serine-threonine kinase, in that it contains both an N-terminal kinase that phosphorylates Rsk substrates, and a C-terminal kinase that is involved in its own activation (Jones and others 1988). The Rsk family is comprised of Rsk1, Rsk2, and Rsk3, each showing unique patterns of tissue expression (Moller and others 1994; Zhao and others 1995). Rsk2 was identified as a Ras-dependent protein kinase that phosphorylates CREB on serine 133 (Ginty and others 1994; Xing and others 1996), thereby regulating its transcriptional activation. Rsk family members are also involved in phosphorylation of the estrogen receptor-α, IκBα/NFκB, and c-fos (Ghoda and others 1997; Joel and others 1998; Schouten and others 1997; Xing and others 1996). Rsks also bind to the transcriptional coactivator CBP (Nakajima and others 1996), and phosphorylate several members of the ribosomal complex (Angenstein and others 1998). Interestingly, Sos is a substrate for Rsk which appears to be negatively regulated by Rsk kinase activity (Douville and Downward 1997), suggesting that Rsk activation downstream from activation of Erk1/2 may feed back to truncate Ras signaling. Recently, all three members of the Rsk family were found to be activated by NGF in PC12 cells, and all were able to phosphorylate CREB at serine 133 (Xing and others 1998). Hence, the Ras pathway is able to sensitively regulate c-fos induction by using a parallel and cooperative pathway in which Erk phosphorylation of Elk-1 converges upon Rsk phosphorylation of CREB (Figure 1.3) (Xing and others 1996). Furthermore, once synthesized, c-fos is subject to regulatory phosphorylation at two serines in its C-terminus. These serines are

located within Rsk and Erk consensus sites, and Rsk and Erk phosphorylate c-fos in a coordinate manner to stabilize the protein and enhance its downstream function (Chen and others 1993; Chen and others 1996). Thus, the Erk pathway is marked by both divergent and convergent signaling, in which an early divergence at the level of Shc versus Gab2 can control the temporal dynamics of Erk activation, and convergence at the level of Elk-1 and CREB regulation of c-fos can control gene transcription and protein translation.

Convergence of signaling is also found between the downstream pathways elicited by PLCγ activation and the Ras pathway. Following binding to tyrosine 785 of TrkA, PLCγ is activated and induced to hydrolyze phosphatidylinositol 4,5-bisphosphate (PI 4,5-P_2). PI 4,5-P_2 is produced by successive phosphorylations of phosphatidylinositol at the D-4 and D-5 positions of the inositol ring (Figure 1.5). PI 4,5-P_2 then serves as a substrate for modification by either PI3 kinase, which will be discussed shortly, or by PLCγ (Figure 1.5) (Kamat and Carpenter 1997). PLCγ-mediated hydrolysis of PI 4,5-P_2 yields two products that each function as intracellular second messengers: inositol 1,4,5-P_3 (IP_3), which interacts with its specific receptor on the endoplasmic reticulum to induce the release of intracellular calcium, and diacylglycerol (DAG), which is a potent activator of protein kinase C (PKC) isoforms (Figure 1.5) (Lee and Rhee 1995). IP_3-mediated release

from intracellular calcium stores leads to the activation of calcium-dependent proteins within the cell and to the generation of further IP derivatives such as IP_4, IP_5, and IP_6, which are able to interact with other intracellular proteins (Menniti and others 1993). DAG produced downstream from PLCγ is a critical activator of several isoforms of the serine-threonine calcium-dependent kinase PKC, including the classical PKC isoforms α, βI, βII, and γ (Bell and Burns 1991; Nishizuka 1988), and the novel PKC isoforms δ, ε, η, and θ (Liyanage and others 1992; Ono and others 1988; Osada and others 1990; Osada and others 1992). DAG may also play a role in activation of the atypical PKC isoforms ζ, ι/λ, and μ (Marais and others 1998). DAG cooperates with calcium, phosphatidylserine, cis-unsaturated fatty acids, and lysophosphatidylcholine to activate the classical PKC isoforms, and it cooperates with phosphatidylserine and cis-unsaturated fatty acids to activate the δ and ε isoforms of novel PKC. Interestingly, PLCγ activation is also involved in the generation of these other modulators, as PLCγ-induced DAG can be further hydrolyzed to arachidonic acid, which can then be converted into eicosanoids such as prostaglandin, that then feedback into the production pathway for factors like cis-unsaturated fatty acid. Furthermore, PLCγ activation is often accompanied by phospholipase A2 (PLA2)-mediated hydrolysis of phosphatidylcholine, directly generating cis-unsaturated fatty acid and lysophosphatidylcholine (Asaoka and others 1992; Nishizuka 1992). These factors, in combination with DAG, serve to sensitively

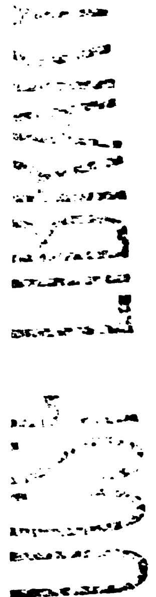
tune PKC activation to signaling downstream from TrkA, leading to phosphorylation of several proteins critical to survival and differentiation (Coleman and Wooten 1994; Wooten and others 1999; Wooten and others 1994; Wooten and others 1997). One such substrate of PKC is Raf, which is directly activated by PKCζ-mediated phosphorylation (van Dijk and others 1997a; van Dijk and others 1997b), as well as by PKCα-, β-, and γ-mediated phosphorylation (Carroll and May 1994; Kolch and others 1993; Schonwasser and others 1998; Sozeri and others 1992). The association of PKCζ with Raf appears to be mediated by binding of the scaffolding protein 14-3-3 (Freed and others 1994; Fu and others 1994; Irie and others 1994; van der Hoeven and others 2000). Such a PKC-(14-3-3)-Raf complex may also contribute to PKCθ- and PKCμ-mediated regulation of the MAP kinase cascade (Hausser and others 1999; Meller and others 1996), and may account for PKCε-mediated activation of Raf (Cacace and others 1996; Ueffing and others 1997). It has also been suggested that PKC mediates activation of the MAP kinase cascade by directly activating Ras, leading to the formation of (Ras-GTP)-Raf complexes independently of an activation pathway that is sensitive to blockade by the N17Ras dominant-negative mutant (Marais and others 1998). This finding is consistent with evidence that PKC-mediated activation of Raf is blocked by mutation in the Ras-binding domain of Raf (Luo and others 1997). Finally, PKC can directly phosphorylate the c-jun protein product, which is also under the control of phosphorylation by Erk, and which is



able to bind to the c-fos protein product to form the transcriptional regulatory complex AP-1 (Oberwetter and others 1993).

Convergence of control over the MAP kinase pathway may also occur between Ras, PKC, and Src. Src was originally identified as the transforming protein tyrosine kinase of the oncogenic retrovirus, Rous sarcoma virus (Brugge and Erikson 1977; Purchio and others 1978), in which it is mutated to a constituitively active form (Brown and Cooper 1996; Collett and Erikson 1978; Stehelin and others 1976). Src was subsequently identified as a member of a large family of non-receptor protein tyrosine kinases that share significant sequence homology, including Fyn, Yes, Yrk, Blk, Fgr, Hck, Lck, Lyn, Frk/Rak, and Iyk/Bsk (Brown and Cooper 1996; Cance and others 1994; Lee and others 1994a; Thomas and Brugge 1997; Thuveson and others 1995; Welch and Maridonneau-Parini 1997). This family of kinases regulates a wide range of cellular events, ranging from cell proliferation, cytoskeletal alterations, and differentiation, to survival, adhesion, and migration. Src family members also contain two modular protein:protein interaction domains that are found in many cellular proteins, including adaptor molecules, transcription factors, cytoskeletal proteins, protein phosphatases, lipid kinases, and lipid phosphatases (Pawson and others 1993). The first such domain is called an SH2 domain (Src homology 2) (Cohen and others 1995; Pawson 1995). SH2 domains in all proteins bind to short contiguous sequences containing phosphotyrosine, with specificity of any

given SH2 domain defined by the 3-5 residues following the phosphotyrosine (Pawson 1995; Songyang and others 1993), as well as residues upstream from the phosphotyrosine (Bibbins and others 1993). Structurally, the SH2 domain is composed of two binding pockets, one of which binds to the phosphotyrosine, and the other of which binds to the third downstream residue (Eck and others 1993; Waksman and others 1993).



The second modular protein:protein interaction domain based upon the Src family prototype is the SH3 domain. This module binds to short contiguous proline-rich regions that contain the core consensus sequence of P-x-x-P, with specificity of binding conferred by surrounding residues (Rickles and others 1995). The SH3 binding pocket contains two hydrophobic grooves that come into contact with the P-x-x-P core, and a second region that contacts the residues either N-terminal or C-terminal to the proline core (Feng and others 1994b; Yu and others 1994). While SH2 and SH3 domains play a role in protein:protein interaction in a host of proteins, within the Src family members, these domains play specific roles in activation of Src catalytic activity. In the basic model of activation, based upon the crystal structures of Src and Hck (Moarefi and others 1997; Sicheri and Kuriyan 1997; Sicheri and others 1997; Xu and others 1997), a C-terminal regulatory tyrosine phosphorylated by the C-terminal Src kinase (Csk) binds to the SH2 domain of the same Src protein. This bond holds the kinase in an inactive conformation that is released upon dephosphorylation of the regulatory tyrosine (Cartwright and others

1987; Imamoto and Soriano 1993; Kmiecik and Shalloway 1987; Nada and others 1993; Piwnica-Worms and others 1987; Reynolds and others 1987). Release of this SH2 interaction results in an extended conformation of the catalytic domain, which may serve both to release the kinase domain from a negative regulatory interaction with the SH3 domain, and to open the kinase to interaction with substrates. Furthermore, a tyrosine autophosphorylation site exists within the catalytic domain of Src, and appears to be important for proper orientation of the N-terminal lobe of the catalytic domain following release from the SH2 and SH3 restraints (Cooper and others 1986; Kmiecik and others 1988; Kmiecik and Shalloway 1987; Parsons and Weber 1989; Piwnica-Worms and others 1987; Snyder and others 1983).

In addition to the recruitment of various adaptors and cascade linkers, RTKs also interact with Src family non-receptor tyrosine kinases, and utilize these kinases for the transduction of several critical pathways (Erpel and Courtneidge 1995). The first RTK found to interact with a Src family kinase was PDGF-Rβ (Ralston and Bishop 1985). Treatment with PDGF-BB was found to increase the catalytic activity of Src, Fyn, and Yes in fibroblasts, and receptor activation induced serine and tyrosine phosphorylation of Src and Fyn (Gould and Hunter 1988; Kypta and others 1990). The Src SH2 domain binds directly to two phosphotyrosines within the juxtamembrane of PDGF-R, leading to the activation of Src. This activation probably occurs via a mechanism in which the Src

SH2 domain releases the Src catalytic domain in order to bind to the phosphotyrosines on PDGF-R, thereby freeing the Src kinase domain of its intramolecular restraints (Alonso and others 1995; Mori and others 1993). PDGF-R may also proactively turn on Src via phosphorylation of two serines and a tyrosine within the N-terminal half of the protein, and phosphorylation of a tyrosine within the catalytic domain (Broome and Hunter 1997; Gould and Hunter 1988; Ralston and Bishop 1985; Stover and others 1996).

EGFR signaling also makes use of Src family kinases. Overexpression of Src enhances several responses downstream from EGF, including tyrosine phosphorylation, DNA synthesis, and tumor formation (Chang and others 1995a; Chang and others 1995b; Luttrell and others 1988; Maa and others 1995; Wilson and others 1989; Wilson and Parsons 1990). Furthermore, EGF stimulation induces a two- to threefold increase in Src kinase activity, as well as a redistribution of Src to specific subcellular fractions indicative of activation (Sato and others 1995; Weernink and Rijksen 1995). Src may interact directly with EGFR, either via an interaction between EGFR and the catalytic domain of Src, or by way of an SH2-mediated interaction (Lombardo and others 1995; Maa and others 1995; Sato and others 1995). Activation may also be mediated indirectly. For example, activation of the SHP-2 tyrosine phosphatase by EGFR may activate Src family kinases by dephosphorylating the negative regulatory tyrosine within the C-terminal region of the kinase (Feng and others 1994a). Finally, several other receptor

families recruit and activate Src family members, including FGFR, the insulin receptor, G-protein coupled receptors, GPI-linked receptors, and cytokine receptors (Thomas and Brugge 1997).

Involvement of Src or a Src family member in NGF-mediated differentiative signaling was first proposed when it was discovered that infection of PC12 cells with the oncogenic form of Src recapitulated the neurite outgrowth induced by NGF (Alema and others 1985). Further analysis showed that neutralization of Ras by microinjection of anti-Ras antibodies blocked the neuritogenic effects of both Src and NGF (Hagag and others 1986; Kremer and others 1991). In contrast, neutralization of Src activity by antibody microinjection did not block neurite outgrowth induced by infection with oncogenic Ras (Bar-Sagi and Feramisco 1985; Kremer and others 1991; Noda and others 1985), but did inhibit NGF-induced neuritogenesis, and did cause retraction of established neurites induced by NGF or FGF treatment (Kremer and others 1991). Finally, both oncogenic Src and oncogenic Ras are able to "prime" PC12 cells, such that subsequent NGF treatment elicits a more rapid and robust neuritogenesis than NGF treatment of unprimed cells (Thomas and others 1991). These data suggest that the role of Src in neurite outgrowth is downstream of TrkA activation, but upstream of Ras signaling. However, evidence from another differentiative signaling cascade argues against a linear relationship between Src and Ras, and suggests that parallel and differentially regulated

pathways are involved. In PC12 cells expressing PDGF-Rβ, treatment with PDGF-BB, like treatment with NGF, induces cessation of growth, neurite outgrowth, induction of sodium channels, persistent activation of the MAP kinase pathway, and persistent phosphorylation of PLCγ (Fanger and others 1995a; Fanger and others 1995b; Gronwald and others 1988; Qui and Green 1992). In cells expressing mutant PDGF-R that is unable to elicit morphological differentiation, persistent activation of the MAPK pathway was still observed (Vaillancourt and others 1995), indicating that, at least in this signaling system, there is a disconnect between neurite outgrowth and persistent MAP kinase activation. Unexpectedly, persistent tyrosine phosphorylation of PLCγ and association of Src with the RTK were correlated to neurite outgrowth (Vaillancourt and others 1995), suggesting that these two signaling elements play critical roles in differentiative signaling. Hence, as persistent activation of the MAP kinase pathway is not predicative of differentiation, while signaling through the Src pathway apparently is, the relationship between Src and Ras discussed above must not be linear (i.e. Src is not simply upstream from Ras), but rather parallel. Further support for a parallel relationship comes from studies showing that in conditionally immortalized E17 hippocampal neurons, oncogenic Src induced cellular differentiation without activation of the Ras-Raf-MAP kinase pathway. However, Src did activate the N-terminal c-jun kinase (JNK) pathway (Kuo and others 1997), another member of the MAP kinase family of serine-threonine kinases.

Hence, one possible explanation for the role that both Src and Ras play in differentiation would be that they share a common convergence factor downstream from each other, but upstream from a differentiative pathway signaled through either Erk1/2 or JNK (Lewis and others 1998). A possible candidate for this factor is one or several of the MEK family members, which control activation of both Erk1/2 and JNK under specific cellular conditions (Ellinger-Ziegelbauer and others 1997). This model is compatible with data showing that pharmacological inhibition of MEK in PC12 cells abrogated neurite outgrowth in response to NGF (Pang and others 1995). Interestingly, MEK activity is also regulated by several PKC isoforms (Berra and others 1993; Berra and others 1995; Schonwasser and others 1998; van Dijk and others 1997b), and overexpression of either PKCι or PKCζ resulted in enhanced NGF-induced neurite outgrowth and enhanced NGF-induced JNK activation (Wooten and others 1999), while inhibition of atypical PKC isoforms blocked NGF-induced activation of JNK (Wooten and others 1999). Furthermore, oncogenic Src enhances JNK activation, and this effect is blocked by expression of a dominant-negative Crk mutant, implicating the Rap pathway in this phenomenon (Dolfi and others 1998). PI3 kinase is also implicated in signaling to JNK, as NGF-induced JNK activation was impaired by either wortmannin or LY294002, and overexpression of PI3 kinase resulted in neurite outgrowth and JNK activation in the absence of Erk activation (Kobayashi and others 1997). Finally, inhibition of JNK activation directly blocked neurite outgrowth in response to NGF (Kita and others 1998).

Thus, a signaling cascade involving Src, PI3 kinase, PKC, and JNK appears to be involved in neurite outgrowth and differentiative signaling, and may either complement or parallel the Ras-Raf-MEK-Erk1/2 cascade.

Signaling through Src, PI3 kinase, PKC, and JNK may also play a role in cell survival signaling. Overexpression of either Src or PKCι enhanced PC12 cell survival in serum-free conditions, and both increased the activation of the transcription factor NFκB (Wooten and others 2000; Wooten and others 1999), apparently via JNK signaling. Moreover, inhibition of Src or atypical PKC isoforms promoted cell death (Seibenhener and others 1999; Wooten and others 2000). Likewise, inhibition of PI3 kinase activity blocked cell survival and reduced NGF-induced NFκB activation (Wooten and others 2000). These findings are compatible with data showing that constituitive activation of NFκB promotes cell survival and resistance to apoptosis (Giri and Aggarwal 1998), and that NGF induction of NFκB is primarily dependent upon signaling through the JNK pathway (Wooten and others 2000). Thus, both differentiative and survival signaling may be controlled in part by a signaling unit that includes Src, PI3 kinase, and PKC.

PI3 kinase and Src are also implicated in survival signaling via the common substrate Akt, a serine-threonine kinase also known as Protein Kinase B (PKB), or Related to A

and C Protein Kinase (RAC-PK). Akt was originally identified as the homolog of the retroviral oncogene v-Akt (Cross and others 1995; Franke and others 1995), and was subsequently found to be regulated by growth factor and serum factor signaling through PI3 kinase (Alessi and others 1996; Andjelkovic and others 1996; Burgering and Coffer 1995; Franke and others 1997; Franke and others 1995; Klippel and others 1997). PI3 kinase is a heterodimer composed of an 85 kDa regulatory subunit and a 110 kDa catalytic subunit. Activation of the kinase involves binding of the regulatory subunit either directly or via adaptors to activated RTKs. This interaction with the cytoplasmic domain of an RTK results in recruitment of the 110 kDa catalytic subunit to the plasma membrane, where it can interact with membrane phosphoinositides. PI3 kinase phosphorylates the D-3 position of the inositol ring of phosphatidylinositol (PI), PI-4-phosphate (PI4-P), and PI-4,5-P_2, to yield, respectively, PI-3-P, PI-3,4-P_2, and PI-3,4,5-P_3 (Figure 1.5) (Franke and others 1997). PI-3-P is constituitively produced by PI3 kinase, in the absence of growth factor signaling, and may play a role in vesicle trafficking via interaction with FYVE domains in proteins like EEA1 (Cockcroft 1999; Leevers and others 1999). PI-3,4-P_2 and PI-3,4,5-P_3 are rapidly induced in response to growth factor signaling, and both signal to activate Akt. In addition, PI-3,4,5-P_3 interacts with specific SH2 and pleckstrin homology domains within proteins such as PLCγ (Bae and others 1998; Falasca and others 1998; Rameh and others 1998) and the Arf-GEFs ARNO, GRP1, and cytohesin-1 (Chardin and others 1996; Klarlund and others 1998; Meacci and

others 1997; Nagel and others 1998; Venkateswarlu and others 1998), proteins that each play a role in vesicular trafficking (Moss and Vaughan 1998; Schimmoller and others 1997). Akt interacts with PI-3,4-P_2 or PI-3,4,5-P_3 via a pleckstrin homology domain located in its N-terminus, but this interaction alone is insufficient to activate the kinase. Rather, the 3-Phosphoinositide-Dependent Kinase PDK1 must also be present and active to get Akt activation (Figure 1.6). PDK1 also contains a pleckstrin homology domain that binds PI-3,4-P_2 or PI-3,4,5-P_3, and this binding is necessary to permit PDK1 to phosphorylate Akt on threonine 308. Threonine 308 is within the activation loop of Akt, and its phosphorylation is required for Akt activity (Alessi and others 1997a; Alessi and others 1997b; Cohen and others 1997; Stephens and others 1998; Stokoe and others 1997). Deletion of the Akt pleckstrin homology domain circumvented the need for PI-3,4-P_2 for activation, while point mutations within the pleckstrin homology domain blocked Akt activation (Stokoe and others 1997). Furthermore, as PDK1 is in a constituitively active conformation, binding of PI-3,4-P_2 or PI-3,4,5-P_3 to Akt must serve to induce a conformational change in Akt that permits phosphorylation of the activation loop (Leevers and others 1999). However, an interaction of PDK1 with PI-3,4-P_2 or PI-3,4,5-P_3 may also serve to control its localization at the plasma membrane, as overexpressed PDK1 was found to be primarily cytoplasmic, moving to the plasma membrane in a PI3 kinase-dependent manner (Anderson and others 1998; Andjelkovic and others 1996). Hence, TrkA signaling via PI3 kinase presumably signals to generate

3-phosphoinositides that conformationally control Akt and permit it to be activated by membrane-associated PDK1. Interestingly, PDK1 has been shown to phosphorylate the activation loop of several other serine-threonine kinases, including certain isoforms of PKC (Chou and others 1998; Le Good and others 1998), suggesting that PI3 kinase-mediated generation of 3-phosphoinositides may also control differentiative or survival signaling via PKC activation.

PI3 kinase mediation of TrkA survival signaling is indicated by experiments showing that two inhibitors of PI3 kinase activity, wortmannin and LY294002, induce apoptosis in PC12 cells and sympathetic neurons supported by NGF. Moreover, PDGF prevented apoptosis in PC12 cells expressing wild-type PDGF-R, but not in cells expressing a mutant PDGF-R that fails to activate PI3 kinase (Crowder and Freeman 1998; Yao and Cooper 1995). The role of Akt in regulation of cell survival downstream from PI3 kinase is suggested by the fact that overexpression of Akt in primary cultures of cerebellar neurons or sympathetic neurons provides protection against death induced by serum withdrawal or inhibition of PI3 kinase, while expression of dominant-interfering forms of Akt blocked NGF-mediated survival (Crowder and Freeman 1998; Dudek and others 1997). The mechanism by which Akt mediates survival is unclear, though Akt has been reported to bind and phosphorylate Bad, a member of the Bcl-2 family of proteins (Figure 1.6) (Datta and others 1997; del Peso and others 1997). Phosphorylation of Bad prevents

it from binding the anti-apoptotic Bcl-2 family members Bcl-2 and Bcl-X_L (Zha and others 1996), shifting the cell to contain more Bcl-2 homodimers than Bcl-2/Bax heterodimers. The Bcl family is composed of two groups of proteins, one which promotes cell survival and includes Bcl-2, Bcl-X_L, Mcl-1, A1, and Bag-1, and the other which promotes cell death and is comprised of Bcl-X_S, Bad, Bid, Bik, Bim, Bax, and Bak (Boise and others 1995; Kroemer 1997; Steller 1995). The members of the Bcl family form homo- and heterodimers, and the balance of each within the cell is considered to regulate the maintenance of survival or the induction of death. In the absence of phosphorylation of Bad on serine 112 and serine 136, Bad signals to promote cell death, apparently by forming heterodimers with Bcl-X_L (Figure 1.6). Formation of these heterodimers leads to the generation of Bax homodimers. Homodimerization of Bax induces its translocation into mitochondria and insertion into the mitochondrial membrane (Gross and others 1998). There it leads to altered mitochondrial membrane potential via ion channel formation, and to generation of cytotoxic reactive oxygen species (Xiang and others 1996). In contrast, the phosphorylation of Bad promotes cell survival by inducing an interaction between Bad and the 14-3-3 protein. This interaction effectively sequesters Bad from any interaction with Bcl-X_L, keeping the balance of Bcl-X_L/Bax heterodimers high, and preventing Bax homodimerization (Figure 1.6) (Zha and others 1996). Hence, TrkA survival signaling involves PI3 kinase-mediated activation of Akt, and the consequent maintenance of Bcl-X_L/Bax heterodimers. Interestingly, Src is

also implicated in the activation of Akt, via a mechanism that involves PI3 kinase and SHP-2 (Datta and others 1996; Hakak and others 2000). This interaction may explain the finding, presented above, that inhibition of Src promotes cell death, and suggests that additional complexity may exist in the mechanism by which TrkA signaling induces cell survival.

3.2 TrkB and TrkC

TrkB, the receptor tyrosine kinase for BDNF and NT-4/5, and TrkC, the RTK for NT-3, exhibit many of the same properties as TrkA (Bothwell 1995; Greene and Kaplan 1995). As described above, TrkA is the only RTK for NGF, while BDNF and NT-4/5 essentially share TrkB (Figure 1.7). NT-3 is the only ligand for TrkC, but NT-3 can also bind and activate TrkA and TrkB, at least within appropriate cellular contexts (Ip and others 1993b; Klein and others 1990; Lamballe and others 1991; Soppet and others 1991; Squinto and others 1991). The Trk family of RTKs all elicit essentially the same signaling cascades, though with varying degrees of intensity and along slightly different time courses, suggesting that these receptors share common adaptor proteins and common mechanisms for activating transcriptional events (Yuen and Mobley 1999). Interestingly, several variant isoforms of TrkB and TrkC are expressed in a cell-specific manner, some of which entirely lack the tyrosine kinase domain. The role of these kinase-less receptors is unclear, though it is hypothesized that they function to scavenge

free neurotrophin or to change the kinetics of binding of BDNF or NT-3 to their fully functional cognate RTKs. Whether these kinase-less receptor isoforms are able to signal following dimerization with the full-length RTK, and how such signaling may modify normal signal transduction in response to BDNF or NT-3, are unanswered questions. It is interesting to speculate that the various isoforms of TrkB and TrkC may function in a manner analogous to the p75 neurotrophin receptor (p75NTR), discussed below.

3.3 p75-Neurotrophin Receptor

3.3.1 Discovery of p75NTR

p75NTR was the first identified NGF receptor, and for many years was believed to be the only such receptor. Initial investigations into the specific binding of NGF to cell-surface receptors identified a wide variety of cell types exhibiting such binding, including sympathetic neurons (Frazier and others 1974; Massague and others 1981), Schwann cells (DiStefano and Johnson 1988b; Taniuchi and others 1986a), neurofibromas (Ross and others 1986), melanoma cells (Fabricant and others 1977), neuroblastoma cells (Marchetti and Perez-Polo 1987; Sonnenfeld and Ishii 1982), and pheochromocytoma (PC12) cells (Green and Greene 1986; Grob and others 1983; Hosang and Shooter 1985; Landreth and Shooter 1980). The identification of the receptor responsible for this binding proceeded with the use of affinity crosslinking of the receptor to iodinated NGF. Isolation of the major radioiodinated complex from several different cell types following

crosslinking identified a protein of 75 kDa (Massague and others 1981). Likewise, immunoprecipitation with monoclonal antibodies directed against the NGF receptor yielded a protein of 75-80 kDa (Green and Greene 1986; Grob and others 1985; Marano and others 1987; Taniuchi and others 1986a; Taniuchi and others 1986b). Finally, affinity chromatography with NGF-sepharose isolated a 75-80 kDa protein from sympathetic neurons and melanoma cells (Kouchalakos and Bradshaw 1986; Marano and others 1987; Puma and others 1983), and metabolic labeling experiments identified the receptor as a glycosylated protein that is processed from a 59 kDa precursor (Grob and others 1985). The receptor was cloned using an elaborate process in which high molecular weight DNA fragments were introduced into mouse fibroblasts, viable transfectants were selected, and cells expressing the receptor were isolated either via a clustering assay or by a cell sorting process using monoclonal antibodies against the receptor (Chao and others 1986; Radeke and others 1987). After selection of a cell line expressing the receptor, the gene was isolated by subtractive cDNA hybridization, and the validity of the cDNA was determined by expression in mammalian cells (Johnson and others 1986b; Radeke and others 1987) and *Xenopus laevis* oocytes (Sehgal and others 1988).

3.3.2 Structure of p75NTR

p75NTR is the first identified member of a superfamily of receptors that includes CD27, CD30, CD40, 4-1BB, OX40, LTβR, Fas (CD95), DR4, DR5, TRID, HVEM, TRAMP, CAR-1, and the tumor necrosis factor receptors (Bazan 1990; Cosman and others 1990; Mallett and Barclay 1991; Smith and others 1994). These receptors share several common signaling features, including the ability to control cell viability via regulation of apoptosis (this will be discussed more fully in the section regarding p75NTR signaling), as well as common structural features that include a highly conserved cysteine-rich motif within the extracellular ligand-binding domain. This motif takes the form of CxxCxxC, and is repeated several times within the extracellular region of each of these receptors (Ware and others 1998). The cysteine-rich motifs within p75NTR define the NGF binding site and play a role in stabilizing the NGF-p75NTR complex (Baldwin and others 1992; Welcher and others 1991; Yan and Chao 1991).

The rat gene for p75NTR encodes a 395 amino acid protein, of which 221 residues comprise the extracellular domain, 22 form the transmembrane region, and 152 residues comprise the intracellular domain (Johnson and others 1986a; Radeke and others 1987). The extracellular domain of p75NTR is glycosylated, and the intracellular domain is palmitoylated (Barker and others 1994), two post-translational modifications that are likely to control the membrane localization and distribution of the receptor. In addition

to the full-length receptor, p75NTR is also expressed as a membrane-anchored cytoplasmic domain that lacks the entire neurotrophin binding region. p75NTR also exists in a soluble form that consists only of the receptor's ligand binding domain, generated by the action of a cell-surface cysteine protease, perhaps in a signal-specific manner (Barker and others 1991; DiStefano and others 1993; DiStefano and others 1991; DiStefano and Johnson 1988a; Zupan and others 1989). Finally, comparison of the p75NTR sequences from rat, human, and chicken indicates that the juxtamembrane, transmembrane, and distal intracellular domains are almost completely conserved, suggesting that these regions are critical to the function of p75NTR (Heuer and others 1990; Large and others 1989).

3.3.3 p75NTR Expression

p75NTR is widely expressed in many tissues, neuronal and non-neuronal. Within the peripheral nervous system, p75NTR is found within DRG sensory neurons, sympathetic neurons, and a subset of enteric and parasympathetic neurons (Carroll and others 1992; Schatteman and others 1993; Verge and others 1992; Yan and Johnson 1988). In the central nervous system, p75NTR is expressed during development within spinal motor neurons, cerebellar deep nuclei neurons, cerebellar Purkinje cells, the cuneate nucleus, the olivary pretectal nucleus, cortical subplate neurons, thalamic nuclei such as the lateral geniculate nucleus, and the amygdala (Buck and others 1988; Ernfors and others 1988;

Heuer and others 1990; Large and others 1989; Schatteman and others 1988). In addition, in the adult, p75NTR is expressed within basal forebrain cholinergic neurons, as well as in neurons of the superchiasmatic, trigeminal, hypoglossal, and raphe nuclei (Hefti and others 1986; Henry and others 1994; Koh and others 1989; Pioro and Cuello 1988; Sofroniew and others 1989; Springer and others 1987). A significant amount of p75NTR expression occurs within non-neuronal tissues, including extremely high levels of expression within the mesenchymal tissue of the pelvis and in the developing limb buds (Heuer and others 1990; Wheeler and Bothwell 1992). Expression is also found within mesenchymal tissue of the kidney, testes, inner ear, hair follicles, and lung, and within myoblasts, endothelial cells, perivascular fibroblasts, and dental pulp cells (Alpers and others 1993; Byers and others 1990; Durbeej and others 1993; von Bartheld and others 1991; Yan and Johnson 1988).

3.3.4 Interactions Between p75NTR and Trk

As discussed earlier, neurotrophins mediate many of their biological effects via Trk receptors. However, while some cells do only express Trk, such as early sympathetic neuronal precursor cells and pyramidal cells of the hippocampus (Ip and others 1993a; Marsh and others 1993; Verdi and Anderson 1994), many cells expressing Trk co-express p75NTR. On these cells, binding studies demonstrate two classes of binding sites. One site, called the low-affinity binding site, accounts for the predominance of neurotrophin

binding, and has a K_d of circa 10^{-9} nM and rapid association and dissociation kinetics. The other site, called the high-affinity binding site, exhibits rapid association but slow dissociation kinetics, and has a K_d of approximately 10^{-11} nM (Rodriguez-Tebar and others 1992; Schechter and Bothwell 1981; Sutter and others 1979). While no consensus currently exists regarding the identification of p75NTR as the receptor responsible for low affinity binding, most data support the idea that it is at least responsible for the majority of such binding (Clary and others 1994; Ibanez and others 1992; Johnson and others 1986a; Radeke and others 1987; Weskamp and Reichardt 1991). p75NTR appears to bind NGF, BDNF, and NT-3 with low affinity (Figure 1.7), while Trks are largely responsible for binding the neurotrophins with high affinity in the presence of p75NTR (Battleman and others 1993; Mahadeo and others 1994; Meakin and others 1992; Rodriguez-Tebar and others 1990; Rodriguez-Tebar and others 1992; Venkatakrishnan and others 1990). Conflicting reports exists regarding the dissociation kinetics of TrkA expressed alone, with most groups reporting a K_d of 10^{-9} nM (Clary and others 1994; Hartman and others 1992; Hempstead and others 1991; Mahadeo and others 1994). This suggests that high affinity binding sites are the result of a cooperative effect between the two receptors, rather than a facet of either receptor by itself. This is further supported by the fact that TrkA alone exhibits a relatively slow association rate, but in the presence of p75NTR expresses the fast association kinetics characteristic of the high affinity binding site (Mahadeo and others 1994; Sutter and others 1979). Furthermore, NGF binding to

p75NTR is a pre-requisite for this enhanced association rate, as specific inhibition of p75NTR binding reduces the amount of NGF that becomes bound to TrkA at limiting concentrations of NGF (Barker and Shooter 1994). These data support the idea that high affinity binding sites are formed via a fast association rate provided by p75NTR and a slow dissociation constant provided by TrkA.

The kinetic argument for the cooperativity between TrkA and p75NTR is supported by biochemical evidence that disruption of p75NTR binding reduces NGF-induced TrkA activation and gene induction, though only at low concentrations of NGF (Barker and Shooter 1994). Furthermore, p75NTR transgenic knockout mice exhibit a progressive loss of sensory and sympathetic innervation, consistent with the idea that a lack of p75NTR reduces the efficacy of neurotrophin binding, and hence reduces the viability of neurons that depend upon neurotrophic retrograde signaling. Sensory neurons from these animals do, in fact, exhibit reduced responsiveness to NGF, though, interestingly, BDNF and NT-3 binding appear to be unaffected (Davies and others 1993; Lee and others 1994b; Lee and others 1994c; Lee and others 1992). This observation suggests that the role p75NTR may play in enhancing neurotrophin binding is selective for sympathetic and sensory neurons that normally co-express TrkA and p75NTR.

The mechanism by which p75NTR cooperates with TrkA to generate high affinity binding is still unresolved. One hypothesis is that p75NTR interacts directly with TrkA to induce an allosteric change in the conformation of the TrkA ligand binding domain such that it binds NGF more efficaciously (Bothwell 1995). An analogous relationship exists between the receptors of the interleukin-2 (IL-2) system, in which the IL-2α subunit enhances the binding characteristics of the IL-2β receptor, without requiring the α subunit to bind IL-2 directly (Grant and others 1992; Lowenthal and Greene 1987; Wang and Smith 1987). Alternatively, p75NTR may enhance TrkA association kinetics by first binding to NGF and then presenting it to TrkA in an altered conformation that supports increased binding. This would require the formation of a heterodimer composed of one p75NTR and one TrkA, cooperatively bound to NGF. While the formation of such a heterodimer is not supported by kinetic data (Jing and others 1992), there is precedence for such a relationship in the transforming growth factor-β (TGF-β) system. In this system, TGF first binds to TGF-RIII, and is then transferred to TGF-RII, whereupon a TGF-RI/TGF-RII heterocomplex forms, resulting in signal transduction (Wrana and others 1994). Finally, a third possibility is that p75NTR acts to concentrate NGF near TrkA, enhancing the association rate with TrkA on a micro-environmental level in much the same way that simply increasing the overall concentration of NGF at the macro-environmental level increases the association rate (Barker and Shooter 1994).

In support of this model, co-expression of p75NTR with TrkA slowed the lateral mobility of p75NTR within the plane of the plasma membrane, suggesting that p75NTR is colocalized with TrkA within the membrane, where it may act to concentrate NGF (Wolf and others 1995).

p75NTR may also interact with TrkA to modify binding specificity. In fibroblasts that express only TrkA, NT-3 and NT-4/5 are able activate the receptor, while in PC12 cells, which express both p75NTR and TrkA, only NGF is able to activate TrkA (Berkemeier and others 1991; Ip and others 1993b). Likewise, mutant PC12 cells that express only very low levels of p75NTR exhibit NT-3-induced TrkA activation (Benedetti and others 1993). Finally, postnatal sympathetic neurons normally exhibit very limited survival in culture in response to NT-3, but these same neurons isolated from p75NTR transgenic knockout mice show a much more robust NT-3-induced survival response (Lee and others 1994c). These data suggest that p75NTR may function to tune individual neurons to specific neurotrophin responsiveness, thereby controlling the ability of such neurons to compete for target-derived neurotrophic support. It is interesting to note that sympathetic neurons normally undergo a switch in trophic dependence, from an early dependence upon NT-3 to a later dependence on NGF, and that this switch is temporally correlated to the onset of p75NTR expression (Birren and others 1993). Furthermore, as NGF signaling via TrkA appears to control p75NTR expression in these cells (Miller and

others 1991; Miller and others 1994; Verdi and Anderson 1994; Verge and others 1992; Wyatt and others 1990), it is likely that first contact between the innervating sympathetic fibers and NGF available from the target field elicits the trophic dependency switch. Also, the expression of p75NTR by cells which have received an NGF signal from the target is likely to increase the sensitivity of those neurons to low levels of target-derived NGF, leading to a situation in which those neurons that express p75NTR are better able to compete for synaptic space within the target. Hence, the ability of p75NTR to sharpen TrkA binding specificity plays a significant role in the maturation of target innervation, and likely controls the competition which defines the adult pattern of innervation. Whether p75NTR plays such a role in synaptic competition within the central nervous system remains to be determined.

3.3.5 p75NTR Signaling

In addition to the role p75NTR plays in modulating and modifying TrkA signaling for survival and differentiation, as discussed above, this receptor may also signal autonomously to induce apoptosis under specific cellular contexts. For example, in the embryonic chick retina, neural precursor cells expressing p75NTR in the absence of TrkA undergo NGF-dependent apoptosis, suggesting that developmentally programmed death in these cells is mediated by p75NTR (Bredesen and Rabizadeh 1997; Carter and Lewin 1997; Frade and others 1996). Furthermore, p75NTR mediates NGF-induced

death of cultured oligodendrocytes (Casaccia-Bonnefil and others 1996; Gu and others 1999; Yoon and others 1998) and cultured hepatic stellate cells (Trim and others 2000), and BDNF signaling via p75NTR was shown to induce apoptosis of postnatal sympathetic neurons in culture (Bamji and others 1998). Moreover, an increased number of sympathetic neurons are found in BDNF-deficient mice, and there is a delay in sympathetic cell death in p75NTR homozygous knockout mice (Bamji and others 1998). Interestingly, BDNF-dependent trigeminal neurons are killed via binding of NT-4 to p75NTR, even though p75NTR is necessary to the cell survival induced by BDNF (Agerman and others 1999). This indicates that p75NTR signaling is not only dependent upon cell context, but also upon neurotrophin binding specificity.

One signal transduction pathway ascribed to p75NTR that may be involved in apoptotic signaling involves generation of the lipid second messenger ceramide via activation of sphingomyelinase. In fibroblasts expressing p75NTR but not TrkA, NGF induced the production of ceramide. Furthermore, in T9 glioma cells, NGF induced the activation of sphingomyelinase and the production of ceramide, and inhibited growth and fiber formation, a process which was mimicked by incubation with membrane-permeant ceramide analogs (Dobrowsky and others 1994). Other members of the p75NTR superfamily, such as TNF-RI and Fas, also signal via ceramide production (Cifone and others 1994). This signaling function appears to be mediated at least in part by a region

within TNF-RI and Fas termed the "death domain," a C-terminal region in the cytoplasmic domain that is necessary for apoptotic signaling downstream from these receptors (Tartaglia and others 1993; Watanabe-Fukunaga and others 1992). Analysis of the p75NTR sequence shows that a homologous "death domain" region exists within the intracellular region of this receptor (Liepinsh and others 1997). Recent experiments suggest that the "death domain" serves to mediate protein:protein interactions. For example, this region mediates Fas and TNF-RI intracellular domain aggregation (Boldin and others 1995a; Song and others 1994), and a homologous region has been found within ankyrin, a protein that anchors transmembranal proteins to the cytoskeleton (Boldin and others 1995b).

Another death signaling domain was recently discovered within the p75NTR juxtamembrane region. This domain, a 29 residue sequence named "chopper," is necessary and sufficient to induce cell death in several cell types, including neurons. Interestingly, a peptide corresponding to the chopper domain only signaled cell death when associated with the plasma membrane via a lipid anchor. Non-anchored chopper peptide did not mediate cell death, and, in fact, acted in a dominant-negative manner to p75NTR-mediated death signaling (Coulson and others 2000), suggesting that palmitoylation of p75NTR is a crucial factor in mediating signaling from the receptor.

This finding also suggests the possibility that proteolytic cleavage of the intracellular domain may play a role in controlling p75NTR signaling.

Another possible mechanism of p75NTR-mediated cell death was suggested by the observation that overexpression of the intracellular domain of p75NTR induced profound cell death in several neuronal populations within the central and peripheral nervous systems (Majdan and others 1997). This finding, plus the observation that immortalized neural cells overexpressing p75NTR exhibit enhanced cell death following serum withdrawal (Rabizadeh and others 1993), suggests that p75NTR may signal pro-apoptotically in the absence of ligand binding. In this model, binding of NGF to p75NTR induces a conformational change which blocks the production of a death signal. Further support for this idea comes from work showing that antisense-induced downregulation of p75NTR in neonatal dorsal root ganglia sensory neurons enhanced survival (Barrett and Bartlett 1994). Moreover, identification of an alternatively spliced isoform of p75NTR lacking the neurotrophin-binding domain supports the model of ligand-independent signaling (Dechant and Barde 1997). The receptor produced by this alternative splice event contains the transmembrane and intracellular domains, but lacks the ability to bind neurotrophin, and may therefore exhibit enhanced cell death signaling consistent with the function of the death domains described above. Finally, p75NTR appears to exhibit ligand-independent signaling through the RhoA pathway. In cells transfected with

p75NTR, RhoA activation was generated in the absence of ligand, and was abolished by addition of ligand, suggesting that p75NTR can signal to reorganize the actin cytoskeleton in a manner that is negatively modulated by the presence of neurotrophin (Yamashita and others 1999b).

Many proteins in the p75NTR superfamily interact with TNF receptor-associated factors (TRAFs) that modulate signaling through the JNK and NFκB pathways. Six such factors have been identified in signaling evoked by TNF-R, CD30, CD40, and the IL-1 receptor (Arch and others 1998; Rothe and others 1995), and recently p75NTR was shown to associate with TRAF-2, TRAF-4, and TRAF-6 following treatment with NGF (Khursigara and others 1999; Ye and others 1999). Interestingly, the association of TRAF-6 with p75NTR is mediated by the receptor's juxtamembrane domain (Khursigara and others 1999), within a sequence that is absolutely conserved between human, rat, and chicken p75NTR (Large and others 1989), suggesting that the interaction with TRAF-6 is critical to p75NTR function. TRAF-6 is recruited to the IL-1 receptor via binding to IRAK, the IL-1 receptor-associated serine-threonine kinase (Cao and others 1996a; Cao and others 1996b), and TRAF-6 has also been shown to signal through NIK, the NFκB inducing kinase (Malinin and others 1997), suggesting that one role of the p75NTR-(TRAF-6) interaction may be to couple p75NTR to several different kinase cascades. The use of adaptor proteins such as TRAF-6 potentially permits p75NTR, which lacks

any intrinsic kinase activity, to recruit and non-catalytically activate several cytoplasmic non-receptor kinases, thereby linking NGF binding to p75NTR to JNK and NFκB activation.

As described above, in addition to apoptosis-related signaling, p75NTR binding of NGF also activates the transcription factor NFκB in neuroblastoma cells (Korner and others 1994), cultured sensory and sympathetic neurons (Maggirwar and others 1998; Wood 1995), Schwann cells (Carter and others 1996; Khursigara and others 1999), and oligodendrocytes (Ladiwala and others 1998; Yoon and others 1998). The activation of NFκB downstream from most inducer proteins involves the degradation of the IκB protein, an inhibitory factor that binds heterodimers of the NFκB p50 and p65 subunits and prevents them from translocating into the nucleus (Ghosh and others 1998). The reduction in IκB levels in response to effector signaling is transient, as the IκB gene contains NFκB binding sites within its promoter. Hence, IκB degradation, resulting in NFκB nuclear translocation, results in upregulated transcription of the IκB gene, and increased production of the IκB protein, which then binds to NFκB in the cytoplasm and inhibits its activity. In oligodendrocytes, in which p75NTR appears to signal via both NFκB and the JNK pathway, expression of TrkA abrogated NGF-induced cell death in a manner that was correlated with cessation of JNK signaling, while the NFκB signal

downstream from p75NTR was unaffected (Yoon and others 1998). This suggests that p75NTR may evoke two separate pathways, one pro-apoptotic, the other anti-apoptotic, and that the balance of these two pathways, modulated by TrkA signaling, controls the ultimate fate of the cell. However, the exact role that NFκB plays is unresolved – in some systems it exhibits anti-apoptotic signaling (Maggirwar and others 1998; Mattson and others 1997), but in others it is associated with pro-apoptotic signaling (Schneider and others 1999; Schwaninger and others 1999). The TNF receptor, generally associated with death signaling, also activates NFκB in a pathway that appears to promote survival of lymphoid cells and fibroblasts (Liu and others 1996; Van Antwerp and others 1996; Wang and others 1996). Likewise, in hippocampal neurons that do not express TrkA, NGF signaling through p75NTR protects these cells from glucose deprivation-induced apoptosis (Cheng and Mattson 1991). Furthermore, p75NTR appears to play a role in protecting Schwann cells following axotomy. In the normal adult animal, Schwann cells do not express p75NTR. However, following nerve injury, Schwann cells distal to the injury site dramatically upregulate p75NTR expression (Heumann and others 1987; Taniuchi and others 1986a), and exhibit increased NFκB activation (Gentry and others 2000). This increase in NFκB activation is correlated to the absence of apoptosis in Schwann cells distal to the injury (Grinspan and others 1996). It is interesting that during development Schwann cells require axonal contact for trophic support, and loss of such

contact results in cell death. Hence, injury induced expression of p75NTR and consequent signaling through NFκB may serve in the adult to maintain Schwann cells in the absence of trophic support from the axon, thereby providing time for the axon to regrow. Whether this survival signaling cascade differentiates post-injury nerve regeneration in the peripheral nervous system from the absence of such regeneration in the central nervous system remains an open question.

4. Endocytosis and Receptor Trafficking

4.1 General Endocytic and Trafficking Mechanisms

4.1.1 Introduction

Endocytosis is defined by the Oxford English Dictionary as "the taking in of material by a living cell," and by the Merriam-Webster dictionary as the "incorporation of substances into a cell." Cells utilize endocytosis for a host of functions, ranging from nutrient acquisition, to maintenance of protein and lipid homeostasis following secretory events, to the transmission of intercellular signals. While the process of endocytosis has been appreciated for more than a hundred years (Mellman 1996), it is only in the last 20 years that the fundamental organization of the endocytic pathways involved in the internalization of cell surface receptors bound by ligands has been delineated (Gruenberg and Maxfield 1995; Helenius and others 1983; Kornfeld and Mellman 1989; Steinman and others 1983; Trowbridge and others 1993). In general, endocytosis is considered to

involve the accumulation of receptor-ligand complexes within clathrin-coated plasma membrane (CCPM) domains at the surface of the cell, the budding and pinching-off of these membranes, and the formation of clathrin coated vesicles (CCVs) within the cytoplasm (Figure 1.9). These CCVs rapidly uncoat and fuse with the early endosome array, comprised of a dynamic and interconnected network of vesicles and tubular structures distributed throughout the cell. These early endosomes are slightly acidic, ranging from pH 6.0 to 6.8 due to the action of an ATP-driven proton pump (Al-Awqati 1986; Forgac 1992; Mellman and others 1986). Within this acidic environment, many receptor-ligand complexes dissociate, with the free receptors selectively accumulating in tubular extensions of the early endosomal network and eventually pinching off to form recycling vesicles that are transported back to the plasma membrane. The dissociated ligands accumulate within the vesicular compartment of the early endosome network, and are eventually pinched off and transported through the cell to the perinuclear region, where they fuse with late endosomes and/or lysosomes. The pH within lysosomes is even more acidic, approximately pH 5.0, and this, plus the high concentration of lysosomal enzymes, leads to the degradation of proteins contained with the lysosomal compartment. Importantly, variations on this theme exist, in which receptors, as well as ligands, are trafficked to the lysosomal compartment and degraded, or in which specially marked receptors are trafficked to specialized degradation machines called proteosomes. Also, some ligand-receptor complexes escape this degradatory pathway and are instead

trafficked through the cell as a cohesive unit, apparently signaling from their location on internal membranes much as other receptors signal from the plasma membrane.

In addition to mediating trafficking from the plasma membrane to the interior of the cell, clathrin-mediated vesicle formation also permits trafficking to occur from the trans-Golgi network to late endosomes and lysosomes. This pathway is necessary for delivering such things as lysosomal enzymes from their site of production to the lysosome, where they are able to function (Kornfeld and Mellman 1989). Thus, CCVs serve in the general role of vesicle formation, both for endocytic trafficking and for intracellular protein and membrane delivery. Therefore, CCVs are effectively a population of transport vesicles specializing in fusion of cargo-containing membranes with endosomes.

The secretory pathway, readily marked by enzymatic activities involved in protein synthesis, folding, and post-translational processing, is clearly organized into discrete functional and physical compartments (Kornfeld and Mellman 1989). The endocytic pathway, as well, is compartmentalized, but, unfortunately, the compartments are not so readily demarcated. Likewise, endocytic compartments tend to be much more heterogeneous than secretory organelles, making it difficult to identify endocytic structures solely on morphological criteria. However, despite the inherent problems in identifying discrete components of the endocytic pathway, it is generally the case that

early endosomes are the site of dissociation and sorting of ligands and receptors, while late endosomes and lysosomes are the sites of accumulation and degradation of proteins and nutrients acquired from outside of the cell or generated within the cell. Furthermore, in the last several years, sophisticated tools for biochemically and functionally identifying and manipulating endocytic organelles have led to determination of many of the mechanisms necessary for the internalization and trafficking of receptors and other protein constituents of the plasma membrane, and this knowledge has permitted better understanding of specializations and exceptions to the general endocytic pathway. The endocytosis and trafficking of TrkA and NGF may be one such exception to the rule of receptor-ligand dissociation and degradation, but an understanding of how the exceptional pathway used by TrkA is relevant to NGF-induced signaling requires understanding of the general endocytic pathway. Hence, the following sections will review the general mechanisms of clathrin-mediated endocytosis, and subsequent sections will focus on the particulars of TrkA endocytosis and trafficking as they relate to the signaling endosome hypothesis.

4.1.2 Evidence for a Role of Clathrin in Endocytosis

Endocytosis is perhaps one of the most active processes exhibited by cells. Macrophages and fibroblasts, for example, have been estimated to internalize the equivalent of more than 200% of their cellular surface area every hour (Steinman and others 1983), and

recycling of membranes at the presynaptic terminals of activated axons is even greater (Marsh and McMahon 1999; Sankaranarayanan and Ryan 2000). Furthermore, most plasma membrane proteins, though endocytosed, exhibit half-lives on the order of 24 hours, suggesting that these proteins must escape degradation for a period of time by recycling back to the surface of the cell. Consistent with this, several different receptors have been shown to undergo approximately 10 cycles of ligand uptake and receptor recycling per hour over the course of their existence (Steinman and others 1983). In general, the mechanism by which these membrane proteins are endocytosed requires the formation of CCVs. The earliest evidence in support of CCV-mediated endocytosis involved an accounting experiment which calculated the volume of fluid contained within CCVs generated within a given time period, balanced against biochemical measurements of the amount of fluid taken up by the cell as a whole (Marsh and Helenius 1980). Further evidence that CCVs were a necessary component of ligand endocytosis was provided by experiments showing the selective localization of receptor-ligand complexes within CCPMs, and a correlation of this localization to ligand uptake, evidenced by a failure of endocytosis of mutant LDL receptors that could not localize to coated pits (Brown and Goldstein 1979). Moreover, the Drosophila mutant shibire was shown to exhibit temperature-dependent paralysis as a result of the loss of endocytosis at motor nerve terminals. This defect was correlated with a loss of CCV production (Koenig and Ikeda 1989; Poodry 1990), later shown to be the result of a mutation in dynamin, a

GTPase necessary for the physical process of budding and pinching-off of CCVs from CCPMs (Chen and others 1991; van der Bliek and Meyerowitz 1991). Hence, CCVs play a clear role in the internalization of plasma membrane receptors, as well as in the recycling of membrane following secretion, and in the uptake of fluid from the extracellular milieu.

4.1.3 CCV Structure and Formation

Biochemical characterization of CCVs isolated from liver or brain led to the identification of two major coat constituents: clathrin and the adaptor protein (AP) complexes (Pearse 1975; Pearse 1976; Pearse 1978). Clathrin is an oligomeric protein comprised of three 180-190 kDa clathrin heavy chains (CHCs) organized in a triskelion array, with each CHC bound to one 30-35 kDa clathrin light chain (CLC) (Figure 1.10) (Schmid 1997). The triskelion is the basic unit of assembly for the polyhedral lattice coat that is characteristic of CCVs. Each leg of the triskelion unit is formed by an extended CHC oriented like the arm of a fan or a pinwheel, with its C-terminus situated at the center of the fan (Figure 1.10). The hub of the fan contains the small globular domain at the C-terminal extreme of each CHC and the trimerization domain of each CHC. These trimerization domains come together to form the center of the pinwheel (Figure 1.10). Also contained within the hub is the proximal leg of each CHC, which serves as the binding site for either CLCa or CLCb. The hub is connected to the distal leg of each

CHC fan arm by a region called the knee, a protease sensitive bend within the extended conformation of CHC that serves to create the characteristic pinwheel shape of the triskelion (Figure 1.10). This region also provides flexibility to the triskelion and allows it to adopt the slightly variable conformations necessary for either pentagonal or hexagonal packing of the triskelions in the overall coat structure (Pearse and others 2000). Under appropriate conditions, clathrin triskelions exhibit self-assembly properties, forming heterogeneously shaped polyhedrons called cages (Crowther and others 1976; Kartenbeck 1978; Woodward and Roth 1978). Electron microscopic analysis of these cages generated a structural model of the clathrin coat that involves a triskelion hub at each vertex of the polygon, with each leg of the triskelion contributing to two edges of the polygon and the knee region of the leg located at the vertex adjacent to the hub vertex (Figure 1.10). Hence, each edge of the polygon is composed of two antiparallel proximal CHC legs and their associated CLCs extending from adjacent vertices along the outer face of the edge, and two antiparallel distal CHC legs (that is, after the knee bend) extending from second-order vertices along the inner surface of the edge (Kirchhausen and others 1986; Vigers and others 1986a; Vigers and others 1986b). The angle of flexion at the knee determines whether the interaction amongst triskelions generates a hexagonal or pentagonal lattice. CCPMs mature into CCVs apparently via gradual addition of pentagons into the initial hexagonal array formed on the cytoplasmic face of the plasma membrane (Heuser 1980; Jin and Nossal 1993; McKinley 1983),

though the exact mechanism driving this interchange remains unclear. CCPMs exhibit a variable degree of curvature when examined in vivo, and the ratio of curved CCPMs to total CCPMs is a function of the rate of endocytosis, suggesting that the interchange of pentagons for hexagons must occur gradually during coating, rather than as a separate step following generation of the coat (Naim and others 1995; Pypaert and others 1987).

Coat assembly onto CCPMs and membrane curvature are insufficient to drive the constriction and vesicle budding necessary to form actual CCVs. These two steps require additional cytosolic factors, as well as ATP and GTP hydrolysis, indicating that these steps are active, energy-driven processes. The process of CCV formation appears to occur sequentially, with the formation of a constricted coated pit attached to the plasma membrane via a narrow neck proceeding as a step separable from pinching-off of the vesicle (Schmid 1997). Constriction is followed by membrane fission (or scission), and both steps are dependent upon GTP hydrolysis, apparently mediated by the 100 kDa GTPase dynamin. The role of dynamin in endocytosis was suggested following the realization that mammalian dynamin and Drosophila shibire exhibit 70% homology (Chen and others 1991; van der Bliek and Meyerowitz 1991). Demonstration that receptor-mediated endocytosis is defective in mammalian cells transiently transfected with a dominant-negative dynamin mutant confirmed the critical role of dynamin in endocytosis (Herskovits and others 1993; van der Bliek and Meyerowitz 1991). Finally,

morphological examination of cells expressing mutant dynamin showed that the protein must act at a step downstream from CCPM formation and invagination, but upstream from constriction and scission (Damke and others 1995; Damke and others 1994). Further analysis of dynamin function showed that dynamin was localized to coated pits on the plasma membrane (Baba and others 1995; Damke and others 1994; Takei and others 1995), that it can self-assemble into helical stacks of rings localized to the necks of invaginated coated pits (Koenig and Ikeda 1989; Takei and others 1995), and that it interacts with AP2 complexes and amphiphysin (David and others 1996; Wang and others 1993). These findings suggested a model for dynamin function in which it is targeted to the nascent clathrin lattice in a GDP-bound, and hence inactive, state. It is likely that amphiphysin, an SH3-domain containing protein, mediates this recruitment via an interaction with the proline-rich C-terminus of dynamin (Simpson and others 1999). Following recruitment to the lattice, dynamin undergoes GTP binding and activation, and then redistributes away from the lattice in a manner that leads to the generation of a neck, coated by a dynamin-formed ring structure, and which separates the coated membrane from the plasma membrane. The helical dynamin rings may be comprised of up to 20 dynamin molecules oriented in a spiral around the neck (Schmid 1997). Formation of the neck is then likely followed by a GTP-hydrolysis dependent constriction and scission of the neck membrane, resulting in detachment of the coated vesicle (Vallis and others 1999).

4.1.4 Adaptor Protein Complexes

In addition to the clathrin hetero-oligomers, clathrin coats also contain a hetero-tetrameric adaptor protein complex (Pearse and Robinson 1990). The proteins in this complex were initially identified as factors that promoted in vitro cage assembly, and were subsequently discovered to mediate recruitment and attachment of clathrin to membranes (Chang and others 1993; Peeler and others 1993; Robinson 1994; Traub and others 1995). Several adaptor complexes have been identified, though only two, the trans-Golgi membrane-specific adaptor complex AP1 and the plasma membrane-specific adaptor complex AP2, have been thoroughly characterized. Both AP1 and AP2 consist of four related subunits called adaptins. AP1 is formed by the association of a 100 kDa γ-adaptin, a 100 kDa β′-adaptin, a 50 kDa μ1-adaptin, and a 20 kDa σ1-adaptin subunit, while AP2 is comprised of either of two 100 kDa α_A or α_C chains, a 100 kDa β-adaptin, a 50 kDa μ2-adaptin, and a 20 kDa σ2-adaptin subunit (Schmid 1997). The 100 kDa subunits show only slight sequence homology, but all have the same domain organization, comprised of a large head domain, a proline-rich hinge region, and a C-terminal ear or appendage domain (Heuser and Keen 1988; Page and Robinson 1995; Ponnambalam and others 1990; Robinson 1993). The four adaptin subunits tetramerize to form a roughly rectangular box

that has the ear domains of the two large subunits extending out from the head domains like the horns of a bull (Schmid 1997).

Adaptor complexes, in addition to recruiting clathrin to membranes, are necessary for recruiting membrane proteins to CCPMs (Glickman and others 1989; Pearse 1988; Sorkin and Carpenter 1993; Sorkin and others 1995). This recruitment is accomplished via recognition of specific sequences within the cytoplasmic domains of membrane proteins. These recognition motifs generally involve aromatic residues, such as tyrosine, within the context of several residues on either side of the aromatic residue that bear large hydrophobic side chains (Trowbridge and others 1993). For example, the LDL receptor contains the cytoplasmic domain sequence FDNPVY, which clearly plays a role in localizing the receptor within coated pits (Mellman 1996). Two general consensus sequences based on tyrosine are found within receptors that associate with coated pits: (F/Y)xNPxY and Yxxϕ, where ϕ represents a large hydrophobic residue like phenylalanine (Marsh and McMahon 1999). Another common recognition motif, especially within immune receptors such as the Fc receptor, CD3, and the MHC class II-associated invariant chain, involves neighboring leucine residues (Hunziker and Fumey 1994; Mellman 1996). Both the tyrosine-based and the dileucine-based motifs are recognized as coated pit localization signals, though it is clear that these motifs can exist within a protein sequence in situations in which the protein is not directed to coated pits.

Furthermore, it has been shown that other residues outside of these narrowly defined motifs are important for internalization, and it is therefore likely that it is the tertiary structure of a protein which determines whether these recognition motifs engage in coated pit localization (Miettinen and others 1989). Finally, two other endocytic sorting motifs that have been identified within some proteins are ubiquitinated lysine residues (Govers and others 1999; Hicke and others 1998) and phosphorylated serine-rich domains, which are found within the C-termini of many G-protein coupled receptors (Ferguson and Caron 1998). However, the exact nature of the interaction of these motifs with adaptor proteins remains to be discovered.

Interestingly, coated pit localization motifs are also found on proteins that are not associated with the plasma membrane. For example, the late endosomal and lysosomal specific glycoproteins lgp and lamp exhibit short cytoplasmic tails that contain a glycine-tyrosine sequence followed two residues later by a hydrophobic residue (Kornfeld and Mellman 1989). In general, lgp and lamp are sorted from the trans-Golgi network to organelles of the endocytic pathway by way of CCVs, indicating that they may be recognized by AP1. However, under conditions in which these glycoproteins are overexpressed, some sorting to the plasma membrane occurs. These cell surface lgps or lamps are rapidly internalized via CCVs derived from the plasma membrane, suggesting that they are also recognized by AP2 complexes (Harter and Mellman 1992).

Other non-receptor proteins are also recognized by the APs. For example, TGN38, a trans-Golgi network resident protein, recycles continuously from the Golgi to the plasma membrane, and back, apparently via an association with early and late endosomes that is mediated by AP1 (Miesenbock and Rothman 1995; Reaves and others 1993). Furthermore, several integral membrane proteins found in synaptic vesicles, such as the secretory carrier protein (SCAMP) 37 and synaptophysin, contain Yxxϕ motifs, and may interact with AP2 during the membrane recycling component of the synaptic vesicle cycle (Singleton and others 1997). Such an interaction may play a role in the synaptic vesicle recycling model that involves recovery of synaptic vesicle membranes at sites outside of the active zone. In this model, a serial progression of exocytosis and complete fusion of the vesicle membrane with the plasma membrane is followed by *de novo* construction of a coated vesicle. Interestingly, synaptophysin also binds to dynamin, an interaction that may mediate clathrin-independent recycling of synaptic vesicle membranes in the so-called "kiss-and-run" model (Daly and others 2000). In this model, the synaptic vesicle never actually integrates its membrane fully into the plasma membrane. Rather, the vesicle retains a discrete identity and is quickly recovered, essentially intact, from the plasma membrane via a clathrin-independent mechanism. Clearly, clathrin and components of the clathrin pathway play vital roles in several cellular functions outside the domain of simple plasma membrane endocytosis.

Another family of proteins that recognize clathrin adaptors is the β-arrestin subfamily of the arrestins. β-arrestin1 and β-arrestin2 are monomeric 45 kDa proteins that bind both to the β2-chain of AP2 and directly to the clathrin heavy chain. These proteins were identified as factors necessary for the activation-dependent surface downregulation of β2-adrenergic receptors. The β2-adrenergic receptor is a G-protein coupled receptor (GPCR) that is removed from the surface of cells following ligand binding via internalization into clathrin-coated vesicles (Tolbert and Lameh 1996; von Zastrow and Kobilka 1992). This internalization may serve either to terminate the β2-adrenergic receptor signaling pathway by directing the receptors to lysosomes, or it may allow for recycling of the receptor back to the plasma membrane for another round of ligand binding following a brief period of desensitization (von Zastrow and Kobilka 1992). Endocytosis of the receptor may also permit interaction with downstream signaling elements located in an endosomal compartment (Luttrell and others 1999). The endocytosis of β2-adrenergic receptors is mediated by the interaction of β-arrestin with clathrin, AP2, and the cytoplasmic face of the GPCR. Hence β-arrestin acts as an adaptor protein, linking clathrin directly to the receptor, as well as an adaptor-adaptor, linking the AP2 complex to the receptor. Both of these functions appear to be necessary for β2-adrenergic receptor internalization, though the interaction with AP2 may, under

appropriate circumstances, be sufficient to mediate downregulation of the GPCR (Laporte and others 1999). The role of β-arrestins in clathrin-mediated endocytosis is explored more fully in chapter 4 of this manuscript, in the context of Cbl as a clathrin-associated protein that may serve a function similar to β-arrestin, but specific for RTKs rather than GPCRs.

4.2 TrkA Endocytosis and Retrograde Trafficking

Receptor tyrosine kinases exhibit ligand-mediated downregulation from the cell surface, generally via clathrin-mediated internalization mechanisms (Sorkin and Carpenter 1993). This downregulation is linked to receptor degradation, and serves, for many receptors, to truncate the signal elicited upon ligand binding (Beguinot and others 1984; Stoscheck and Carpenter 1984). Early studies on TrkA suggested that it, too, was downregulated from the cell surface following NGF treatment (Hosang and Shooter 1987; Layer and Shooter 1983). Such downregulation was marked by a pronounced decrease in the ability to cross-link radiolabeled NGF to TrkA following 60 minutes of NGF treatment (Zhou and others 1995), and by an increase in protected biotinylated TrkA inside the cell following 10 minutes of NGF treatment (Beattie and others 1996). This downregulation was correlated with receptor degradation, as 60 minutes of NGF treatment led to the loss of two-thirds of total cellular TrkA (Zhou and others 1995).

While downregulation and subsequent degradation is the fate of most mitogenic RTKs, differentiative RTKs like TrkA may utilize receptor internalization for an additional purpose: to communicate the neurotrophic signal from axon terminals in the target region back to the neuron cell body (Figure 1.11). This communication requires the formation of a signal that can be coherently transmitted through the axon. Three mechanisms have been proposed to explain retrograde signaling (Campenot 1994; Hendry and Crouch 1993): 1) the target-derived neurotrophin is internalized and transported from the axon tip to the cell body, where it binds receptors localized in the cell body to initiate a signaling cascade; 2) the target-derived neurotrophin activates presynaptic neurotrophin receptors which initiate signaling cascades that reach the cell body in a wavelike fashion; 3) the target-derived neurotrophin binds to and activates presynaptic neurotrophin receptors, inducing internalization of ligand-receptor complexes into endosomes that are retrogradely trafficked to the cell body, where they initiate local signal transduction cascades, including those that mediate transcriptional events. Evidence from several sources supports the third model, the "Signaling Endosome Hypothesis." First, while target-derived neurotrophin is internalized and retrogradely transported (DiStefano and others 1992; Hendry and others 1974a; Hendry and others 1974b; Johnson and others 1987), injection of neurotrophin into the cell body does not mimic the neurotrophic signal initiated at the axon terminal (Heumann and others 1981),

suggesting that the neurotrophin by itself is unlikely to carry the signal. Second, as mentioned above, NGF induces the endocytosis of TrkA (Zhou and others 1995), and it is bound to TrkA within endosomes (Grimes and others 1997; Grimes and others 1996). Interestingly, TrkA was found to colocalize with clathrin on the surface of NGF-treated PC12 cells, suggesting that TrkA and NGF utilize the clathrin-coated vesicle pathway for endocytosis, and that the endosomes containing NGF bound to TrkA may be derived from such vesicles. Moreover, these endosomes are associated with activated Shc and PLCγ, downstream components of the NGF signaling cascade, suggesting that they carry the neurotrophin signal (Beattie and others 1996; Grimes and others 1996).

The third piece of evidence in support of the signaling endosome hypothesis comes from studies showing that complexes containing NGF and activated TrkA are found in sciatic axons (Tsui-Pierchala and Ginty 1999), and that these complexes are retrogradely transported to sympathetic neurons (Bhattacharyya and others 1997; Ehlers and others 1995). It has been recognized for 25 years that radiolabeled NGF is retrogradely transported at a rate of approximately 2.5 mm per hour from the terminals of axons that innervate the iris to the cell bodies of sympathetic neurons within the superior cervical ganglion (Hendry and others 1974b; Korsching and Thoenen 1983). Furthermore, in compartmentalized cultures of sympathetic neurons, radiolabeled NGF is transported at a rate of 3 to 20 mm per hour from distal axon tips to cell bodies (Claude and others 1982;

Ure and Campenot 1997). Hence, as mentioned above, NGF is certainly retrogradely transported. Evidence that activated TrkA is also transported was provided by studies showing that phosphorylated TrkA accumulates distal to a sciatic nerve ligation or crush, and that injection of excess exogenous NGF into the footpad increases the amount of phosphorylated TrkA that accumulates at the ligature site, while injection of anti-NGF antibodies blocks such accumulation (Ehlers and others 1995; Johanson and others 1995). Moreover, phosphorylated TrkA accumulates in the cell bodies of sympathetic neurons grown in compartmentalized cultures following treatment of the distal axons and terminals with NGF (Riccio and others 1997; Senger and Campenot 1997). Finally, an NGF-TrkA complex is transported from axon terminals to sympathetic neuron cell bodies in compartmentalized cultures, and this TrkA is active as judged by tyrosine phosphorylation (Tsui-Pierchala and Ginty 1999). Interestingly, retrogradely transported phosphorylated TrkA is associated with at least 3 tyrosine-phosphorylated proteins (Tsui-Pierchala and Ginty 1999), suggesting that it may form a complex with associated signaling molecules that are carried with the receptor down the axon.

The formation of signaling complexes associated with retrogradely transported TrkA would suggest that this TrkA may signal in the cell body. In fact, the fourth piece of evidence in support of the signaling endosome hypothesis is that the arrival of NGF-TrkA complexes in neuron cell bodies coincides with the phosphorylation of CREB in the cell

bodies (Riccio and others 1997; Watson and others 1999). Serine phosphorylation of CREB on its transcriptional regulatory site is a modification required to drive transcriptional events necessary for the survival and maintenance of sympathetic neurons (Ginty and others 1994). While application of NGF directly to the cell resulted in phosphorylation of nuclear CREB within 5 minutes, application of NGF to the distal chamber of the compartmentalized cultures resulted in CREB phosphorylation in the cell bodies only after 20 minutes, generating a rate of signal conduction that is on the order of 2 to 4 mm per hour (Riccio and others 1997). This transport rate is virtually identical to the rate of NGF transport measured in sympathetic neurons (Hendry and others 1974b). Importantly, the retrograde signal to phosphorylate CREB depended upon internalization and retrograde transport of NGF at the axon terminals. This was shown by experiments in which NGF was covalently coupled to microspheres. NGF coupled in this fashion is still competent to induce activation of TrkA and downstream signaling cascades, but is not internalized (Riccio and others 1997). Application of bead-coupled NGF to cell bodies resulted in CREB phosphorylation, suggesting that internalization of NGF is not necessary to transmit the TrkA signal over relatively short distances, such as from the cell body plasma membrane to the nucleus. However, bead-coupled NGF completely failed to elicit CREB phosphorylation when applied to the distal chamber of compartmentalized cultures (Riccio and others 1997). Hence, internalization and retrograde transport are necessary to move the TrkA signal from axon terminals to the cell body, suggesting that a

wave of signal generated by the receptor is not sufficient to transduce nuclear events at a distance. Such a wave-like retrograde movement of the neurotrophin signal is also not supported by the known kinetics of signal transport, which support neither a diffusion-mediated mechanism, nor a regenerating wave phenomenon (Fitzsimonds and Poo 1998). Thus, the available evidence supports the signaling endosome hypothesis and suggests that activated TrkA is internalized into an endocytic organelle that is retrogradely transported from the target to the neuron cell body (Figure 1.11).

5. Implications of the Signaling Endosome Hypothesis

Perhaps one of the most interesting applications of the signaling endosome hypothesis is to our understanding of the mechanisms of neurodegeneration. In particular, the signaling endosome hypothesis may be a useful organizing principle for furthering our understanding of the pathogenetic mechanisms of Alzheimer's disease. Alzheimer's disease is an age-related disorder of unknown etiology and pathogenesis that causes widespread but specific degenerative changes in several neuronal populations within the central nervous system. The disease is characterized by a progressive dementia that begins with a variable declarative memory impairment, and ends in virtually systemic failure of cognitive, comportmental, and psychiatric function. Alzheimer's disease currently accounts for up to 80% of the cases of dementia in the elderly, and with the shift in age demographics that is expected during the next several decades, Alzheimer's

disease may become essentially pandemic. Current therapeutic interventions are limited to modest, short-term enhancements of cognitive dysfunction and symptomatic relief of behavioral problems. No therapy currently exists to alter the eventual outcome of the disease or to prevent its most serious clinical manifestations (Drouet and others 2000).

The development of effective therapeutic strategies requires a deeper understanding of the key pathogenetic events involved in Alzheimer's disease. In particular, more information is needed about disease-related defects in neurotrophin receptor trafficking and signaling, and a detailed account must be made of the state of neurotrophic support communication from the target to neuron cell bodies in Alzheimer's patients. In Alzheimer's disease, loss of cholinergic function is a key factor in the early forgetfulness that marks the disease (Bartus 2000). Progressive and widespread synaptic loss eventually leads to severe dementia. The etiology of this synaptic loss is complicated and largely not understood. However, it is becoming increasingly clear that loss of neurotrophic support for cholinergic neurons is an early and critical component of the disease progression. One hypothesis regarding this cholinergic synaptic loss holds that a failure in retrograde transport of NGF and TrkA from basal forebrain cholinergic axon terminals in hippocampal and cortical target regions leads to progressive synaptic dysfunction and eventually to synaptic retraction and death of cells within the basal forebrain (see chapter 5 of this manuscript). Support for this hypothesis comes from data

showing that NGF levels in the hippocampus and cortex of Alzheimer's disease brains are increased 2-fold compared to normal, while NGF levels in the basal forebrain are reduced by half (Scott and others 1995). These data suggest a failure in retrograde axonal transport of the neurotrophin signal. This failure may be due to many causes, though several primary possibilities exist: 1) NGF, though produced, is not released by the target; 2) NGF is released, but does not bind to TrkA receptors on the surface of cholinergic axon terminals; 3) NGF binds to TrkA, but the ligand-receptor complex is not internalized; 4) the NGF-TrkA complex is internalized, but is aberrantly degraded by lysosomes that are ectopically present within axons of cholinergic neurons in Alzheimer's disease patients; or 5) the NGF-TrkA complex is internalized, but a defect in some component of the cytoskeletal transport system prevents efficient retrograde transport of the signaling endosome. It is currently unclear which of these mechanisms plays a role in the pathogenesis of Alzheimer's disease, and further experimentation will be necessary to isolate the root cause of the failure to transport NGF and the neurotrophin signal from target regions to the cell bodies within the basal forebrain. However, the experiments addressed within the remainder of this manuscript provide not only the first proof of the existence of neurotrophic signaling endosomes, but also a deeper mechanistic understanding of the role that endocytosis itself plays in the propagation and control of the neurotrophin signal. This manuscript documents the generation of an exquisite tool for the further analysis of the signaling endosome hypothesis: isolation of

the nascent signaling endosome. Application of this tool to real neurons and animal models will extend our knowledge of the pathogenetic mechanisms that underlie Alzheimer's disease.

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Table 1

Neurotrophin	Receptive Neurons
Nerve Growth Factor	Sympathetic Neural-Crest Derived Sensory (Nociceptive) Basal Forebrain Cholinergic Striatal Cholinergic Cerebellar Purkinje
Brain-Derived Neurotrophin Factor	Neural-Crest Derived Sensory Placode-Derived Sensory Basal Forebrain Cholinergic Trigeminal Mesencephalic Substantia Nigra Dopaminergic Cerebellar Granule Retinal Ganglion Motor Hippocampal and Cortical
Neurotrophin-3	Sympathetic Sensory (Proprioceptive) Enteric Basal Forebrain Cholinergic Locus Coeruleus Adrenergic Motor
Glial-Derived Neurotrophic Factor	Sympathetic Sensory Enteric Substantia Nigra Dopaminergic Locus Coeruleus Adrenergic Motor
Ciliary Neurotrophic Factor	Sympathetic Parasympathetic (Ciliary) Sensory Striatal Cholinergic Motor

Figure 1.1 Neurotrophic Factor Hypothesis.

The Neurotrophic Factor Hypothesis states that during development, innervating neurons compete for a limiting supply of target-derived neurotrophic factor. This figure schematically represents this process. In the early stage of development, many projecting neurons exhibit exuberent connectivity with the target field. As development progresses and these innervating fibers compete for neurotrophic factor released by the target, some connections are maintained, while others are retracted. Process retraction and failure to acquire sufficient target-derived trophic support, at least from a developmental perspective, is correlated with cell death, yielding a final pattern of innervation that is sculpted by the size of the target.

Figure 1.1

Neurotrophic Factor Hypothesis

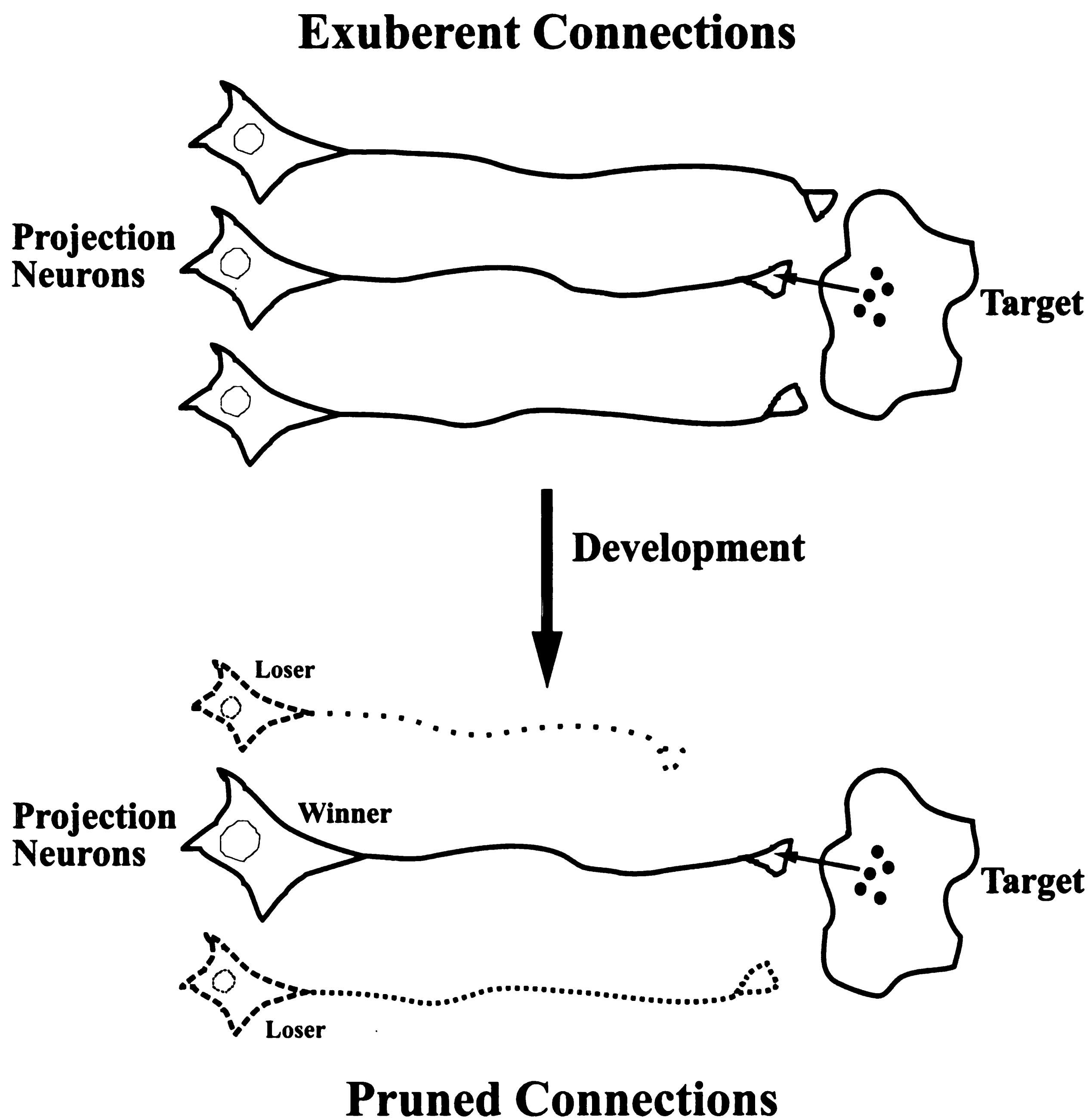


Figure 1.2 TrkA Structural Elements

TrkA is a 140 kDa glycoprotein comprised of a single polypeptide chain that crosses the plasma membrane once (TM = transmembrane domain). Following ligand binding, the receptor dimerizes, leading to activation of the intrinsic kinase within the receptor. The NH_2-terminal extracellular domain is comprised of several cysteine-rich (Cys-rich) and immunoglobulin-like (Ig-like) domains that participate in defining the extended NGF binding site. The COOH-terminal intracellular domain contains several tyrosine residues that are necessary for kinase activity and downstream signaling. Tyrosines 670, 674, and 675 form the core of the kinase domain in TrkA, and participate in auto- and trans-phosphorylation of the receptor following ligand binding and dimerization. These tyrosines may also participate in binding to accessory proteins such as rAPS and SH2-B. Tyrosine 490 serves as a docking site for several signaling adaptors, including Shc, Gab, and FRS-2. Tyrosine 785 is a docking site for PLCγ, CHK, and the adaptor protein Cbl.

Figure 1.2

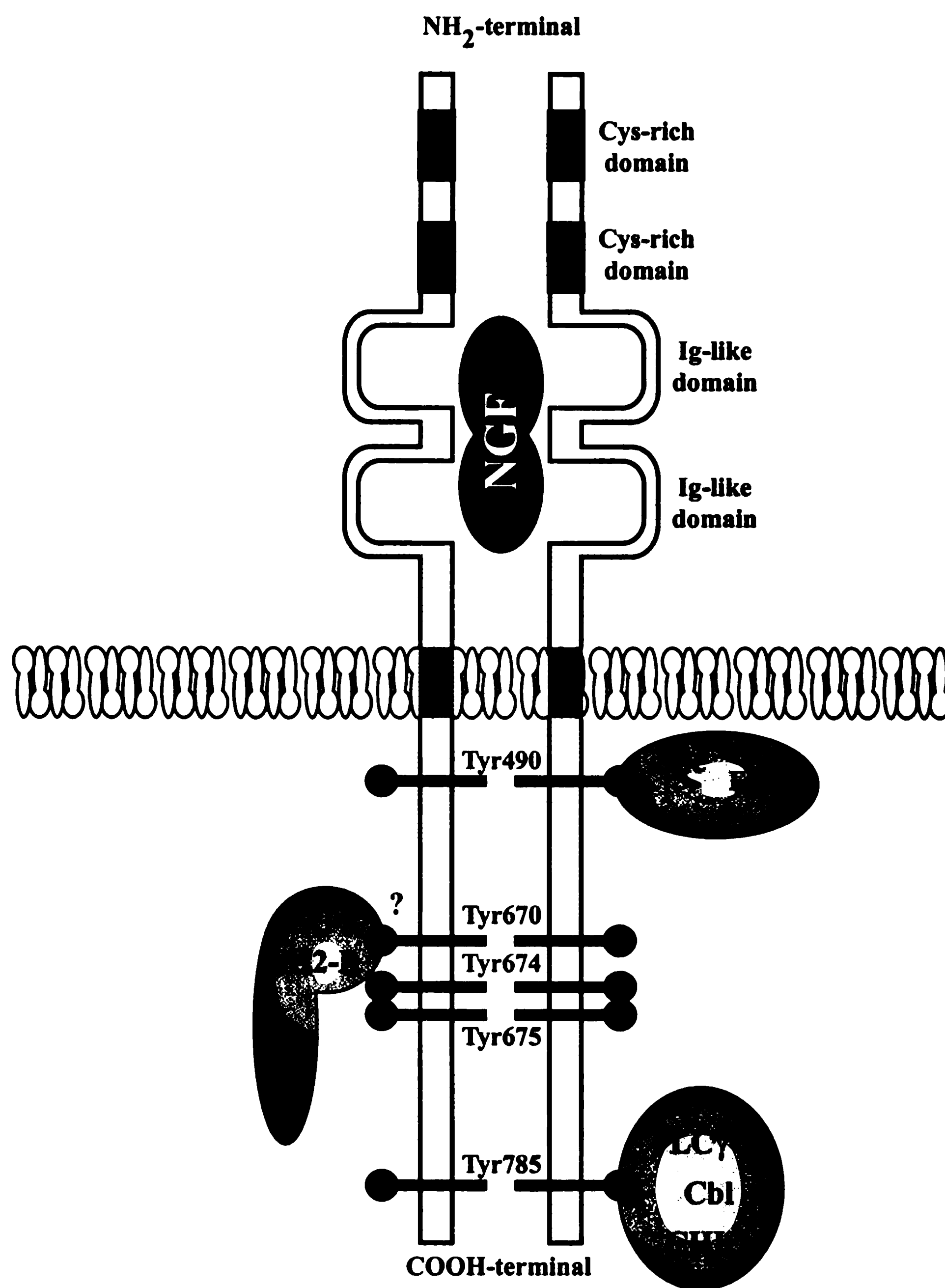


Figure 1.3 Ras-mediated MAP Kinase Cascade Downstream From TrkA.

Following phosphorylation of tyrosine 490 within TrkA, Shc is recruited to the receptor via either an SH2- or PTB-based interaction. Consequently, Shc is bound by a Grb2-SOS complex. Recruitment of SOS to the membrane brings it into proximity of Ras, where it functions as a GTP-exchange factor, activating Ras. Activated Ras recruits and activates Raf. Raf is a serine-threonine kinase that phosphorylates the MAP kinase kinase MEK on 2 serines. This phosphorylation event initiates activity of the dual-specificity kinase, leading to activation of the MAP kinases Erk1/2 via phosphorylation of threonine 202 and tyrosine 204. Phosphorylated Erk1/2 then participate in at least two cascades. Erk1/2 may translocate into the nucleus, where it phosphorylates the transcription factor Elk-1 on 2 serines, or it may phosphorylate the kinase Rsk. Phosphorylation of Elk-1 allows it to interact with the accessory transcription factor SRF, after which it binds to the Serum Response Element (SRE) within the c-fos promoter region and contributes to initiation of transcription. Phosphorylation of Rsk leads to its nuclear translocation and consequent phosphorylation of CREB on serine 133. Phosphorylated CREB is bound by the transcriptional coactivator protein CPB, which also binds to the SRF-Elk complex, creating an extended transcriptional factor complex that leads to c-fos transcription.

Figure 1.3

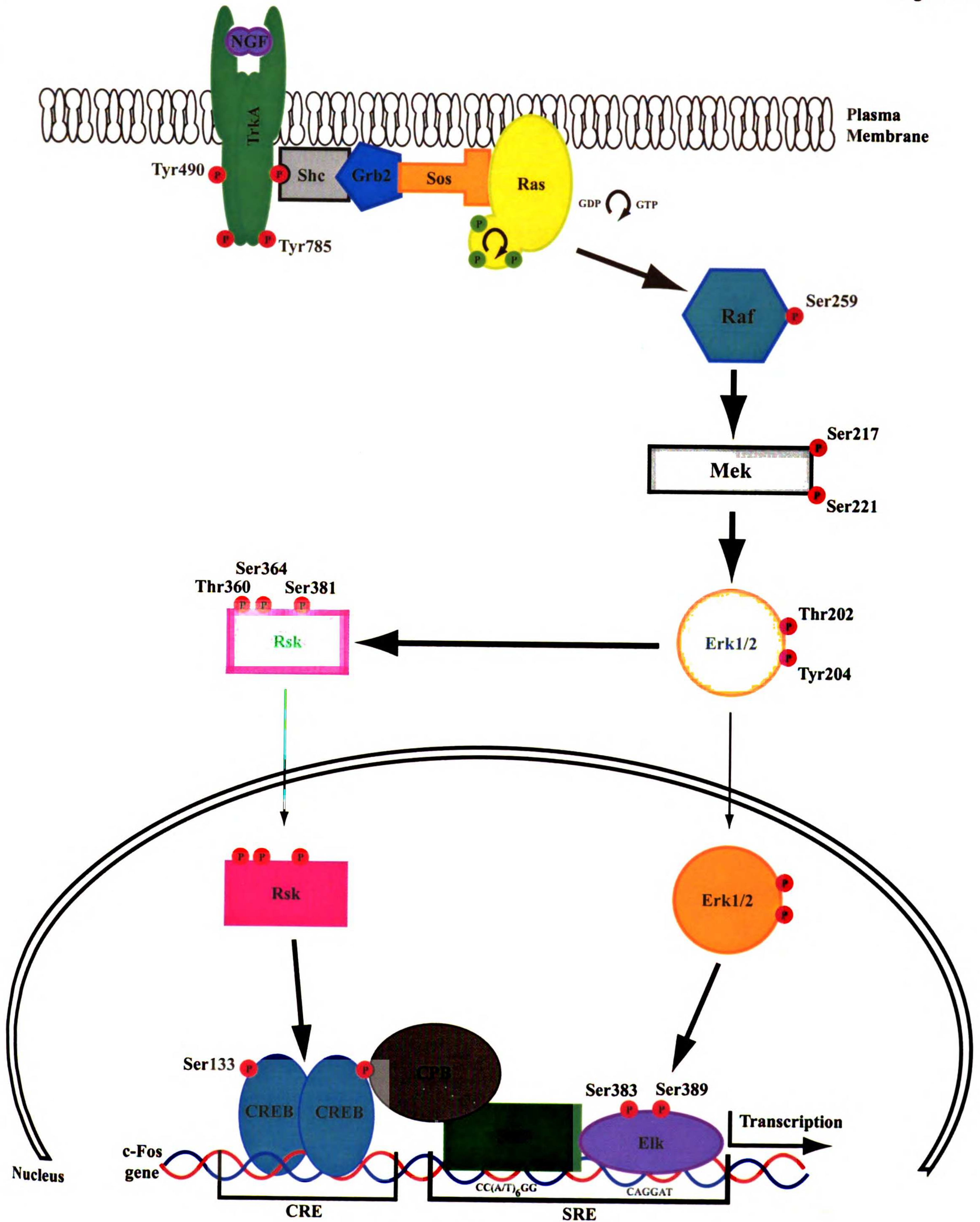


Figure 1.4 Rap-mediated MAP Kinase Cascade Downstream From TrkA

In parallel with the Ras pathway described in Figure 1.3, TrkA activation can lead to signaling through Rap. Phosphorylated tyrosine 490 within TrkA is bound by the adaptor protein Gab2. Activated Gab2 recruits Crk and C3G to the plasma membrane, bringing the Rap GTP-exchange factor C3G into proximity of Rap. Activated Rap recruits and activates B-Raf, which then phosphorylates Mek, leading to Erk1/2 and Rsk activation in parallel with such activation initiated by Ras.

Figure 1.4

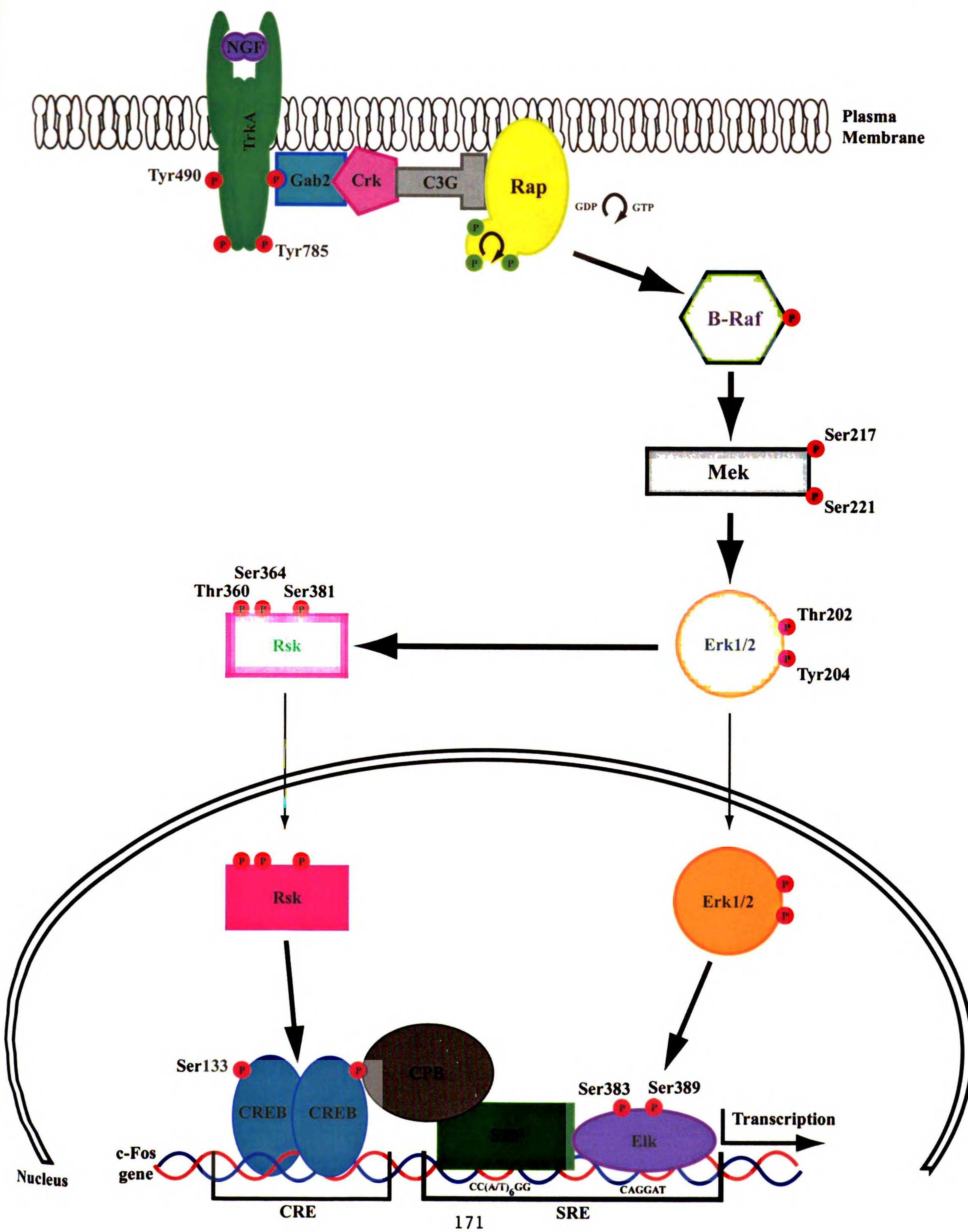
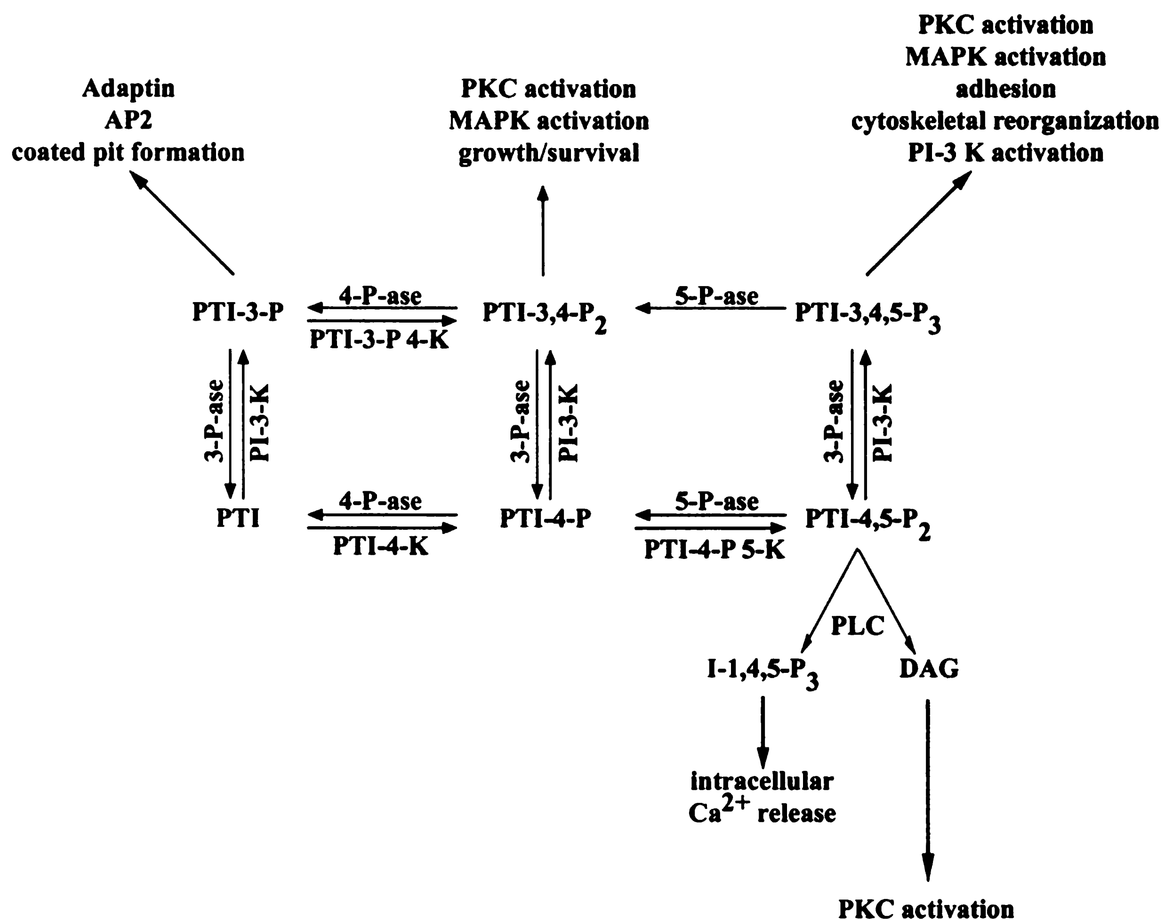


Figure 1.5 PI3 Kinase and PLCγ Effectors and Signaling Cascades

Phosphatidylinositol metabolism is schematized in this figure. PTI-3,4-P_2 is a key activator of the PLCγ pathway, serving as a substrate that leads to the generation of IP_3 and DAG. These factors then participate in release of intracellular calcium and activation of the serine-threonine kinase PKC. PTI-3,4-P_2 is also a critical substrate for PI3 kinase, leading to the generation of PTI-3,4,5-P_3, a factor that leads to the activation of PKC and the MAP kinases, as well as to adhesion and cytoskeletal reorganization. Other PTIs participate in pleiotropic cellular functions.



PTI = phosphatidylinositol
PI = phosphoinositide
PTI-n-P = phosphatidylinositol-n-phosphate
PTI-m-P n-K = phosphatidylinositol-m-phosphate n-kinase
PI-3-K = phosphoinositide 3-kinase
n-P-ase = n-phosphatase

Figure 1.6 TrkA Survival Signaling

TrkA phosphorylation leads to the activation of PI3 kinase. PI3 kinase catalyzes the production of 3-phosphoinositides, including PI-3,4,5-P_3, which binds to and activates PDK1. PDK1 associates with and phosphorylates the serine-threonine kinase Akt. Akt then phosphorylates BAD, inducing its association with the 14-3-3 protein and sequestering it from heterodimerization with Bcl-X_L. As a result of BAD sequesteration, Bcl-X_L is able to heterodimerize with Bax, preventing Bax homodimerization. Homodimerized Bax is a key element in apoptotic signaling, via its role in altering mitochondrial membrane potential, and the balance of Bax:Bax homodimers versus Bax:Bcl-X_L heterodimers may determine whether the cell lives or dies.

Figure 1.6

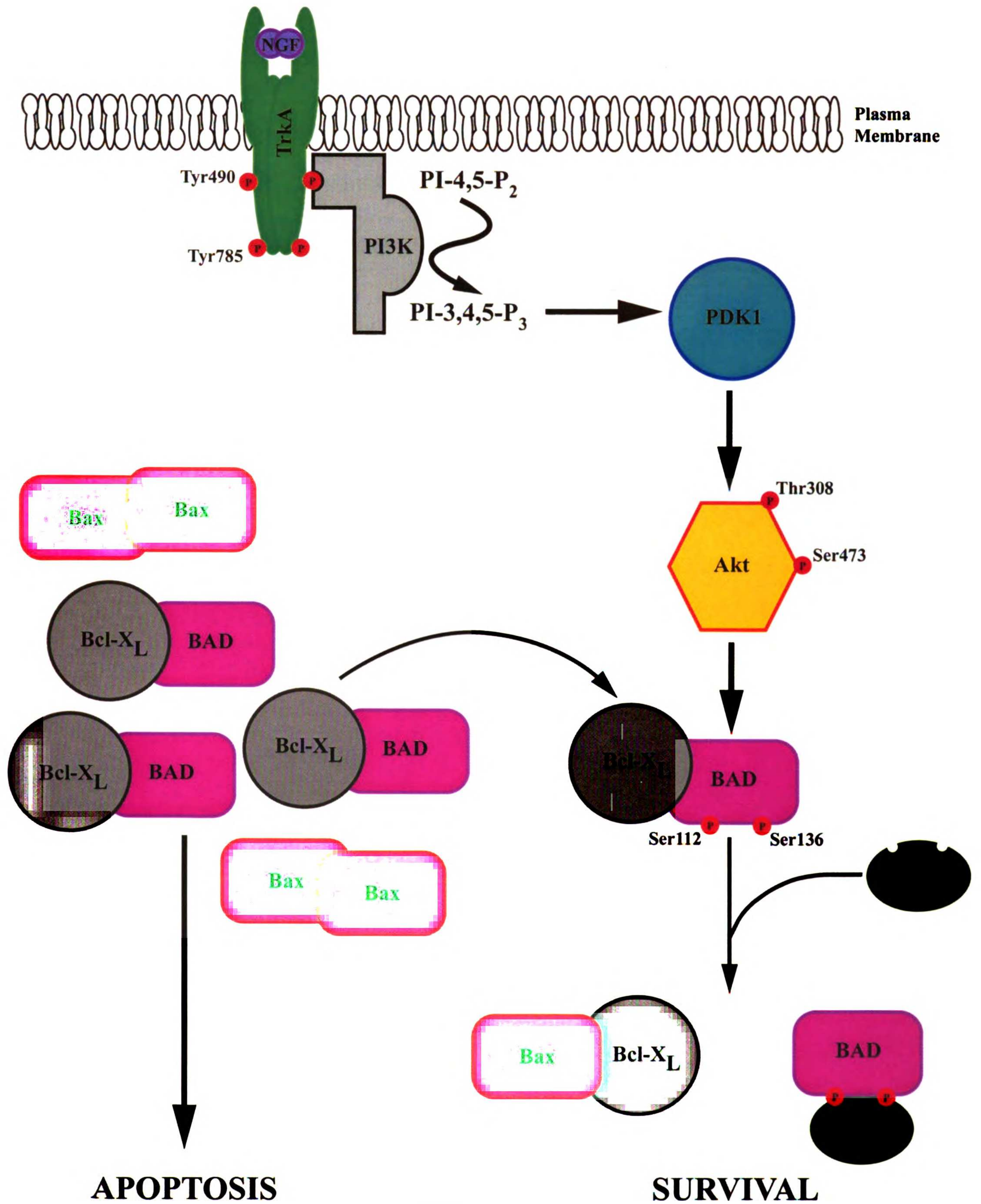


Figure 1.7 Trk and p75 NTR Binding Specificities

TrkA is the only receptor tyrosine kinase for NGF, and TrkB is the receptor tyrosine kinase for BDNF. NT3 binds predominantly to TrkC, but in appropriate cellular and receptor contexts, NT3 is able to bind to both TrkA and TrkB. All three neurotrophins bind to p75NTR with approximately equal kinetics.

Figure 1.7

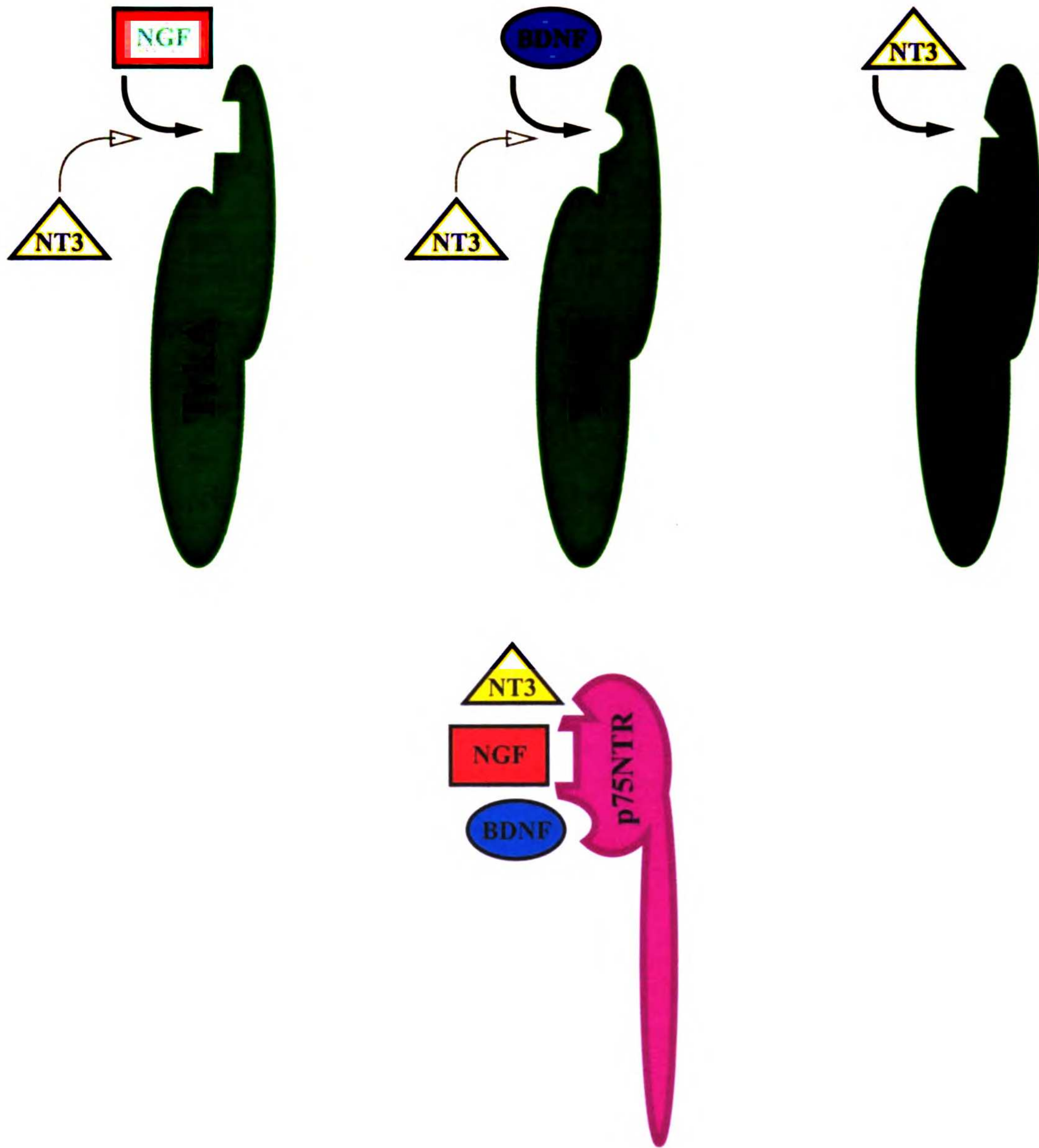


Figure 1.8 Trafficking Within the Endocytic Pathway

Many plasma membrane receptors are endocytosed via the clathrin coated vesicle (CCV) pathway. Following internalization, CCVs rapidly uncoat and fuse with elements of the endosomal compartment, including early endosomes. Early endosomes may mature into late endosomes, and/or sort into secretory vesicles to permit recycling of receptors back to the plasma membrane. Receptors and/or ligands that are trafficked into the late endosome compartment may eventually graduate into the lysosomal compartment, where they are degraded. Lysosomal enzymes are specifically trafficked from their site of production and processing in the ER and Golgi apparatus to lysosomes via coated vesicles.

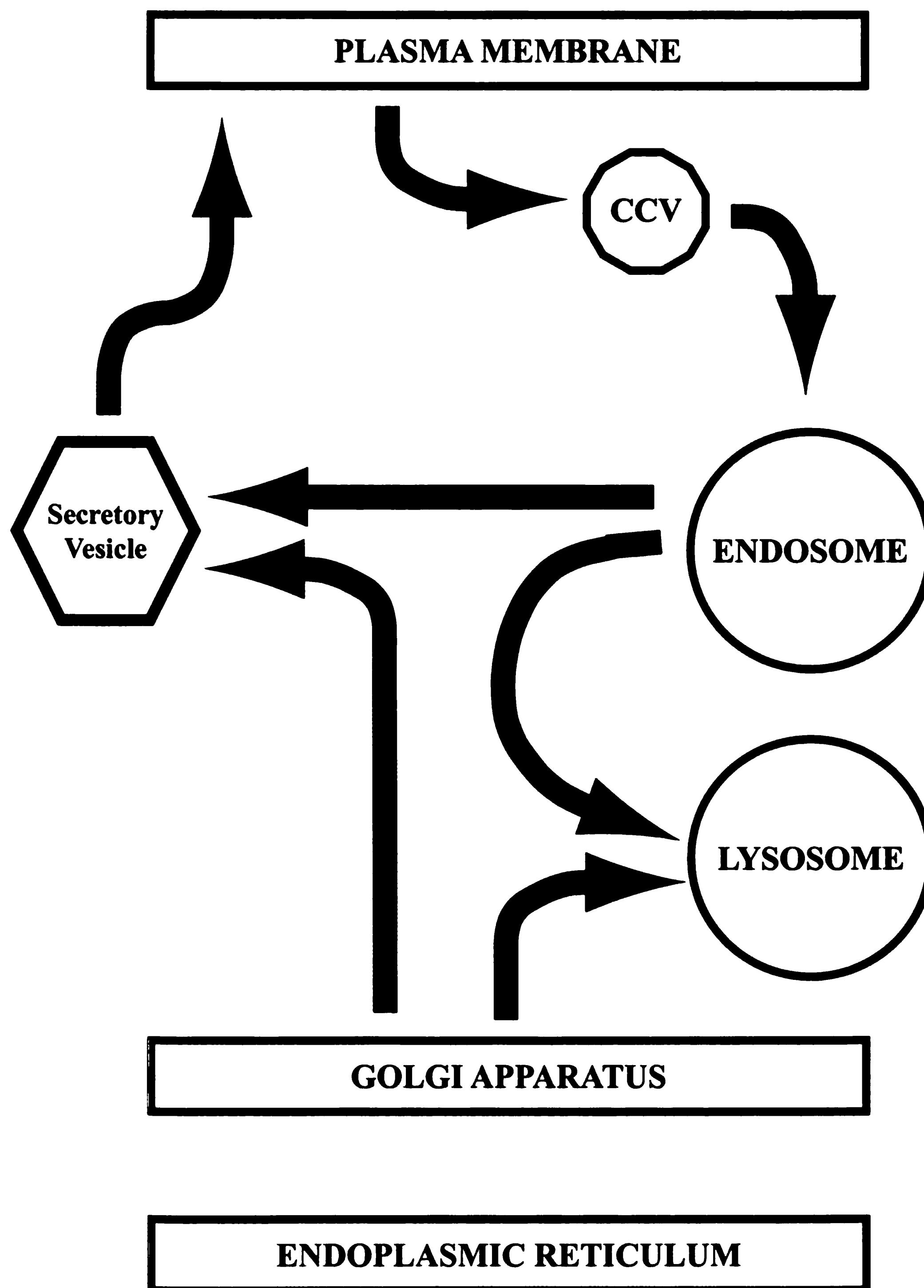

PLASMA MEMBRANE
CCV
Secretory
Vesicle
ENDOSOME
LYSOSOME
GOLGI APPARATUS
ENDOPLASMIC RETICULUM

Figure 1.9 Clathrin-Mediated Growth Factor Internalization

1) Following binding of growth factor to its receptor tyrosine kinase, 2) the receptor is phosphorylated and recruits the AP2 adaptor complex to specific internalization motifs. These motifs either involve phosphotyrosine or are exposed for AP2 binding by conformational changes induced by receptor activation. 3) Following AP2 binding to the receptor, clathrin triskelions are recruited and begin to coat the plasma membrane. Alternatively, clathrin may coat the membrane at specific "seed" sites, and AP2-bound receptors may migrate into these coated pits. 4) Coated membranes acquire greater curvature as the clathrin triskelions incorporate more pentagonal elements into the initially hexagonal array. Curvature is likely to be assisted by accessory proteins, and is certainly promoted by dynamin. Dynamin functions to generate a neck that connects the nascent clathrin-coated vesicle (CCV) to the plasma membrane. Dynamin then participates in scission of this neck, leading to 5) release of the CCV from the plasma membrane. 6) CCVs are rapidly uncoated, freeing the clathrin and AP2 components to participate in new rounds of internalization. Uncoated vesicles containing the growth factor bound to its receptor may then undergo specific sorting events that lead to incorporation into early endosomes and eventual degradation, or these vesicles may mature into elements of the signaling endosome pathway.

Figure 1.9

Extracellular

1

2

3

4

5

6

Intracellular

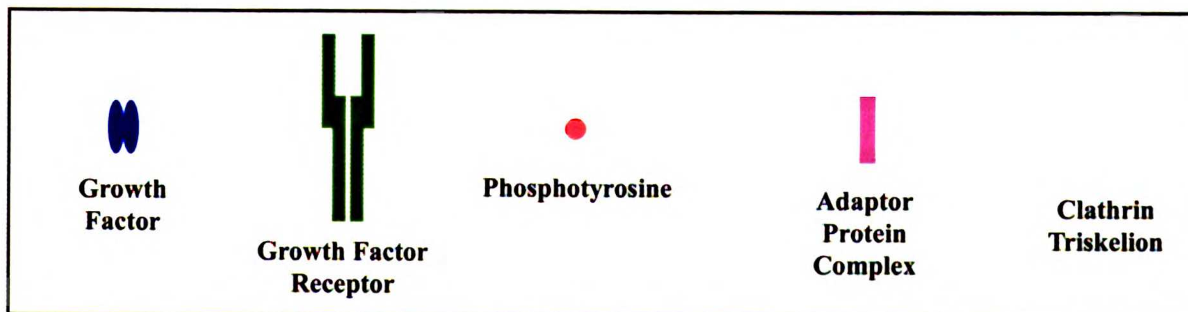


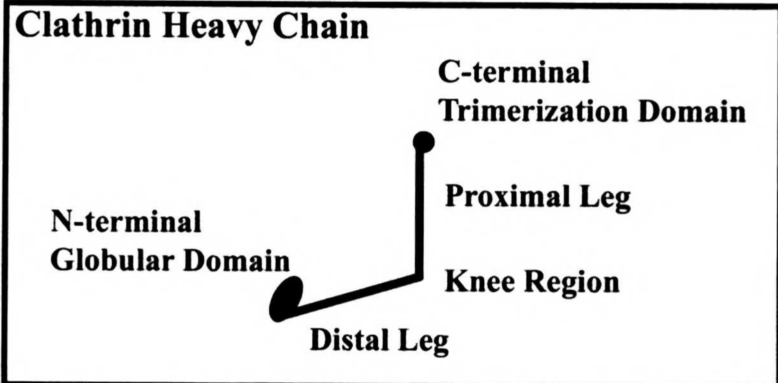
Figure 1.10 Clathrin

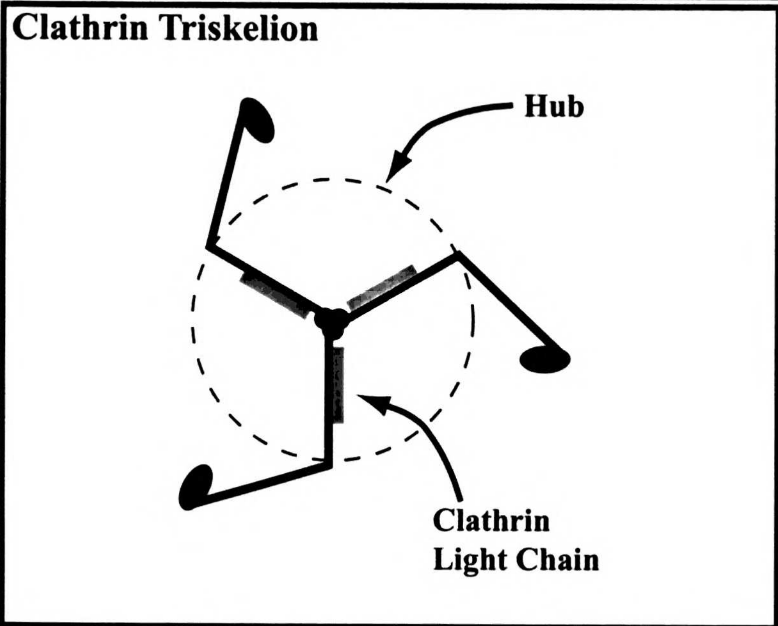
The upper panel shows the structural elements of one clathrin heavy chain. The protein is comprised of an N-terminal globular domain that may participate in binding to other proteins involved in clathrin coat formation. The distal leg and proximal leg are separated by a flexible knee region that permits the heavy chain to adopt a variety of conformations within the coat, thereby permitting the formation of hexagons or pentagons. The C-terminal trimerization domain participates in bringing 3 heavy chains together to form the triskelion.

The middle panel depicts a single triskelion. The hub is defined by the knee region and proximal elements of the heavy chain. A clathrin light chain binds to the proximal leg of each heavy chain within the triskelion hub.

The lower panel shows the formation of an hexagonal packing lattice composed of triskelions. The triskelions aggregate via highly specific interactions between the hubs and the distal legs of other triskelions (these interactions are discussed at length in the text).

Figure 1.10





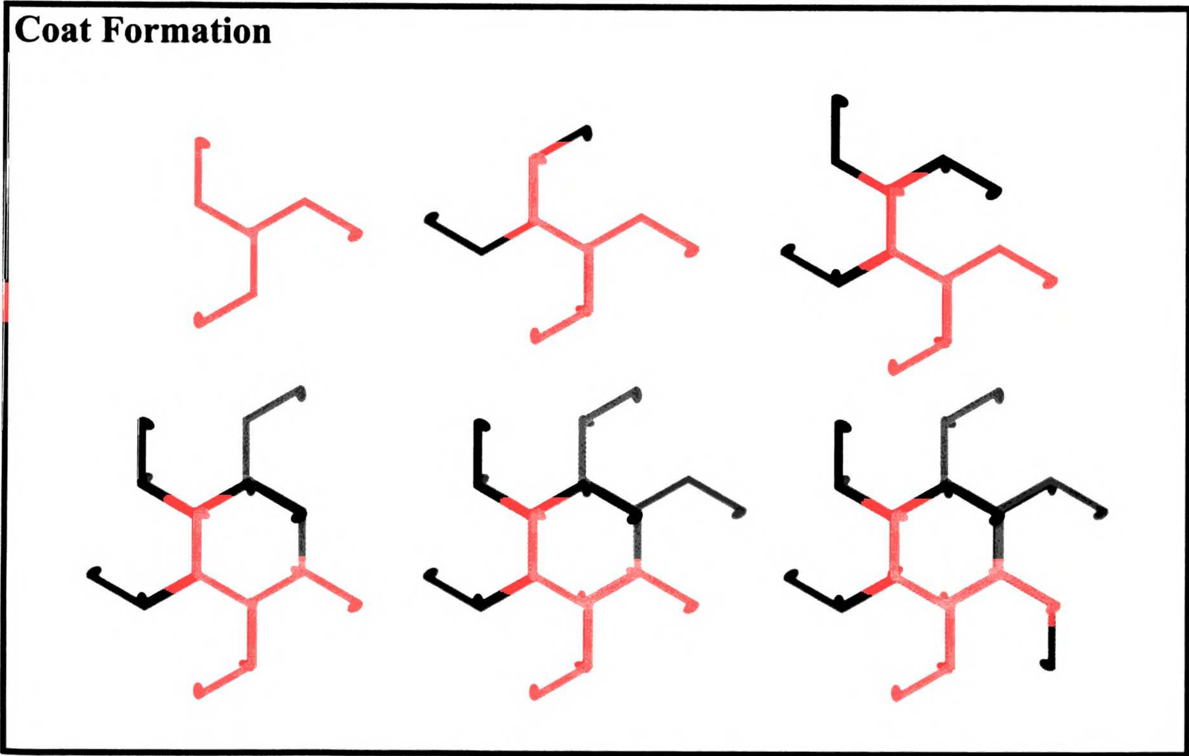
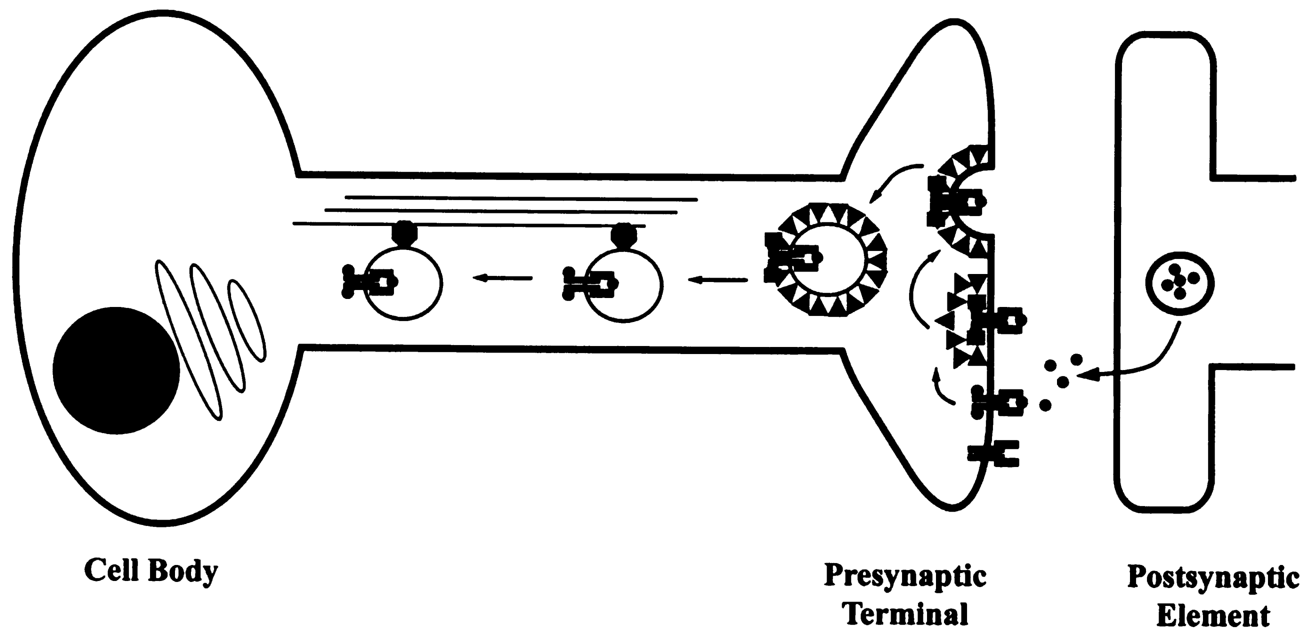
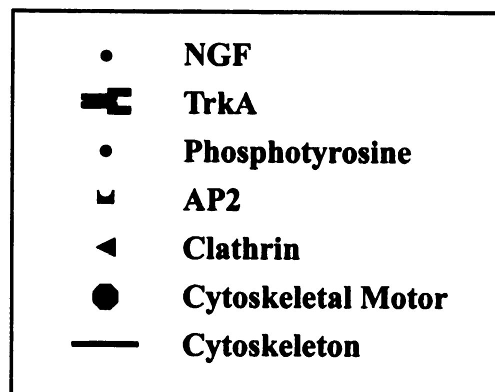


Figure 1.11 The Signaling Endosome Hypothesis

Binding of NGF to TrkA at the presynaptic terminal leads to the internalization of a TrkA:NGF complex into clathrin coated vesicles, as described in Figure 1.9. These vesicles rapidly uncoat, generating endosomes that contain NGF bound to activated TrkA. Such endosomes carry the NGF signal from the terminal to the cell body via active transport along the axonal cytoskeleton. In the cell body, signaling endosomes initiate local signaling cascades that result in transcriptional and translational events.

Figure 1.11





Chapter 2

NGF Signals Through TrkA To Increase Clathrin at the Plasma Membrane and Enhance Clathrin-Mediated Membrane Trafficking

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Summary

Neurotrophin (NT) signals may be moved from axon terminals to neuron cell bodies via signaling endosomes - organelles in which NTs continue to be bound to their activated receptors. Suggesting that clathrin-coated membranes serve as one source of signaling endosomes, in earlier studies we showed that nerve growth factor (NGF) treatment increased clathrin at the plasma membrane and resulted in colocalization of clathrin with TrkA, the receptor tyrosine kinase for NGF. Strikingly, however, we also noted that most clathrin puncta at the surface of NGF-treated cells did not colocalize with TrkA, raising the possibility that NGF induces a general increase in clathrin-coated membrane formation. To explore further this possibility, we examined the distribution of clathrin in NGF- and BDNF-treated cells. NGF signaling in PC12 cells robustly redistributed the adaptor protein AP2 and the clathrin heavy chain (CHC) to surface membranes. Using confocal and epifluorescence microscopy, as well as biochemical assays, the redistribution of clathrin was shown to be due to activation of TrkA. Significantly, NGF signaled through TrkA to induce an increase in clathrin-mediated membrane trafficking, as revealed in increased endocytosis of transferrin. In that BDNF treatment increased AP2 and clathrin at the surface membranes of hippocampal neurons, these findings may represent a physiologically significant response to NTs. We conclude that NT signaling increases clathrin-coated membrane formation and clathrin-mediated membrane

trafficking and speculate that this effect contributes to their trophic actions through increased internalization of receptors and other proteins present in clathrin-coated membranes.

Introduction

The neurotrophins (NTs) regulate the trophic state of neurons (Yuen and Mobley, 1996; Kaplan and Miller, 1997; Casaccia-Bonnefil et al., 1999). An interesting question is how signals generated at the terminals of axons are communicated retrogradely to neuronal cell bodies. That such communication occurs is strongly supported by studies showing that NTs are produced in target tissues and that this source of NTs is critical for the survival of responsive neurons (Snider, 1994; Li et al., 1995; Silos-Santiago et al., 1995; Francis et al., 1999). We (Beattie et al., 1996; Grimes et al., 1996; Grimes et al., 1997) and others (Ehlers et al., 1995; Bhattacharyya et al., 1997; Riccio et al., 1997; Senger and Campenot, 1997; Tsui-Pierchala and Ginty, 1999; Watson et al., 1999) have provided evidence that retrograde NT signals are transmitted through the formation of signaling endosomes, organelles that arise through endocytosis of complexes in which NTs are bound to their Trk receptors.

Earlier findings in this laboratory suggested that clathrin-coated membranes may be used to move NTs and their receptors into signaling endosomes. Nerve growth factor (NGF) treatment of PC12 cells resulted in a more than 10-fold increase in the colocalization of TrkA, the receptor tyrosine kinase for NGF, with the clathrin heavy chain (CHC) at or near the cell surface (Beattie et al, 1996; Grimes et al, 1996; Grimes et al, 1997). CHC is a constituent of clathrin used to mark the presence of clathrin-coated membranes (Nathke

et al., 1992; Schmid, 1997; Marsh and McMahon, 1999). Thus, as is the case for a number of other cell surface receptors (e.g. the transferrin receptor (TfnR), and the EGF receptor (EGFR)) (Schmid, 1997), clathrin-coated membranes may mediate the endocytosis of Trk receptors. In the same experiments, we noted that NGF treatment markedly increased clathrin-immunostained puncta at or near the plasma membrane (Grimes et al. 1996). Though consistent with earlier, as well as more recent, observations on the effect of NGF, EGF and insulin (Connolly et al., 1981; Connolly et al., 1984; Corvera, 1990, Wilde et al., 1999), the extent of the change and its rapidity were impressive. Quite unexpectedly, we also found that most (~ 80%) clathrin puncta near the surface of NGF-treated cells failed to stain for TrkA, suggesting that clathrin was recruited to membranes containing little or no TrkA. Our observations gave evidence that NGF signaling regulates clathrin-coated membrane formation. They predicted that NGF increases endocytic trafficking of TrkA and other proteins found in these membranes.

We have now tested the suggested link between NGF signaling and clathrin-mediated membrane trafficking. NGF signaled through TrkA to increase the formation of clathrin-coated membranes. Significantly, TrkA activation also increased clathrin-mediated membrane endocytic traffic, as revealed by increased uptake of transferrin (Tfn). These findings may reflect a physiologic action of NTs since BDNF increased clathrin

association with the surface membranes of hippocampal neurons. We speculate that enhanced clathrin-mediated membrane trafficking may be a common feature of NT actions that supports their trophic properties.

Experimental Procedures

Reagents

X22, a mouse monoclonal antibody against CHC (Brodsky, 1985), was used for immunoprecipitation and immunostaining. TD.1, another mouse monoclonal antibody to CHC (Nathke et al., 1992), was used for probing Western blots. AP.6 (Chin et al.,1989), a monoclonal antibody to the clathrin adaptor protein AP2 (Schmidt, 1997), was used in immunostaining experiments. Immunoprecipitation of tyrosine phosphorylated proteins was accomplished using mouse monoclonal antibody 4G10 as an agarose conjugate (UBI, Lake Placid, NY). Blotting for tyrosine phosphorylated proteins also used 4G10. To detect the presence of mouse antibody binding to blots, we used HRP-conjugated goat anti-mouse IgG (Santa Cruz Biotechnology, Santa Cruz, CA). For immunofluorescence studies, goat-anti-mouse IgG antibodies conjugated to FITC or to rhodamine were obtained from Cappel (Costa Mesa, CA). The membrane marker DiI C-7000 was from Molecular Probes (Eugene, Ore.).

PC12 nnr5 cells and nnr5 derivatives stably transfected with wild type human TrkA or with the TrkA mutants 22.7 (activation loop mutant, YY674/675FF) or M1 (kinase inactive, K538N), were obtained from Robert Kupta in the Louis Reichardt laboratory. The mutant constructs were created by David Kaplan and colleagues (Ferrari et al., 1995; Cunningham et al., 1997) and were used with permission. For PC12 cells, we used KB PC12 cells (Grimes et al., 1996). Parental 3T3 cells and 3T3 cells expressing either TrkA or $p75^{NTR}$ were supplied by Chin-shiou Huang and maintained as indicated (Huang et al., 1999).

NGF was isolated from the mouse submaxillary gland (Mobley et al., 1976). BDNF was a gift of Regeneron Inc. (Tarrytown, NY). Papain was from Worthington Biochemical Corporation. (Lakewood, NJ). Serum extender and poly-D-lysine were from Collaborative Research (Bedford, MA). Collagen was obtained from Cohesion (Palo Alto, CA). Normal goat serum was from Jackson Immuno Research Laboratories Inc. (West Grove, PA). Geneticin, Neurobasal medium and B-27 serum-free supplement were from Gibco BRL Inc. (Rockville, MD). FITC-dextran was from Molecular Probes. Tfn, K252a, saponin and TX-100 were from Sigma Chemicals (St. Louis, Mo.). Unless otherwise stated, all other reagents were from Sigma. Tissue culture media and media additions were supplied by the UCSF Cell Culture Facility.

Preparation of Cultured Hippocampal Neurons.

A modification of a previously described procedure was used (Lester et al., 1989). The hippocampi of six to nine P0 Sprague-Dawley rats were removed and placed in a dissecting solution (161 mM NaCl, 5.0 mM KCl, 530 uM $MgSO_4$, 2.9 mM $CaCl_2$, 5.0 mM HEPES, 5.5 mM glucose, 5.6 uM phenol red, pH 7.4). The dentate gyri were dissected and discarded. The remaining tissue was treated with papain (20 units/ml) in 10 ml of the same solution containing 1.7 mM cysteine, 1mM $CaCl_2$, and 0.5 mM EDTA for 45 minutes at 37^{0}C. The digestion was stopped by decanting the solution and by adding 10 ml of complete medium (minimal essential medium with Earle's Salts, without L-glutamine, and with 20 mM glucose, serum extender [1:1000], and 5% heat-inactivated fetal calf serum) containing 25 mg BSA and 25 mg trypsin inhibitor type 3-0. The tissue was then triturated in a small volume of this solution using a fire-polished Pasteur pipette. Using coverslips coated with poly-D-lysine (0.1 mg/ml) and collagen (0.06 mg/ml), the cells were plated overnight in Neurobasal medium containing the additives B-27 serum-free supplement (1x) and L-alanyl-L-glutamine (2 mM). One-half the medium was replaced with the Neurobasal medium plus additives the following day. Cultures were refed by replacing one-half the volume of medium at weekly intervals. At week one they were refed with complete medium containing B-27. At week two and beyond, they were fed with the Neurobasal medium plus additives. Astrocyte growth was inhibited at day

12 by adding 5-fluoro-2'-deoxyuridine (0.3 mM) plus uridine (0.7 mM). Cultures were used for experiments between weeks three and four.

Immunofluorescence Studies

All cells were grown and maintained at 37°C with 5% CO_2. PC12 cells, PC12 nnr5 cells and nnr5 variants were cultured in DME-H21, 10% horse serum, and 5% fetal calf serum on collagen-coated plates. The medium used for maintaining TrkA-variant expressing nnr5 PC12 cell lines included 100 ug/ml Geneticin. Priming of PC12 cells was accomplished by adding NGF (2 nM) for seven days. 3T3 cells were grown on plastic in DME-H21 in 10% horse serum.

In preparation for immunostaining experiments on primed PC12 cells, the cells were washed at 37^{0}C with serum-free medium (minus NGF) in three changes (30 minutes each). They were then treated with NGF or the vehicle in serum-free medium, as indicated below. For experiments on unprimed PC12 cells, PC12 nnr5 cells and 3T3 cells, cells were first incubated in DME-H21 containing 1% horse serum overnight prior to treatment in the same medium. Hippocampal neurons were maintained and treated in the medium described above. To examine the effects of NGF treatment, cells were usually first incubated with NGF under conditions in which NGF would bind to its

receptors without inducing membrane trafficking events. Thus, cells were incubated at 4°C in medium with NGF (2 nM), or with the vehicle (0.2% acetic acid in the same small volume as used to add NGF), for one hour prior to warming at 37°C. In some experiments, NGF (2 nM) or BDNF (2 nM) was added to cells at 37°C. After NGF treatment, cells were quickly chilled to 4°C, fixed with 4% paraformaldehyde in phosphate buffered saline (PBS) for 20 minutes at 4°C, permeabilized in PBS containing saponin (1ug/ml), and blocked in 10% normal goat serum. To visualize clathrin (i.e. CHC), X22 (10ug/ml) was incubated with cells overnight at 4°C and the signal was developed with either rhodamine- or FITC-conjugated goat anti-mouse IgG antibody. To stain AP2, AP.6 (6ug/ml) was incubated with cells using the same protocol and the signal was developed with rhodamine-conjugated goat anti-mouse IgG. For plasma membrane demarcation, DiI (0.5 ug/ml in PBS) was applied to fixed and immunostained cells for 30 minutes at room temperature in the dark, followed by a brief wash with PBS.

Confocal microscopic analysis of clathrin distribution was accomplished using a MRC 1000 Laser Scanning Confocal Microscope (Bio-Rad, Hercules, CA) equipped with a krypton/argon laser and attached to a Zeiss Axiovert microscope. Care was taken to ensure data were collected at a point midway between the substrate-attached plasma membrane and the top of the cell. Immunostained puncta were located near the apparent

margin of all cells stained for CHC or AP2. Though puncta were fewer in number and less intense in vehicle-treated cells, the staining was adequate to delineate the cell margin. The surface of cells was defined as a line that linked the outermost puncta. Figure 2 shows that this method defined the margins of both NGF- and vehicle-treated cells. The length of the line that marked the cell surface was measured using the measurement analysis tool provided with the public domain NIH Image program (developed at the U.S. National Institutes of Health and available on the internet at http://rsb.info.NIH.gov/NIH-image/) and this value was used as the cell perimeter. Immunostained puncta were counted as described (Grimes et al., 1996). Briefly, after defining the edge of the cell, a second line was drawn 0.5um interior to the first, and all immunostained puncta between the lines were counted. The results were expressed as the number of puncta per um of cell surface or the number per cell.

Epifluorescence microscopy was performed using a Nikon Diaphot 300 inverted microscope with a PlanApo 60 Nikon objective. Images were collected and processed using a Princeton Instruments Micro Max CCD camera and IP Lab Spectrum Image Processing software from Signal Analytics (Vienna, Virginia). To define the margins of cells and the number of puncta at or near the cell surface, the same methods were used as for confocal microscopy.

Clathrin Membrane Association Studies

For biochemical analyses, all cells were cultured and prepared for experiments as described above. However, in some cases serum deprivation for 4 hours replaced overnight incubation in 1% horse serum. We used two methods to measure membrane-associated clathrin. In each, gentle conditions were used that favored maintaining the association of clathrin with membranes (Wilde and Brodsky, 1996). The first method produces a cell ghost depleted of cytosol and internal membranes; importantly, however, the plasma membrane remains associated with the cell ghost (Grimes et al., 1996). Cells ($5x10^7$ per condition) were harvested at 37°C in calcium- and magnesium-free (CMF)-PBS. They were pelleted at 1000 x g and resuspended in binding buffer (PBS containing glucose (1mg/ml), BSA (1mg/ml) and HEPES (10mM), pH =7.4). Cells were then treated with NGF (2nM) or vehicle for 2 minutes at 37 ^{0}C. The suspensions were then rapidly chilled to 4°C in an ice water bath, pelleted at 1000 x g at 4^0 C, and resuspended in 1 ml of cold (4°C) MES buffer (100 mM MES, pH 6.8, 0.5 mM $MgCl_2$, 0.2 mM DTT, 1 mM NaOrthovandate, 1mM PMSF, and 0.1 ug/ml each leupeptin and aprotinin). Cells were then gently disrupted using a ball homogenizer, as described previously (Grimes et al., 1996). To separate the cell ghost from the cytosol, the preparation was centrifuged at 8000 x g for 35 minutes, a procedure that also pelleted the heaviest membranes released

from the cell ghost. After washing the pellet once in MES buffer and repelleting at 8000 x g for 35 minutes, the pellet was lysed with lysis buffer (20mM Tris, pH 8.0, 137 mM NaCl, 1mM NaOrthovandate, 1% NP-40, 0.5% DOC, 10% glycerol, 1mM PMSF, and 1 ug/ml each of leupeptin, and aprotinin). The samples, which represented equivalent numbers of cells, were immunoprecipitated for CHC with X22 (10ug/ml). Immunoprecipitates were processed by SDS-PAGE and transferred to nitrocellulose as described (Grimes et al., 1996). The blot was probed with TD.1 (3ug/ml). After incubating with HRP-conjugated goat anti-mouse IgG, the signal was visualized by ECL phosphorescence (Amersham, Buckinghamshire, England). Data were quantified using NIH Image software.

In the second method, we more thoroughly disrupted cells in an attempt to eliminate any possible contamination of membrane fractions by cytosol. One 15cm plate (5×10^7 cells) per condition was harvested with CMF-PBS. The cells were pelleted at 1000 x g for 5 minutes and then resuspended in 5 ml of cold (4°C) DME containing 25mM HEPES buffer and either NGF (2 nM) or the vehicle. The cell suspensions, in 15 ml conical tubes, were rotated for 1 hour at 4 °C, then warmed for the time indicated in a 37°C water bath with periodic gentle mixing. Samples were then chilled in an ice bath for 3 minutes and the cells were pelleted at 1000 x g for 5 minutes. They were then washed once with

cold PBS (4°C) and resuspended in 1 ml cold (4°C) MES buffer. Membranes were disrupted by three cycles of freezing followed by thawing, after each of which a 25-gauge needle was used to further disrupt the material. Samples were then spun at 1000 x g for 5 minutes to remove nuclei and intact cells and the supernatant was centrifuged at 100,000 x g for 40 minutes, essentially as described (Grimes et al., 1996). On the basis of results from earlier studies (Grimes et al., 1996), the resulting pellet (P2') was predicted to contain small and large fragments of the plasma membrane, internal membranes derived from the plasma membranes, other cellular membranes, and organelles such as mitochondria and ribosomes; S2' contained the cytosol. P2' was resuspended in the lysis buffer. The supernatant (S2') was diluted 1:2 in a solution of 0.5 M Tris buffer containing 1% TX-100. Lysed P2' fractions were equalized for protein, as were S2' fractions, and they were subjected to SDS-PAGE, transferred to nitrocellulose and immunoblotted for clathrin (i.e. CHC) with TD.1 (3ug/ml) as described (Grimes et al., 1996). A modification of this method was used to examine PC12 nnr5 cells and PC12nrr5 cells expressing wild-type TrkA and mutant TrkA receptors. After disrupting cells as indicated above, they were centrifuged at 10,000 xg for 10 minutes at 4°C. This produced a pellet containing cell ghosts, cell ghost fragments and heavy membranes that was similar to that collected with the first method. The pellet was suspended in the lysis buffer and processed as above, except that samples were slot blotted to nitrocellulose and immunoblotted for CHC using the same antibody. After incubating blots with HRP-

conjugated goat anti-mouse IgG, the signal was detected by ECL phosphorescence and quantified using NIH Image software. In control studies we verified that protein loads and exposures produced signals in the linear range.

CHC Phosphorylation Assay

PC12 cells were used in the clathrin phosphorylation assay. Cells (3 x 10^7 per condition) were treated with NGF (2 nM) or the vehicle at 37°C for 2 minutes. The medium was removed and cold (4°C) PBS was used to wash cells while the plates were transferred onto ice. Cells were then lysed in the lysis buffer and immunoprecipitated with X22 (10ug/ml). The immunoprecipitates were subjected to SDS-PAGE and transfer, and were immunoblotted as described (Grimes et al., 1996). Antibodies against CHC (TD.1; 3ug/ml), and phosphorylated tyrosine (4G10; 1/2000), were used to sequentially probe the blot. To prepare for reprobing, the blot was acid-stripped using TBS (pH 2.0) for 30 minutes at room temperature. After incubating blots with HRP-conjugated goat anti-mouse IgG, the signal was detected by ECL phosphorescence and quantified using NIH Image software.

FITC-Dextran and ^{125}I Transferrin Uptake Assays

In FITC-dextran uptake studies, PC12 cells ($5x10^7$ cells per condition) were removed from culture plates in warm (37°C) CMF-PBS and incubated, rotating, for 30 min in serum-free PBS-HEPES (10 mM, pH 7.4) at 37°C. Cells were then pelleted for 2 minutes at 1000 x g and resuspended in 1 mL HEPES-PBS. FITC-dextran (1 nM), with or without NGF (2 nM), was then added to the suspension and cells were incubated, rotating, at 37°C for 5 or 10 minutes. Cells were chilled (4°C) and washed three times with ice-cold PBS. They were then lysed in the lysis buffer. After sedimenting nuclei and insoluble debris at 1000 x g, absorbance was measured at 490 nm.

In Tfn uptake timecourse experiments, PC12 cells from four 15 cm plates ($20x10^7$ cells) were pooled and suspended in 16 ml of CMF-PBS. One ml aliquots were incubated at 4°C for 30 minutes in PBS-HEPES. 125I-Tfn (10 ng/mL), with or without NGF (2 nM), was added to cells at 4°C and the mixtures were then incubated at 37°C for the time intervals indicated. Cells were then chilled and spun down (1000 x g for 1 minute) prior to acid-stripping as described (Zhou et al., 1995) for 10 min at 4°C. Cells were then quickly pelleted and washed once with cold (4°C) PBS. Radioactivity of the cell pellet was measured in a Beckman gamma counter. In samples treated with 125I-Tfn or with 125I-Tfn and NGF, but not warmed, the counts in the pellet following stripping were less than 20% of the warmed values, and did not differ between NGF-treated and vehicle-

treated samples. In experiments testing the role of TrkA kinase activity on 125I-Tfn uptake, PC12 cells or PC12nnr5 cells were treated for 5 minutes with 125I-Tfn or with 125I-Tfn and NGF, essentially as described above. For K252a experiments, cells were pretreated at __°C in suspension with 200 nM K252a for 30 minutes, and chilled to 4°C. They were then treated with 125I-Tfn or 125I-Tfn plus NGF for 5 minutes, as above.

Results

NGF signaling recruited clathrin to the plasma membrane.

To investigate further the NGF effect on clathrin-coated membrane formation, we examined the cellular localization of clathrin in PC12 cells incubated with NGF (2 nM), or the vehicle, for one hour at 4° followed by warming for 2 minutes at 37°C. After treatment, cells were quickly chilled, and then washed, fixed and permeabilized before immunostaining for CHC. Confocal microscopy of cells not treated with NGF showed that CHC staining was seen in small puncta distributed diffusely throughout cells, with most staining in the cytosol (Figure 2.1). Staining was noted in the perinuclear region, but there was relatively little associated with the plasma membrane. Following NGF treatment there was a redistribution of staining with a marked increase at or near the plasma membrane. There was also a consistent increase in staining in the perinuclear region. The puncta near the cell surface consistently demonstrated a "picket-fence"

pattern in which they appeared to line up near the edge of the cell. We quantified the increase in puncta within 0.5 um of the cell surface following NGF treatment. In NGF-treated cells, the average number of these puncta was 0.76 per um. With an average cell perimeter of 56 um, the average number of puncta per cell was 43. The number of puncta per um in NGF-treated cells was 244 % of the vehicle-treated control (+/- 6.8 %[SEM], n= 15 cells in two separate experiments), a result that was significant ($p<0.01$). We conclude that NGF acted to induce redistribution of clathrin.

The change in CHC staining suggested that NGF redistributed clathrin to the plasma membrane. To better define the locus of clathrin near the cell surface, we asked if CHC staining would colocalize with a lipophilic membrane marker, DiI. Figure 2.2 B and E show that DiI effectively marked surface membranes in both vehicle-treated and NGF-treated cells. Permeabilization was required to immunostain for CHC. As a result, DiI sometimes also marked membranes near the cell surface. The redistribution of CHC staining that followed NGF treatment (compare panels A and D) resulted in increased co-localization of CHC with DiI at the cell surface. Note the marked increase in the number of CHC puncta colocalized with DiI (yellow denotes co-localization) following NGF treatment (compare panels C and F). Since brief (3 minute) NGF treatment of PC12 cells has been shown not to increase plasma membrane surface area (Connolly et al., 1984),

the NGF-induced increase in CHC immunostaining reflects an increase in clathrin at the plasma membrane.

The change in CHC staining that followed NGF treatment was consistent with an increase in membrane-associated clathrin. To confirm this prediction, we used two methods. In each case cells were treated with NGF for 2 minutes. In the first method, we gently disrupted cells using a ball homogenizer (Grimes et al., 1996). This method depletes cells of cytosol, internal membranes and organelles under conditions that do not fragment plasma membrane and that favor the continued association of clathrin with membranes. By harvesting the 8000g pellet, clathrin in the cell ghost was separated from cytoplasmic clathrin and from clathrin associated with all but the heaviest membranes released from cells. CHC in the pellet was examined by SDS-PAGE, and transfer to nitrocellulose followed by immunoblotting. In NGF treated cells, CHC was increased to 158% of untreated controls (Figure 2.3A).

In the second method, we used 3 cycles of freezing and thawing to more thoroughly disrupt cells to ensure that trapping of cytosolic clathrin in cell ghosts could not contribute to the findings for membrane-associated clathrin. After pelleting the remaining cell ghosts, we quantified the amount of CHC associated with fragments of the

plasma membrane and with membranes released from disrupted cells. These membranes (P2') were separated from cytosol (S2') using a 100,000g spin. CHC was present in both fractions in both untreated and treated cells. Following NGF, the amount of CHC in P2' increased while the amount in S2' decreased (Figure 2.3 B and C). In NGF treated cells, the amount of CHC in P2' was 166% of the vehicle-treated control. There was a small decrease in CHC in S2'.

These observations show that NGF induced movement of clathrin to membranes, with changes that were comparable using the two methods. In that plasma membrane was a major constituent of the membrane fractions produced by both methods, the findings point to an NGF-induced increase in clathrin at the plasma membrane. The change in CHC immunostaining at the surface of NGF-treated cells is consistent with this view, as is an earlier EM study showing an NGF effect on clathrin-coated membranes (Connolly et al., 1984). The possibility exists that NGF induced an increase in clathrin association with other membranes, including those derived from the plasma membrane.

NGF signaled through TrkA to increase the amount of clathrin at the plasma membrane.

NGF signals through two receptors, TrkA and $p75^{NTR}$. To ask which NGF receptor(s) was responsible for the redistribution of clathrin, we carried out experiments in a number of different cell types. To examine a contribution by TrkA in the absence of $p75^{NTR}$, we tested 3T3 cells that express TrkA (3T3-TrkA cells). In the absence of NGF treatment, CHC was distributed in the pattern seen in untreated PC12 cells (Figure 2.4A). Addition of NGF to these cultures resulted in redistribution of CHC such that the number of CHC puncta at or near the plasma membrane was significantly greater (Figure 2.4 B). Quantification of puncta within 0.5um of the cell surface showed that NGF treatment for 2 minutes resulted in a value that was 250% of the vehicle-treated control (n=10 cells, $p < 0.01$). Corresponding to this increase, we found that in 3T3-TrkA cells treated with NGF the amount of membrane-associated CHC in the P2' fraction was increased (at 1 minute = 170%, at 2 minutes =136%, and at 10 minutes =137% of the vehicle –treated controls). Very similar results for clathrin redistribution were obtained when 3T3-TrkB cells were treated with BDNF (data not shown). In the 3T3 parental cells, the number of puncta at the plasma membrane was unchanged after NGF treatment (3% below vehicle-treated control, n=10 cells, $p=0.38$) (Figure 2.4 E & F). NGF did not increase CHC-positive puncta near the plasma membrane in 3T3 cells expressing $p75^{NTR}$ (Fig 2.4 C & D). Indeed, NGF actually decreased the number of such puncta (30% below vehicle treated control, n=10 cells, $p<0.05$). Further studies are needed to characterize $p75^{NTR}$ effects on

CHC distribution. We conclude that the NGF increased clathrin-coated membrane formation through TrkA.

We tested further the role of TrkA signaling in the clathrin redistribution by carrying out studies in normal PC12 cells and in a series of PC12 cell variants (i.e. nnr5 PC12 cells) carrying wild-type and mutant TrkA receptors. In all untreated cells, clathrin was distributed diffusely and in a cytosolic pattern. In PC12 cells, as expected, NGF signaling resulted in an increase in clathrin staining at or near the plasma membrane (Figure 2.5 A and B). In PC12nnr5 cells, which have extremely low levels of TrkA but normal levels of $p75^{NTR}$ (Loeb and Greene, 1993), there was no evident change in the distribution of clathrin with NGF treatment (Figure 2.5 E and F). Redistribution of CHC staining was seen in a variant of PC12 nnr5 cells transfected with the wild-type TrkA receptor (Figure 2.5 C and D). When these cells were treated with NGF, many brightly stained puncta were found in at the cell surface. We also examined PC12nnr5 cells stably transfected with the M1 TrkA mutant, in which substituting N for K at residue 538 inactivates the kinase domain, and with the 22.7 mutant, in which two activation loop tyrosines (Y674 and 675) are replaced with phenylalanine. Earlier studies documented markedly decreased TrkA signaling in cells expressing these mutants (Ferrari et al., 1995; Cunningham et al., 1997). We found that NGF treatment failed to induce clathrin redistribution in cells expressing the mutant TrkA receptors (Figure 2.5 G through J).

Consistent with these findings, while CHC association with membranes was induced by NGF in the nnr5 TrkA cells (% of vehicle = 113 +/- 1.9%, n=3, $p<0.05$), there was no increase in the 22.7 mutant cells (% of vehicle = 96.2 +/- 2.1, n=3, p=0.26). In the M1 cells, NGF treatment caused a decrease that was not significant (% of vehicle = 82 +/- 8, n=3, p=0.18). Taken together, the data provide strong evidence that NGF signaled through activation of TrkA kinase to induce formation of clathrin-coated membranes.

NTs signaled to redistribute AP2 and clathrin in PC12 cells and in hippocampal neurons.

The marked effect of NGF on clathrin at the plasma membrane suggested that other components of clathrin-coated membranes would also be recruited. We tested this by examining the distribution of AP2, a major constituent of clathrin coats at the plasma membrane (Beck et al., 1992; Wilde and Brodsky, 1996; Marsh and McMahon, 1999). By epifluorescence microscopy, AP2 was distributed in a cytosolic pattern in untreated PC12 cells (Figure 2.1C). Following NGF treatment, much more AP2 was present at or near the plasma membrane (Figure 2.1D). The pattern of staining was essentially identical to that seen for CHC. Surface puncta were counted, as described above, in epifluorescence micrographs. In NGF-treated cells, the number of puncta per um was 225% (+/-15%; n= 12; p<0.01) of the vehicle-treated control. Thus, as was true for clathrin, NGF signaling recruited AP2 to the plasma membrane of PC12 cells.

To ask whether or not NTs influence the distribution of clathrin and AP2 in neurons, we carried out studies in hippocampal primary cultures. Hippocampal neurons express little if any TrkA, but they do express TrkB and respond to BDNF (Ip et al., 1993a; Minichiello et al., 1999). In vehicle-treated cells, AP2 staining was distributed more or less uniformly in the cytosol (Figure 2.6A [confocal image] and B [epifluorescent image]). There was no apparent change in the distribution of AP2 with NGF (not

shown). However, with BDNF treatment there was a clear increase in staining near the plasma membrane (Figure 2.6C and D). The surface membranes of both cell bodies and processes showed the change. The change in AP2 staining was quantified in epifluorescence micrographs. In BDNF-treated cells the number of surface puncta was 306% (+/- 23%; n=5; p<0.01) of the vehicle-treated control. To show whether or not clathrin was also redistributed by BDNF treatment, hippocampal neurons were examined after staining for CHC. In vehicle-treated cells there was a diffuse cytosolic pattern of staining (Figure 2.6E [confocal image] and F, I [epifluorescent images]). BDNF treatment for 2 minutes resulted in marked redistribution of CHC staining to the plasma membrane (Fig 2.6G [confocal] and H, J [epifluorescence]); the number of puncta was 241% (+/- 25%; n=5, p<0.01) of that in vehicle-treated cells. The redistribution of AP2 and CHC induced by BDNF acting on hippocampal neurons suggests that increased movement of clathrin to surface membranes may represent a physiological response to NT signaling.

NGF increases phosphorylation of CHC.

Recently, we showed that the redistribution of clathrin seen with EGF signaling was associated with the phosphorylation of CHC (Wilde et al., 1999). To determine whether NGF signaling also induced an increase in the phosphorylation of CHC, we quantified tyrosine phosphorylated CHC in immunoprecipitates from NGF-treated and untreated

PC12 cells. Figure 2.7 shows that phosphorylated CHC was present in untreated cells. The amount was significantly increased following NGF treatment for 2 minutes; the value averaged 223% (+/- 19 %, n=3, p<0.01) of the vehicle-treated control. Similar results were obtained in experiments in which the 4G10 antiphosphotyrosine antibody was used for immunoprecipitation followed by probing with the antibody for CHC (data not shown). To evaluate further the effect of NGF on CHC phosphorylation, we carried out timecourse studies. NGF treatment increased CHC phosphorylation as early as 1 minute (NGF treated = 141% (+/- 9%) of control, n=3, p< 0.01). The effect was maximal at 2 minutes (see above) and lasted through 15 minutes (at 5 minutes: 174% (+/- 14%), n=3; at 15 minutes: 143% (+/- 5%), n=3; for both p<0.01). These data show that NGF actions on CHC phosphorylation are rapid and robust and that they were correlated in time with clathrin redistribution.

NGF increases endocytosis and trafficking through clathrin-coated membranes. The NGF-induced increase in AP2 and clathrin at the plasma membrane suggested that NGF could signal to induce increased endocytosis through clathrin-coated membranes. To test this idea we examined NGF effects on two markers of endocytosis. FITC-dextran provides a marker of fluid-phase endocytosis. PC12 cells were incubated in the presence of FITC-dextran for 0 to 10 minutes at 37° C. After 5 minutes NGF treatment, uptake was increased by 20% over baseline; by 10 minutes the increase was nearly 60% (Figure

2.8A). These findings show that NGF increases uptake of solutes that enter the cell by bulk flow.

To test the idea that NGF influences trafficking through clathrin-coated membranes, we examined NGF actions on the uptake of Tfn. This ligand is internalized through clathrin-coated pits following binding to TfnR (Schmid, 1997). To measure the uptake of Tfn, we treated PC12 cells with radiolabelled Tfn in either the presence or absence of NGF. With NGF treatment there was a marked increase in Tfn endocytosis over vehicle-treated controls. The increase was marked by 5 minutes, measuring about 2-fold. The increase in uptake persisted through 15 minutes (Figure 2.8B). By 30 minutes, the level of Tfn uptake in NGF treated cells was the same as in vehicle-treated cells. The increase in endocytosis was not seen following NGF treatment of PC12 nnr5 cells or in PC12 cells pretreated with K252a, an inhibitor of Trk activation (Koizumi et al., 1988) (Figure 2.8C). These findings show that TrkA activation was required to induce endocytosis. That NGF-treated cells more rapidly reached the same plateau for Tfn endocytosis as vehicle-treated cells suggests that NGF acted to allow stimulated cells to more quickly reach an equilibrium with respect to Tfn trafficking. Whether the plateau for Tfn endocytosis reflects the presence of a limiting number of TfnRs or was due to an NGF effect on TfnR recycling is unknown. In either case, these data are evidence that NGF increases trafficking through clathrin-coated membranes.

Discussion

Endocytosis plays a critical role in cellular functions ranging from nutrient acquisition to synaptic transmission. It is important to elucidate the mechanisms that underlie endocytosis and how they are regulated (Marsh and McMahon, 1999). The current study shows that NT signaling increased the formation of clathrin-coated membranes. NGF acted through its receptor tyrosine kinase TrkA to increase clathrin on the surface membranes of PC12 cells. In concert, there was increased activity of the clathrin-coated pit pathway, as evidenced by enhanced endocytosis of Tfn. In that BDNF induced clathrin recruitment to the surface membranes of hippocampal neurons, our findings suggest that NTs may act normally to regulate clathrin-coated membrane formation and to increase clathrin-mediated membrane traffic.

A number of protein-protein and protein-lipid interactions underlie the assembly of the clathrin-based endocytic machine. In addition to clathrin [i.e. clathrin heavy chains (CHC) and light chain chains], AP-2 and, in neurons, AP180 (Schmid, 1997), the proteins include dynamin and amphiphysin (Damke et al., 1994; McMahon et al., 1997). Accessory cytosolic proteins include synaptojanin I, an inositol 5-phosphatase, and Eps15 (Benmerah et al., 1995; Tebar et al., 1996; Haffner et al., 1997; van Delft et al., 1997). In what appears to be a critical step for vesicle formation, dynamin binds and possibly activates endophilin I, a lysophosphatidic acid acyl transferase that catalyses the

conversion of lysophosphatidic acid to phosphatidic acid (Schmidt et al., 1999). How the interaction of these components is regulated is of considerable interest. It has been known for some time that polypeptide growth factor signals influence the formation of coated membranes (Connolly et al., 1981; Connolly et al., 1984). Greene and colleagues showed in PC12 cells treated with NGF that the number of clathrin-coated plasma membrane densities increased two to threefold within 30 seconds of NGF addition (Connolly et al., 1981). Similar results were seen in sympathetic neurons treated with NGF and in PC12 cells treated with EGF (Connolly et al., 1984). In related studies, insulin treatment of adipocytes caused a 3-fold increase in the amount of CHC associated with plasma membrane (Corvera, 1990). Recently, we showed that EGFR activation resulted in a dramatic redistribution of clathrin to the plasma membrane of A431 cells, as judged by confocal microscopy and by quantification of membrane-associated CHC (Wilde et al., 1999). The current study extends these observations by showing that NGF signaled through TrkA to robustly regulate clathrin coating of the plasma membrane. NGF treatment resulted in a prominent increase in membrane-associated CHC and AP2. This was revealed by immunostaining studies of these proteins and in biochemical studies in which we measured the amount of CHC in membrane fractions.

Our findings, and those for EGF and insulin (Connolly et al., 1984; Corvera, 1990; Wilde et al., 1999), suggest that a common mechanism may link activation of receptor tyrosine

kinases to induction of clathrin-coated membrane formation. However, beyond the requirement for TrkA kinase activation, the mechanism by which TrkA signaling induces increased clathrin-coated membranes is yet to be defined. Of note, several of the proteins that make up clathrin-coated membranes are subject to phosphorylation and dephosphorylation and it has been shown that such modifications contribute significantly to the regulation of endocytic function (Slepnev et al., 1998). As was seen with EGF (Wilde et al., 1999), NGF effects on clathrin redistribution were associated with changes in the phosphorylation of CHC. Whether, as is the case for EGFR, pp60src is required downstream of TrkA activation to increase clathrin-coated membrane formation and CHC phosphorylation is yet to be determined. Furthermore, it is uncertain as to whether the increase in clathrin at surface membranes was due to local changes in signaling (i.e. due to local recruitment of clathrin and AP-2 by activated TrkA receptors) or to signaling events not spatially contiguous with activated TrkA receptors. However, we have seen TrkA in complex with CHC and AP-2 in PC12 cells and that the amount of such complexes increases with NGF treatment (C. Howe, E. Beattie and W.Mobley, unpublished observations). This suggests that some membrane-associated clathrin is complexed with activated Trks

To demonstrate the physiological relevance of our findings, we asked whether or not BDNF would influence the distribution of clathrin in hippocampal neurons. The changes

induced by BDNF were identical to those seen for NGF, in that both AP-2 and clathrin were rapidly recruited to surface membranes. Surface membranes were prominently decorated with immunostained puncta. Remarkably, the changes seen with BDNF were registered on cell bodies and processes. These findings show that BDNF signaling induces widespread effects on clathrin-coated membrane formation and suggest that much of the surface of neurons is responsive to this aspect of BDNF actions. The similarity of the findings for BDNF and NGF suggests that each of the NTs will be shown to act through Trk receptors to increase the production of clathrin-coated membranes, a suggestion that is consistent with the existence of some signaling mechanisms shown to be common for the Trk receptors (Ip et al., 1993b; Kaplan and Miller, 1997; Yuen and Mobley, 1999).

Endocytic trafficking of cell surface receptors through clathrin-coated membranes follows from their concentration in clathrin-coated membranes on either a constitutive basis (e.g. the TfnR, the low density lipoprotein receptor [LDL-R]) or in response to ligand binding (e.g. EGF-R) (Schmid, 1997). Our findings for NGF and EGF suggest that regulated formation of clathrin-coated membranes also contributes to endocytosis. One consequence would be increased endocytosis of the receptors for NGF and EGF. Evidence that this is the case are data showing that when EGF signaling through pp60src was inhibited, there was inhibition of clathrin redistribution and a delay in EGF

endocytosis. In studies on PC12 cells, we found that NGF treatment increased endocytosis of TrkA and that TrkA at or near the surface of treated cells was colocalized with clathrin (Grimes et al. 1996). Additional recent findings also support the view that endocytosis of TrkA is via clathrin-coated membranes (C. Howe and W. Mobley, unpublished observations). Thus, it is likely that NGF acts to enhance the endocytosis of TrkA receptors through clathrin-coated membranes. We speculate that clathrin-coated vesicles may serve as a source of signaling endosomes.

We entertained the novel possibility that NGF signaling effects on clathrin-mediated membrane formation would result in a general increase in clathrin-mediated endocytosis, augmenting the uptake of markers unrelated to NGF or its receptors. This possibility was suggested, in part, by the finding that many of the clathrin-positive puncta appearing near the surface of NGF-treated cells did not stain for TrkA. Indeed, we discovered that even though NGF induced more than a 10-fold increase in the number of TrkA puncta that colocalized with CHC, TrkA was detected in only ~20% of CHC puncta (Grimes et al., 1996). In the current study we showed that NGF treatment increased the endocytosis of FITC-dextran and Tfn. The data for Tfn are especially important; they show that NGF acted through TrkA activation to cause increased clathrin-mediated membrane trafficking of a receptor unrelated to the NGF receptors and whose endocytosis is constitutive. The endocytosis of other receptors, including those whose internalization is normally induced

by ligand binding, may also be regulated by NGF. Indeed, in preliminary studies the endocytosis of EGFR has been shown to increase following NGF treatment (C. Howe, W. Mobley, unpublished observations). Our findings suggest that NGF may regulate the endocytosis of many receptors present in clathrin-coated membranes. If so, increased clathrin-coated membrane formation and trafficking may play an important role in mediating the trophic effects of NGF and other NTs. One such effect might involve increased delivery of receptors carrying nutrients, thus rapidly supplying neurons with substrates important for growth and differentiation. The enhanced uptake of Tfn shown here may supply iron needed for the function of iron-containing proteins under conditions of NGF stimulation. Through increased endocytosis of plasma membrane receptors for neurotransmitters and growth factors, NTs may impact a neuron's ability to respond to such influences. The interesting possibility arises that NTs could exert indirect but important influences on signaling through nonNT signaling pathways. Finally, it is tempting to speculate that NT signaling might enhance the uptake of synaptic vesicle proteins through clathrin-coated membranes, an action that could directly support neurotransmission (Berninger et al., 1999; Schinder et al., 2000). It will be important to explore further the significance for neuronal function of the NT-induced increase in clathrin-mediated membrane trafficking.

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Figure 2.1

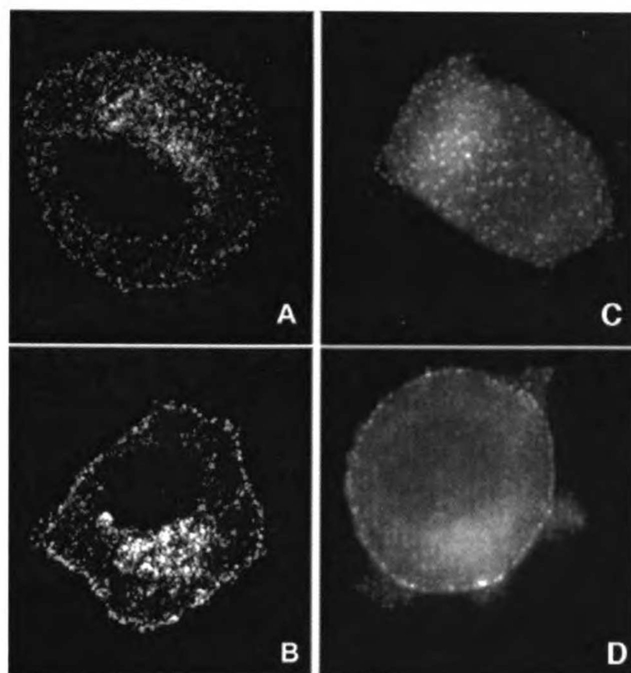


Figure 2.1. NGF Treatment Caused Clathrin and AP2 Redistribution in PC12 Cells.

Panels A and B: PC12 cells were cultured (i.e. primed) in the presence of NGF (2nM) for seven days. After washing three times with fresh serum-free medium without NGF, cells were chilled to 4°C and incubated for one hour in either the absence (A) (i.e. with vehicle alone) or presence (B) of NGF (2nM) in serum-free medium. Cells were then warmed at 37°C for 2 minutes, quickly chilled (4°C), fixed and processed for CHC immunostaining using X22. The panels show confocal micrographs. The width of each panel is 45um.
Panels C and D: The localization of the adaptor protein, AP2, was examined by epifluorescence microscopy. Unprimed PC12 cells were treated with vehicle (C) or NGF (2nM) (D) at 37°C for 2 minutes. They were then chilled, fixed and processed for immunostaining for AP2 with AP.6. NGF increased AP2 near the plasma membrane. The width of each panel is 45um.

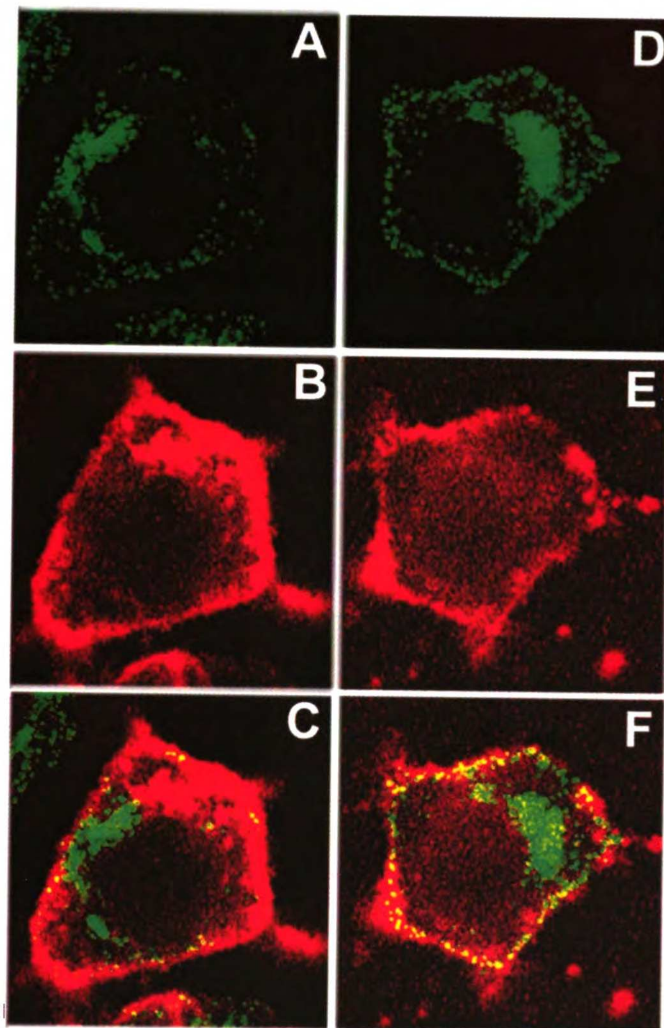

A
D
B
E
C
F

Figure 2.2. NGF Treatment Resulted In Increased Movement of Clathrin To The Plasma Membrane.

Confocal microscopy was used to show that CHC immunostaining colocalized with that for the membrane marker DiI. PC12 cells were chilled to 4°C and incubated with the vehicle (A, B, and C) or with NGF (2nM) (D, E, and F). Cells were then warmed to 37°C for 2 minutes, chilled, fixed and prepared for CHC immunostaining with X22 (A and D) and stained with DiI (B and E). The merged images for a control (C) and NGF-treated cell (F) show that CHC colocalized with DiI at the surface of both cells and that the extent of colocalization was much greater in the NGF-treated cell. The width of each panel is 55um.

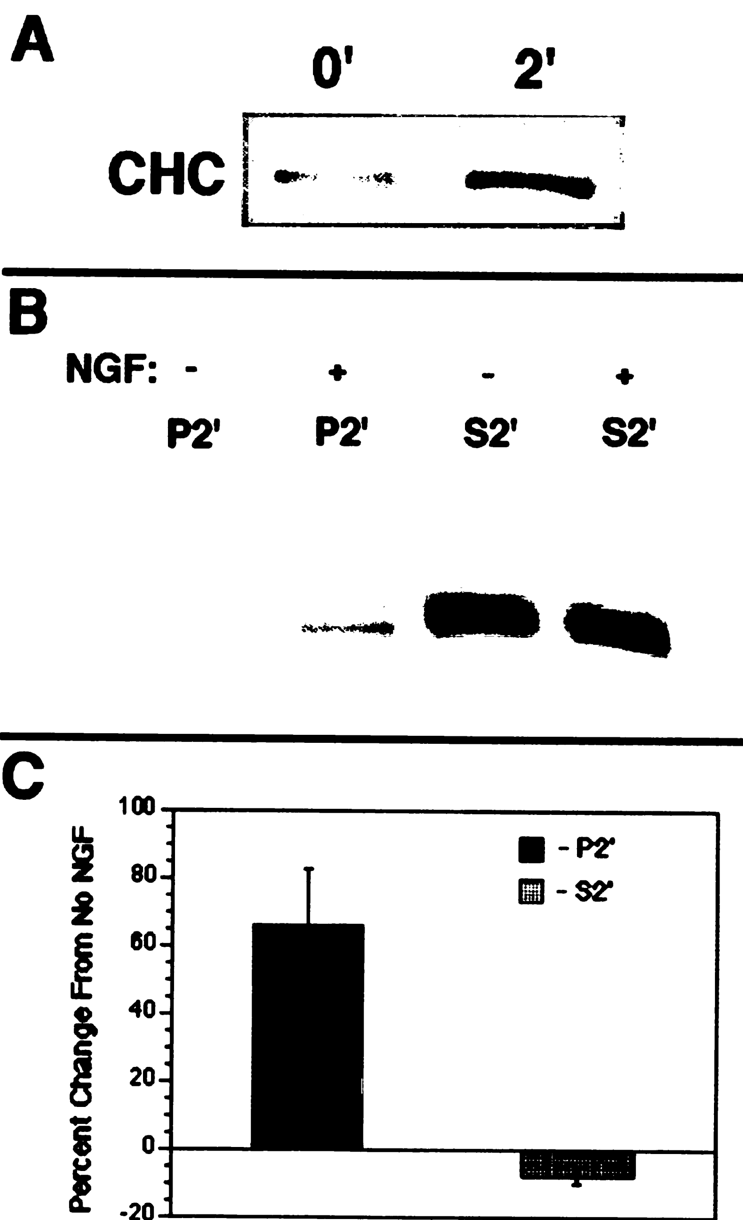

A
0'
2'
CHC
B
NGF: -
+
-
+
P2'
P2'
S2'
S2'
C
100
80
60
40
20
0
-20
Percent Change From No NGF
- P2'
- S2'

Figure 2.3. NGF Induced an Increase In Membrane-Associated Clathrin.

To quantify the amount of clathrin that was associated with membranes, we examined CHC in membrane and cytosolic fractions using two methods. (A): In the first method, equal numbers of PC12 cells were treated with either NGF (2nM) or with the vehicle for 2 minutes at 37°C and the ghost of gently disrupted cells was separated from the cytosol by pelleting at 8000 x g. CHC was immunoprecipitated with X22 and submitted to SDS-PAGE followed by transfer to nitrocellulose and immunoblotting with TD.1. NGF treatment caused a 158% increase (+/- 9 %, n =4; $p<0.01$) in membrane-associated clathrin. (B) In the second method, cells were more thoroughly disrupted by 3 cycles of free/thaw. Using samples normalized for protein from the P2' (membrane-associated) fraction or from the S2' (cytosolic) fraction, CHC was immunoprecipitated with X22, submitted to SDS-PAGE, transferred to nitrocellulose and immunoblotted with TD.1. NGF treatment caused a significant increase in CHC in P2' (166% of the vehicle-treated control +/- 18%, n=3, $p<0.05$). There was a concomitant small decrease in CHC in S2'(92% of the vehicle-treated control +/- 3.5 %, n=3, $p=0.06$). (C) The bands developed in (B) were quantified using NIH-Image and the data from 3 separate experiments are shown. Error bars represent S.E.M.

Figure 2.4

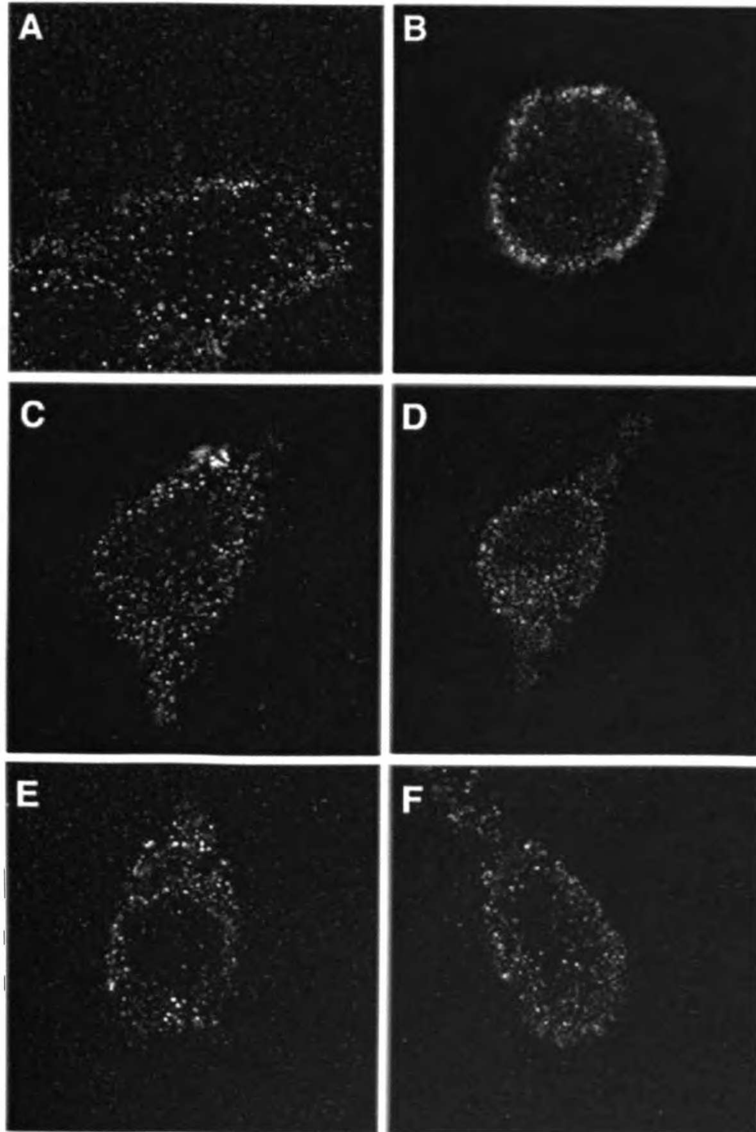


Figure 2.4. NGF Induced Redistribution of Clathrin to the Surface of 3T3 Cells Expressing TrkA, But Not $p75^{NTR}$.

NIH 3T3 fibroblasts were examined. Parental cells (i.e. cells without Trk or $p75^{NTR}$) are shown (panels E and F), as are cells transfected with $p75^{NTR}$ (C and D), or with TrkA (A and B). Cells were treated with NGF (2nM) (B, D, and F) or vehicle (A, C, and E) for 2 minutes at 37°C. They were then chilled at 4°C, fixed, and processed to show the distribution of clathrin by CHC immunostaining. The panels shown are confocal micrographs and their width is 65um. Only the TrkA-expressing cell line displayed an increase in clathrin at the plasma membrane (panel B).

Figure 2.5

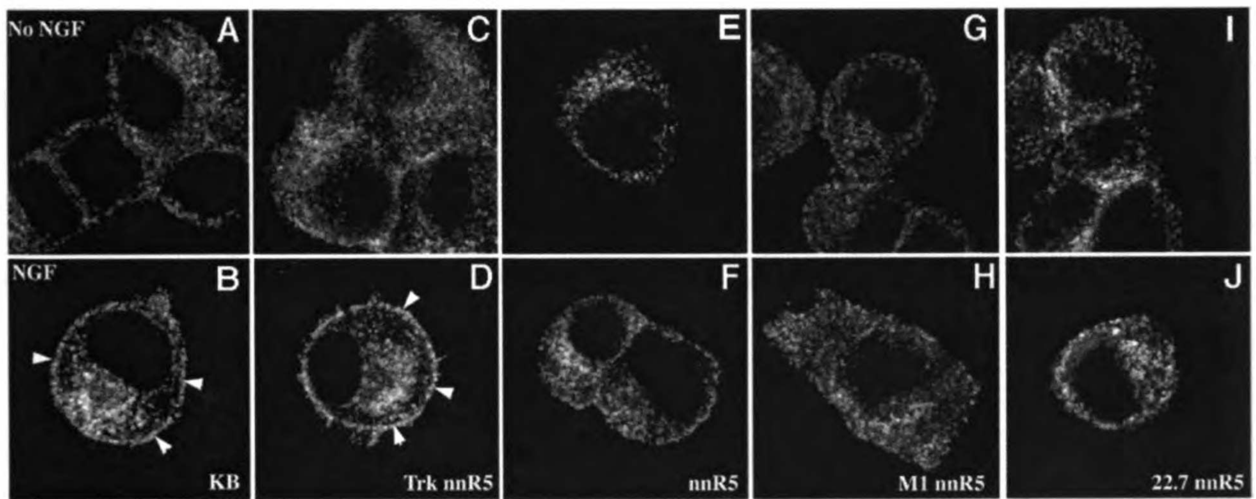


Figure 2.5. NGF Induced Clathrin Redistribution In PC12 Cells Expressing Wildtype TrkA.

PC12 cells, PC12 nnr5 cells and nnr5 variants were chilled (4°C) and then incubated with vehicle or NGF (2nM) for one hour before warming at 37°C for 2 minutes. Following treatment, cells were quickly chilled, fixed, and processed to determine the distribution of clathrin by immunostaining for CHC with X22. The cell lines examined were KB PC12 cells expressing endogenous wild type TrkA (KB) (A and B), nnr5 cells transfected with wildtype TrkA (TrkA nnr5) (C and D), nnr5 parental cells (nnr5) (E and F), nnr5 cells transfected with kinase-inactivated TrkA (M1 nnr5) (G and H), and nnr5 cells transfected with activation-loop mutated TrkA (22.7 nnr5) (I and J). Confocal microscopy was used to assess the distribution of clathrin in the vehicle-treated (top row) and NGF-treated (bottom row) conditions. Only cells with wild type TrkA (B and D) responded to NGF with an increase in clathrin near the plasma membrane (arrowheads). The width of each panel is 55um.

Figure 2.6

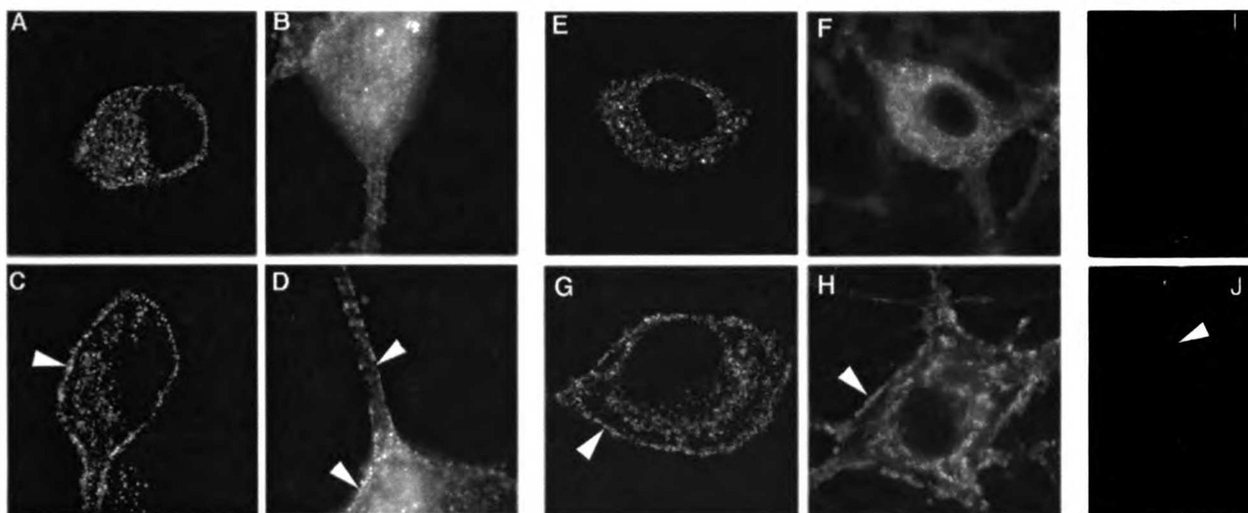


Figure 2.6. BDNF Induced An Increase In AP2 and Clathrin At the Surface of Hippocampal Neurons.

To show whether or not NT treatment induced an increase in AP2 and clathrin associated with surface membranes in primary neurons, BDNF was used to treat rat hippocampal neurons. The distribution of AP2 was assessed by confocal (A and C) and epifluorescence (B and D) microscopy of neurons immunostained with AP.6. Clathrin distribution was assessed using confocal (E and G) and epifluorescence (F, H, I, and J) microscopy of neurons immunostained for CHC with X22. BDNF (2 nM) (C, D, G, H, and J) or vehicle (A, B, E, F and I), were applied to cultured neurons for 2 minutes at 37°C prior to chilling, fixing and processing for immunostaining. BDNF increased staining for AP2 (C and D) and CHC (G and H) at the plasma membrane. Panels I and J show sections of neuronal processes and indicate that the BDNF effect was also registered here (J). The width of all panels is 55um.

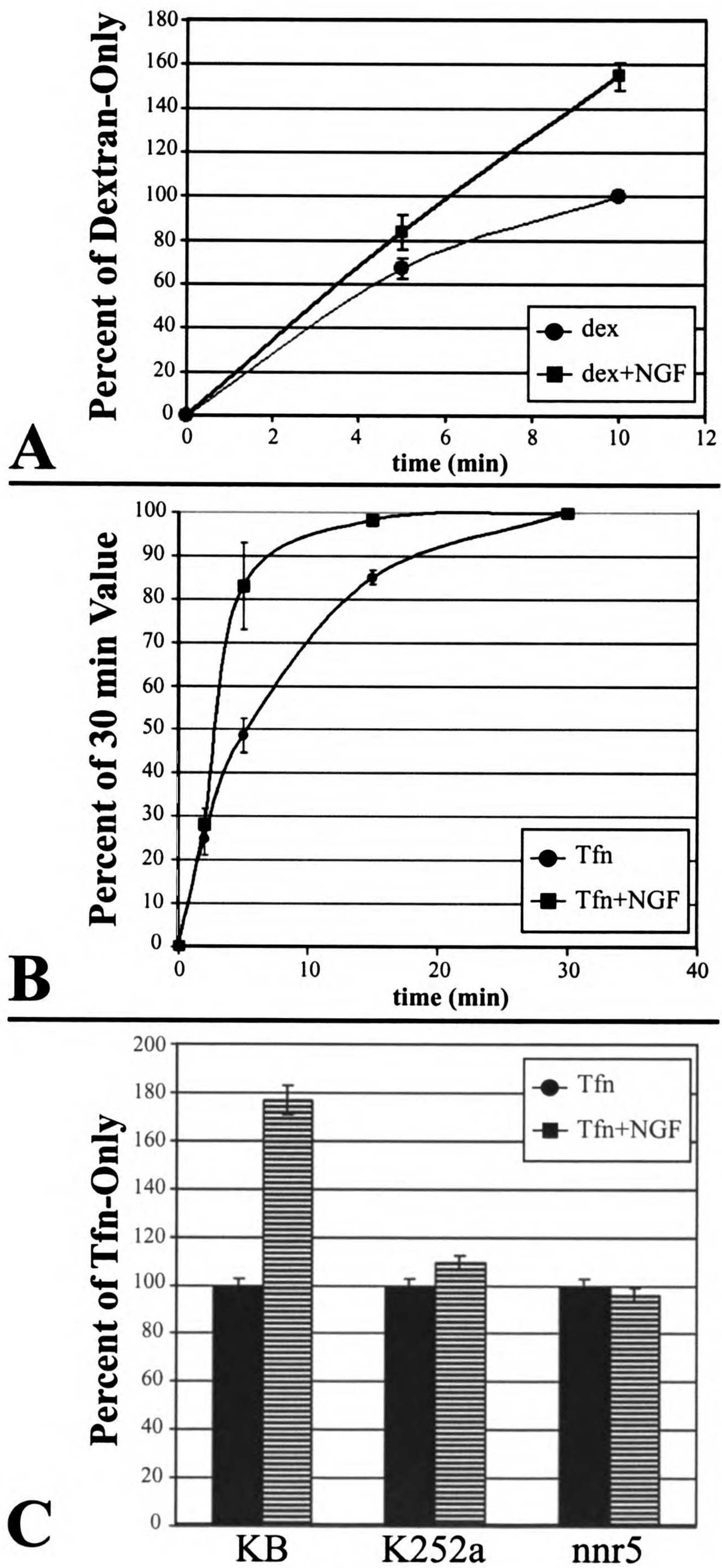
Figure 2.7

Figure 2.7. NGF Treatment Increased Phosphorylation of CHC.

PC12 cells were treated with NGF or vehicle for 2 min. at 37°C. Cells were then quickly chilled (4°C), and lysed in lysis buffer. Samples equalized for protein were immunoprecipitated with X22 and subjected to SDS-PAGE, transferred to nitrocellulose, and immunoblotted (IB) with the anti-phosphotyrosine antibody 4G10. The p-tyr panel shows that CHC phosphorylation was increased by NGF. The CHC panel confirms that equal amounts of CHC were present in the NGF and vehicle-treated samples.

Figure 2.8. NGF Enhanced The Uptake of FITC-dextran and of ^{125}I Tfn In PC12 Cells.

Panel A: PC12 cells were incubated with FITC-dextran for 0 to 10 minutes at 37° in the absence or presence of NGF (2nM). The amount of internalized FITC-dextran was determined by measuring the absorbance at 490nm of the lysates of washed cell pellets. The values are expressed as percent of the vehicle-treated samples warmed for 10 minutes. NGF increased the uptake of FITC-dextran by 20% (+/- 3%, n=3, p=0.02) at 5 minutes and by 60%(+/- 6%, n=3, p=0.01) at 10 minutes. The increase at 10 minutes resulted in a value that was 157% of the vehicle-treated control. The error bars represent SEM. Panel B: PC12 cells were incubated with125I-Tfn in the absence or presence of NGF (2nM) for 2, 5, 15, or 30 minutes at 37°. They were then chilled (4°C) and quickly pelleted prior to acid-stripping of surface-bound Tfn. Cell-associated counts represent internalized 125I-Tfn. The values are expressed as percent of the vehicle-treated samples at 30 minutes. NGF treatment increased uptake of 125I-Tfn by 35% (+/- 10%, n=3, p=0.001) at 5 minutes, to a value that was approximately twice the control. By 15 minutes the increase was 13% (+/- 1%, n=3, p=0.001). Error bars are SEM. (C) TrkA activation was required for the NGF effect on increased endocytosis of 125I-Tfn. Cells were incubated with 125I-Tfn in the absence or presence of NGF (2nM) for 5 minutes at 37°C. While NGF induced increased endocytosis of 125I-Tfn in KB PC12 cells (177%


A
Percent of Dextran-Only
time (min)
dex
dex+NGF
B
Percent of 30 min Value
time (min)
Tfn
Tfn+NGF
C
Percent of Tfn-Only
Tfn
Tfn+NGF
KB
K252a
nnr5

+/- 6% of the vehicle-treated, n=3, p= 0.001), it had no significant effect in KB PC12 cells pretreated with K252a (200 nM) (110% +/- 3%, n=3, p=0.08) or in PC12 nnr5 cells (96% +/- 3%, n=3, p=0.38).

Chapter 3

Clathrin Coated Vesicles are Signaling Endosomes Containing the Nerve Growth Factor Signal

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This chapter represents work submitted for publication.

Summary

The target-derived neurotrophic factor, nerve growth factor (NGF), signals through TrkA to promote the survival, differentiation, and maintenance of neurons. How the NGF signal in axon terminals is conveyed to the cell body is unknown. The 'signaling endosome hypothesis' envisions that NGF-TrkA complexes are internalized at axon terminals and retrogradely transported to the cell body. We found that NGF treatment induced the formation of clathrin coated vesicles (CCVs) in which NGF was bound to activated TrkA. Also, Shc, Ras, and activated Erk were recruited to CCVs in NGF treated cells, and these vesicles signaled in vitro to activate Elk, a downstream physiological target of Erk. Our results are evidence of NGF-induced signaling endosomes derived from clathrin coated membranes.

Introduction

That the targets of neuronal innervation play a vital role in regulating the survival and differentiation of innervating neurotrophin-responsive neurons has been appreciated for many years. Early studies documenting this phenomenon pointed to a direct correlation between the number of peripheral sensory and sympathetic neurons and the size of the targets they innervate (Shorey 1909; Detwiler 1920; Hamburger 1934; Hamburger 1939; Bueker 1948; Hamburger and Levi-Montalcini 1949). An important clue to the mechanism by which targets mediate neuronal function and survival came with the discovery of nerve growth factor (NGF) by Levi-Montalcini and Hamburger (1953). Subsequent investigators have demonstrated that NGF and other neurotrophic factors are produced and released in target tissues to activate receptors on the presynaptic elements of innervating neurons, thereby signaling to regulate the survival and differentiation of these neurons.

An unresolved issue is the mechanism by which such signals are communicated from axon terminals to neuron cell bodies. We and others (Ehlers et al. 1995; Grimes et al. 1996; Bhattacharya et al. 1997; Grimes et al. 1997; Riccio et al. 1997; Senger and Campenot 1999; Tsui-Pierchala and Ginty 1999; Watson et al. 1999; Zhang et al. 2000) have suggested that such signals are transmitted via endocytosis of complexes containing NGF bound to its activated receptor tyrosine kinase (RTK) TrkA, followed by retrograde

transport of the 'signaling endosomes' thus formed. In vitro and in vivo data support this hypothesis. TrkA endocytosis is enhanced by NGF, and endosomal fractions from NGF-treated cells contain NGF, activated TrkA, and activated PLC-γ and Shc (Grimes et al. 1996; Grimes et al. 1997). Significantly, NGF is still bound to TrkA in endosomes, raising the possibility that persistent binding of NGF to TrkA provides a source of continuous signaling (Grimes et al. 1996; Grimes et al. 1997). Additional support for the "signaling endosome hypothesis" has been provided by Campenot and colleagues (Senger and Campenot 1997), by Ginty and colleagues (Riccio et al. 1997; Tsui-Pierchala and Ginty 1999), and by Segal and colleagues (Bhattacharyya et al. 1997; Watson et al. 1999; Zhang et al. 2000). Using cultures in which sympathetic neuron cell bodies and their processes were compartmentalized, Senger and Campenot (1997) showed that NGF treatment of distal axons resulted in the accumulation in cell bodies of tyrosine phosphorylated TrkA and a number of additional tyrosine phosphorylated proteins. Furthermore, Riccio et al. (1997) showed that NGF treatment of axon terminals and distal neurites resulted in the activation of cell body loacalized CREB, a transcription factor important for transmitting the NGF signal to the nucleus. Consistent with the signaling endosome hypothesis, NGF internalization and persistent activation of TrkA were required for transmission of the signal to the cell bodies. Using the same cultures, it was recently shown that a complex containing NGF bound to TrkA was moved from distal axons to cell bodies, and that retrogradely transported TrkA was activated (Tsui-Pierchala

and Ginty 1999). Similar findings have been documented for compartmentalized cultures of DRG neurons (Watson et al. 1999). Recent work has also shown that activated TrkA within endosomes enhances neuronal differentiation in response to NGF treatment (Zhang et al. 2000). Finally, *in vivo* studies have shown that activated TrkA as well as Erk and several downstream effectors of the Erk cascade are retrogradely transported in sciatic nerve (Ehlers et al. 1995; Johanson et al. 1995), and that NGF regulates the retrograde axonal transport of activated ATF-2, a transcription factor activated by MAP kinase (Delcroix et al. 1999). Taken together, this evidence favors the existence of signaling endosomes. Importantly, however, no well-defined preparation of endosomes has been isolated and shown to propagate the neurotrophin signal(s).

One clue as to the source of signaling endosomes is that TrkA is colocalized with clathrin on the plasma membrane of NGF-treated PC12 cells (Grimes et al. 1997; Beattie et al. 2000). This suggested that clathrin-coated plasma membranes (CCPMs) may give rise to signaling endosomes containing the NGF-TrkA complex. CCPMs mediate the endocytosis of receptors that are either constitutively internalized, such as the transferrin receptor, or that are endocytosed in response to ligand binding, such as epidermal growth factor receptor (EGFR) and insulin receptor (Harding, Heuser, and Stahl 1983; Hanover, Willingham, and Pastan 1984; Sorkin and Waters 1993; Ceresa et al. 1998). Interestingly, while studies examining a role for coated membranes in receptor trafficking

are extensive, little attention has been paid to the possibility that clathrin-coated vesicles (CCVs) derived from CCPMs could be used to transmit an RTK signal. Stimulated by observations that link NGF signaling to CCPM formation and TrkA trafficking, (Grimes et al. 1996; Grimes et al. 1997; Beattie et al. 2000), we asked whether NGF signaling induced the formation of CCVs that carry the NGF signal. Herein, we show that NGF increased the association of clathrin with membranes and induced the formation of complexes containing activated TrkA, clathrin heavy chain (CHC), and the plasma membrane specific adaptor complex, AP2. Linking complex formation to endocytosis via CCPMs, CCVs isolated from NGF-treated cells contained NGF bound to activated TrkA. Shc, a critical component of the NGF signaling cascade was recruited to these membranes, as was Ras and activated ERK1/2. Importantly, we found that NGF-induced CCVs were competent in an in vitro kinase assay to convey the NGF signal from the ERKs to Elk, a downstream target of activated ERK. Our findings indicate that NGF does signal from endosomes. They suggest that CCVs are one source of signaling endosomes produced in response to NGF treatment.

Results

NGF Induced Recruitment of CHC to Membranes.

We recently showed that NGF signaled to increase the association of CHC with the plasma membrane of PC12 cells (Grimes et al. 1997; Beattie et al. 2000). To confirm this finding, we examined the cellular localization of CHC after loading PC12 cells with 2 nM NGF for 1 hour at 4°C, and then warming for 0, 2, or 15 minutes at 37°C. After treatment, cells were chilled and washed, and then fixed, permeabilized, and immunostained for CHC (X.22). By confocal microscopy, CHC staining in unwarmed PC12 cells was seen in small puncta distributed diffusely throughout cells, with most staining in the cytosol (Figure 3.1A). This is further demonstrated in Figure 3.1D-E, which shows the transmitted light image of an unwarmed cell (Figure 3.1D), the CHC immunostaining in the same cell (Figure 3.1E), and the digital colocalization (Figure 3.1F), indicating that most of the staining is concentrated in the cytoplasm. In contrast, following 2 minutes of warming there was a marked redistribution of punctate CHC staining to the plasma membrane (Figure 3.1B). Such a plasma membrane localization is confirmed in Figure 3.1G-I, showing the transmitted light image of a cell following 2 minutes of warming (Figure 3.1G), the CHC staining in the same cell (Figure 3.1H), and the colocalization overlay (Figure 3.1I), indicating a robust increase in CHC immunostaining at the periphery of the cell. By 15 minutes of warming, the CHC immunostaining was less punctate and was concentrated in the perinuclear region, with a

commensurate decrease in staining proximal to the plasma membrane (Figure 3.1C). Importantly, warming of cells did not itself induce a redistribution of CHC immunostaining (data not shown).

The change in CHC staining following NGF treatment suggested that there was an increase in membrane-associated CHC. To test this prediction we examined the amount of CHC in membranes of cells treated with NGF over several time intervals (Figure 3.2). Following treatment, cells were resuspended in 4°C MES buffer and homogenized by several passages through a Balch homogenizer, essentially as described in our previous work (Grimes et al. 1997; Beattie et al. 2000). Membranes were isolated using two different methods. In the first method, membranes corresponding to cell ghosts and to the heaviest membranes released from cells were isolated by an 8000g centrifugation (corresponding to P1+P2 from Grimes et al. 1996). In the second method, the suspensions of permeabilized cells were first centrifuged at 1000g to remove cell ghosts and unpermeabilized cells, and the resulting supernatant was then subjected to a 100000g centrifugation to isolate released vesicular membranes (corresponding to P2+P3 from Grimes et al. 1996). CHC in these membrane fractions was examined by SDS-PAGE and western blotting. NGF treatment increased the amount of membrane-associated CHC in both fractions, with the peak of association occurring at 2 minutes in the 8000g preparation (Figure 3.2A), and at 1 minute in the 100000g membrane fraction (Figure

3.2B). This result shows that more CHC was transiently associated with a variety of cellular membranes following NGF treatment. Furthermore, the redistribution of CHC was dependent upon TrkA tyrosine kinase activity, as membranes isolated by 8000g centrifugation from cells expressing either a kinase-defective TrkA (nnr5-M1, K538N mutation) or an extremely low level of TrkA (nnr5) do not show NGF-induced increases in membrane-associated CHC, while cells overexpressing TrkA (PC12 6.24) exhibited a robust redistribution (Figure 3.2C). In agreement with our previous work (Beattie et al. 2000), we conclude that NGF acts via TrkA to increase the association of clathrin with membranes, including the plasma membrane.

TrkA was Found in Complexes with AP2 and CHC Following NGF Treatment.

RTKs undergo endocytosis via recruitment of ligand-bound receptors to clathrin-coated pits at the plasma membrane. These events are influenced by structural changes that facilitate interaction with adaptor proteins, as well as by downstream signaling events. The recruitment of clathrin to the plasma membrane and intracellular membranes following NGF treatment, suggested that activated TrkA would be found in complex with CHC and AP2. To test this prediction, cells were treated with NGF over intervals ranging from 0 to 60 minutes and then lysed. Lysates were immunoprecipitated with an antibody to TrkA (06574) prior to SDS-PAGE and transfer. The blots were probed with antibodies to clathrin, AP2, and phosphotyrosine (p-tyr). As evidenced in the p-tyr blot,

NGF treatment induced a robust increase in tyrosine-phosphorylated TrkA (Figure 3.3A). Increased TrkA phosphorylation was associated with an increase in the amount of CHC and AP2 in the TrkA immunoprecipitates. The increases for both AP2 and CHC were maximal between 1 and 2 minutes. The levels of AP2 and CHC returned to baseline by 15 minutes. Interestingly, we also observed an increase in the association of tyrosine phosphorylated CHC with TrkA. The peak in association occured following 5 minutes of NGF treatment, after the peak in association of total CHC with TrkA, suggesting that CHC phosphorylation may be the result of complex formation, not a predicative factor.

To determine whether or not activated TrkA receptors were present in NGF-induced complexes, we immunoprecipitated phosphorylated TrkA from NGF-treated cells (Figure 3.3B). We found that, as in the total TrkA immunoprecipitates, CHC and AP2 were in complex with activated TrkA increased following NGF treatment, peaking at 2 minutes and rapidly returning to baseline. This result is consistent with published data showing by confocal microscopy that CHC redistribution to the plasma membrane did not occur in response to NGF in PC12 cell variants expressing kinase-defective TrkA or expressing extremely low levels of TrkA (Beattie et al. 2000), and with the biochemical analyses described above. We conclude that NGF signaling results not only in increased formation of CCPMs, but also in the formation of complexes containing activated TrkA and the molecular scaffolding that mediates receptor endocytosis.

NGF Signaling Increased the Amount of CCVs.

In earlier studies, we discovered that NGF increased general trafficking through the clathrin pathway (Beattie et al. 2000). These data, together with the increase in membrane-associated clathrin and the formation of complexes containing activated TrkA, AP2, and CHC, suggested that NGF might itself be internalized via the clathrin pathway. To test this prediction, we measured the internalization of ^{125}I-NGF in the presence of two inhibitors of clathrin-mediated endocytosis: chlorpromazine (Wang , Rothberg, and Anderson 1993; Subtil, Hemar, and Dautry-Varsat 1994) and monodansylcadaverine (Davies et al. 1980; Davies et al. 1984). As shown in Figure 3.5A, preincubation with either 100 uM chlorpromazine or 50 uM monodansylcadaverine significantly blunted or blocked the internalization of NGF under high affinity binding conditions. Importantly, these concentrations of chlorpromazine and monodansylcadaverine did not significantly alter TrkA phosphorylation in response to NGF (data not shown). Hence, we conclude that the internalization of NGF under conditions which primarily involve TrkA binding is mediated by the clathrin pathway.

To further address the role of clathrin-mediated endocytosis in the internalization of NGF, we asked if NGF signaled to increase the number of CCVs, the organelle derived from CCPMs. To answer this question, we isolated CCVs using a modification of an

established protocol (Maycox et al. 1992). Briefly, PC12 cells were chilled, washed, and resuspended in a MES-based CCV buffer at pH 6.5, and then submitted to permeabilization with a Balch homogenizer. The P2 plus P3 fraction was isolated using our previously described protocol (Grimes et al. 1996; Grimes et al. 1997), and submitted to a series of gradient fractionations to produce a pellet (P7) containing CCVs (Figure 3.4A). This fraction was examined by EM and found to contain electron-dense particles that were approximately 65 nm in diameter with the characteristic structure of CCVs (Figure 3.4B). Fragmented CCVs were also present. Biochemical analysis showed that the CCV fraction was highly enriched in CHC, AP2, and other characteristic markers of this organelle, such as internalized transferrin, the transferrin receptor, EGF, and EGFR (data not shown).

To examine NGF effects on CCV production, PC12 cells were loaded with NGF (2 nM) for 1 hour at 4°C, and then warmed for 0, 2, 5, or 15 minutes. The amount of total protein in the CCV fractions obtained from an identical number of cells was measured (Figure 3.5B). Following 2 minutes of warming there was a 164% increase in the amount of isolated CCVs (±6.1%; n=6; $p<0.000001$). After 5 minutes there was a 154% increase (±5.7%; n=7; $p<0.000001$), and by 15 minutes the number of CCVs had returned almost to the level found in untreated cells (114% ± 2.4%; n=6; $p<0.0001$). Importantly, warming alone did not contribute to the formation of CCVs (data not shown). The

temporal pattern for the increase in CCVs paralleled that for membrane association of CHC and for complex formation between TrkA, CHC, and AP2. We conclude that NGF acted to increase the number of CCVs.

If NGF signals from CCVs, our hypothesis predicts that NGF should be contained within these vesicles. To test this prediction, we isolated CCVs from PC12 cells treated with radiolabeled NGF and assayed the amount of internalization (Figure 3.5C). Briefly, PC12 cells were treated with ^{125}I-NGF (2 nM) for 1 hour at 4°C, and then warmed for 0, 2, 5, or 15 minutes. Following warming, cells were chilled and washed, and CCVs were isolated. The specific activity of internalized NGF was measured as cpm per ug CCVs. Figure 3.5C indicates that the specific activity of internalized NGF increased throughout the timecourse, with a 124% increase in internalized ^{125}I-NGF after 2 minutes of warming (±5.7%; n=3; $p<0.001$), a 128% increase after 5 minutes (±7.7%; n=3; $p<0.001$), and a 182% increase following 15 minutes of warming (±3.9%; n=3; $p<0.00001$). These findings indicate that NGF is internalized via CCVs, and that NGF is enriched in these organelles over time. Furthermore, chlorpromazine and monodansylcadaverine both inhibited the NGF-induced increase in CCV production (Figure 3.5D), and inhibited the internalization of ^{125}I-NGF into CCVs (Figure 3.5E), providing further evidence that NGF is internalized via a clathrin-mediated pathway.

Since activated TrkA is recruited to complexes containing CHC and AP2, and since NGF is internalized through CCVs, we predicted that NGF would be bound to TrkA in CCVs. CCVs were isolated from PC12 cells following 0, 2, and 5 minutes of NGF treatment, then lysed, immunoprecipitated for TrkA (06-574), and submitted to SDS-PAGE and transfer. Blots were probed with an anti-TrkA antibody (RTA). Figure 3.6A shows a clear increase in TrkA in CCVs following 2 and 5 minutes of NGF treatment. To determine whether NGF in CCVs was bound to TrkA, CCVs isolated from cells warmed in the presence of radiolabeled NGF were lysed, and the lysates were immunoprecipitated with an antibody against TrkA (G158A). Figure 3.6B shows that radiolabeled bands corresponding to the monomer weight of NGF were present in autoradiographs. Note that the presence of NGF was due to specific binding to TrkA since there was little or no signal when cells were treated with radiolabeled NGF in the presence of 500-fold excess cold NGF (Figure 3.6B 'cc'). In one experiment, following autoradiography, bands were excised from the gel and counted in a Beckman Gamma 4000 gamma counter. Compared to the unwarmed sample, the amount of ^{125}I-NGF was increased by 296% at 5 min, and 562% at 15 min. These data indicate that NGF is bound to TrkA in CCVs.

Since NGF is bound to TrkA in CCVs, these receptors should be activated. To test this, we loaded PC12 cells with NGF (2 nM) at 4°C for one hour and then warmed them for 0, 2 or 5 minutes. They were then chilled and permeabilized, and isolated CCVs were

lysed and immunoprecipitated with an anti-TrkA antibody (06574). Blots were then probed with an antibody to phosphotyrosine. As expected, there was essentially no tyrosine phosphorylated TrkA in the unwarmed sample. In contrast, increasing amounts of activated TrkA were present in the CCVs of cells warmed for either 2 or 5 minutes (Figure 3.6C), and the increases were proportional to the increases in total TrkA. We conclude that NGF is bound to activated TrkA in CCVs.

An NGF Signal is Contained in CCVs.

The signaling endosome hypothesis states that internalized activated receptors are capable of transmitting a signal. To assess the ability of CCVs to signal, we asked whether they were competent to signal via the MAP kinase pathway. Shc is an adaptor protein that participates in a multi-component complex to link TrkA to Ras and the MAP kinases, ERK1 and 2. CCVs isolated from NGF treated and untreated cells were lysed, and the constituent proteins were examined by SDS-PAGE and blotting. We found that after 2 and 5 minutes of NGF treatment there was a robust increase in the amount of Shc associated with CCVs (Figure 3.6D). Furthermore, we found that while some Ras was present in the CCVs of untreated cells, the amount was greatly increased after NGF treatment for 2 or 5 minutes (Figure 3.6E). These results suggested that CCVs could signal to activate ERK 1 and 2. Moreover, they raised the possibility that activated ERK1 and 2 might be associated with CCV membranes. To test this prediction we asked

if ERK1 and 2 were present in CCVs. We discovered that they were present in both treated and untreated cells (Figure 3.6F). The amount of these proteins was influenced very little, if at all, by NGF treatment. However, phosphorylated (that is, activated) ERK 1 and 2 were only present in the CCVs of NGF-treated cells (Figure 3.6G). The degree of phosphorylation of both ERKs increased between 2 and 5 minutes, a pattern that was highly reminiscent of that seen for Ras (Figure 3.6E).

Our hypothesis states that NGF signals from endosomes. The presence of activated Erk suggested that the basic machinery to propagate such a signal is associated with NGF-induced CCVs. To test the *in vitro* signaling ability of CCVs, we examined the capacity of isolated CCVs to phosphorylate Elk, a well-known substrate of ERK signaling. We used as substrate an Elk-GST fusion protein that includes the Elk domain normally phosphorylated by ERK 1 and 2 *in vivo*. CCVs isolated from treated or untreated PC12 cells were incubated in kinase buffer with Elk-GST in the presence of ATP. Following a 30 minute incubation at 30°C, the reaction was quenched with sample buffer and submitted to SDS-PAGE and tranfer. Blots were probed with an anti-phospho-Elk antibody (9181), and a band corresponding to the molecular weight of the fusion protein was identified (40 kDa). Consistent with our findings for the phosphorylation state of the ERKs, there was a marked increase in activity associated with the CCVs isolated from PC12 cells treated with NGF for 2 or 5 minutes (Figure 3.6H). We conclude that NGF

acts to increase the activity of ERK1 and 2 in CCVs, and that these CCVs are signaling endosomes.

Discussion

Neurotrophin-induced signals must be conveyed from the presynaptic terminal to the cell body to induce pleiotropic effects on survival and differentiation. In the studies reported herein, we addressed the possibility that clathrin-mediated pathways play an important role in facilitating the movement of the neurotrophin signal from the cell surface to internal membranes, thereby creating signaling endosomes that are capable of undergoing retrograde transport from the axon tip to the cell body. In concert with earlier studies we showed that NGF signaling through its receptor tyrosine kinase TrkA regulates the extent to which clathrin assembles on surface membranes and induces the formation of complexes containing TrkA together with AP2 and CHC. In a highly purified CCV fraction, we demonstrated that NGF signaling results in the formation of CCVs containing NGF bound to activated TrkA receptors. Significantly, the activated TrkA receptors present in CCVs were found together with activated ERK1 and 2. The NGF-TrkA complex in CCVs signaled in a cell-free assay to phosphorylate Elk, a downstream target of the ERKs. We conclude that NGF signaling induces the formation of CCVs that serve as signaling endosomes. We speculate that CCVs are one source of signaling endosomes for moving the NGF signal from axon terminals to neuron cell bodies.

Three mechanisms have been proposed to explain retrograde signaling (Hendry and Crouch 1993; Campenot 1994): 1) the target-derived neurotrophin is internalized and transported from the axon tip to the cell body, where it binds receptors localized in the cell body to initiate a signaling cascade; 2) the target-derived neurotrophin activates presynaptic neurotrophin receptors which initiate signaling cascades that reach the cell body in a wavelike fashion; 3) the target-derived neurotrophin binds to and activates presynaptic neurotrophin receptors, inducing internalization of ligand-receptor complexes into endosomes that are retrogradely trafficked to the cell body, and initiating local signal transduction cascades that mediate transcriptional events. Evidence from several sources supports the third model. First, activated Trk receptors have been found along the length of sciatic axons, suggesting that they must play a role in carrying the neurotrophin signal (Ehlers et al. 1995; Bhattacharyya et al. 1997). Second, a wave of second messengers cannot be the only mechanism for transmitting the neurotrophic signal, as the speed at which the signal arrives at the cell body and the concentration of signal generated at the axon terminal are inconsistent with such a mechanism (Bhattacharyya et al. 1997; but see Senger and Campenot 1997). Third, neurotrophin receptors, like other growth factor receptors, are internalized and downregulated from the cell surface following activation (Hosang and Shooter 1987; Kahle et al. 1994). Fourth, neurotrophin receptors are retrogradely transported (Johnson et al. 1987; Raivich, Hellweg, and Kreutzberger 1991;

Loy et al. 1994; Ehlers et al. 1995; Bhattacharyya et al. 1997; Tsui-Pierchala and Ginty 1999). Finally, internalized complexes containing NGF and activated TrkA are retrogradely transported in sympathetic neurons, and the arrival of such complexes in neuron cell bodies coincides with the phosphorylation of CREB, a modification required to drive transcriptional events necessary for the survival and maintenance of these neurons (Riccio et al. 1997; Watson et al. 1999). This evidence supports the "signaling endosome hypothesis," but proof requires the isolation of discrete, endocytic, membrane-bound organelles containing the neurotrophin signal.

The formation of CCVs from the plasma membrane is a complex process that involves the assembly of a number of protein components including the coat proteins clathrin and AP2. How the assembly of these components is regulated and how they are coordinated to support endocytosis is uncertain. The signaling endosome hypothesis suggests that the formation of endosomes is regulated by trophic factor signaling, and in recent studies, activation of EGFR, TrkA, or TrkB was shown to result in a dramatic redistribution of clathrin to the plasma membrane, as judged by confocal microscopy and by quantitation of membrane-associated CHC and AP2 (Wilde et al. 1999; Beattie et al. 2000). NGF signaling induced changes in CHC distribution through activation of TrkA, and the recruitment of AP2 and CHC to the plasma membrane in response to NGF suggested not only that TrkA signaling would increase TrkA endocytosis, but that activated TrkA

would be found in complex with these proteins. While AP2 and CHC were present in complexes with inactive TrkA prior to NGF treament, we found that brief NGF treatment significantly increased the amount of complexed AP2 and CHC. These data suggest either that activated receptors are trafficked to clathrin coated pits or that they are preferentially retained in these membranes.

The molecular details that specify how proteins like TrkA interact with CCPMs are poorly understood. One contributing factor is the receptor itself. Membrane proteins that are internalized from CCPMs are known to contain one or more sequences that specify their localization in such membranes. The exposure of these motifs in activated receptors contributes to their endocytosis. That AP2 was co-immunoprecipitated with TrkA suggests the existence of one or more endocytic codes within the TrkA cytoplasmic domain whose binding to AP2 is enhanced by kinase activation. Of the several sorting signals identified, the TrkA cytoplasmic domain contains five dileucine motifs (including two which fit the criteria for the D/ExxxLL AP2 recognition motif), three potential YxxΦ motifs, 12 lysines which may participate in ubiquitination, and an IxNPxY motif that may be a variant of FxNPxY (Marsh and McMahon 1999). The carboxyl terminus of EGFR also contains multiple endocytic codes. Of these, one containing Y974 was shown to mediate high-affinity binding of the receptor to AP2 (Sorkin et al. 1996). What residues consititute sorting signals for TrkA must be determined. As was demonstrated for EGFR

activation, downstream signaling events, including those regulated by Ras, may also influence the ability to localize TrkA in CCPMs. Given the roughly 2-fold increase in the amount of CHC associated with membranes, one might expect this effect alone to double TrkA endocytosis. In previous studies we have shown that the increase in TrkA internalization was about 2-fold at 5 minutes (Grimes et al. 1996), suggesting that increases in CCPM formation and changes in the binding of activated TrkA receptors to AP2 may both contribute to internalization of the receptor. Consistent with the recruitment of AP2 and CHC to the plasma membrane, we documented an increase in the formation of CCVs in NGF-treated cells. The increase was about 160% above baseline at two minutes. The magnitude of this change was the same as the increases in CHC recruitment measured by confocal microscopy and by biochemical measurements of clathrin recruitment to membranes (Beattie et al. 2000). These findings suggest that the CCPMs formed in response to TrkA activation are engaged in TrkA endocytosis.

Our findings suggested that NGF signaling would be carried into cells via CCVs. In a series of studies we showed that CCVs could serve as signaling platforms for NGF. First we showed that NGF was present in this fraction. Indeed, the specific activity of NGF in CCVs (i.e. the amount of NGF per ug protein) exceeded the specific activity of NGF bound to the surface of unwarmed PC12 cells (data not shown). The specific activity was increased at two minutes, relative to unwarmed cells, and continued to increase with

further warming; at 15 minutes the specific activity was increased further. Next we showed that NGF in CCVs was specifically bound to TrkA. We used TrkA immunoprecipitation to demonstrate this, a method that precluded any interference with TrkA trafficking that might arise from crosslinking NGF to its receptors. That NGF is bound to TrkA in CCVs is consistent with a persistent tight association between ligand and receptor. Third, we showed that TrkA was activated in CCVs isolated from NGF-treated cells, as revealed by the tyrosine phosphorylation of the receptor.

To test the signaling potential of CCVs we investigated downstream signaling proteins. Significantly, Shc and Ras were both found associated with CCVs isolated from NGF-treated cells, indicating that these signal mediators were recruited to activated TrkA that was internalized via the clathrin pathway. Furthermore, phosphorylated ERK 1 and 2 were found in the CCVs from NGF treated cells, and these kinases were active as judged by their ability to phosphorylate a substrate consisting of an Elk-GST fusion protein. Taken together, our findings are evidence that NGF acts upon the clathrin coated pit pathway to accomplish two effects: 1) an increase in CCPM-mediated trafficking of TrkA; 2) the creation of membranes that serve as a platform to deliver the NGF signal to the cell interior. Though we and others have previously provided evidence in support of signaling from internalized membranes, the findings reported here are the first to document the existence of endosomes that signal. Importantly, our findings suggest that

in addition to the classical model of the NGF signaling cascade, endosomes are created that act as coherent signaling platforms capable of moving the neurotrophic signal within the cell (Figure 3.7). The most compelling context for this model is in the retrograde transport of the neurotrophic signal from the axon terminal to the cell body. Unanswered questions include the nature of the endosome that actually carries the signal through the axon, how the endosome is moved through the axon, and what differential signals may be generated in the axon versus the cell body by such endosomes.

Experimental Procedures

Antibodies

TD.1, a mouse monoclonal antibody against CHC, was from the Berkeley Antibody Company (Richmond, CA). X22, a mouse monoclonal against CHC, was from Affinity Bioreagents (Golden, CO). 100/2, a mouse monoclonal against the α-subunit of AP2, was from Sigma (St. Louis, MO). 9141, a rabbit polyclonal antibody against TrkA specifically phosphorylated at tyrosine 490, 9181, a rabbit polyclonal antibody that recognizes only Elk-1 phosphorylated at serine 383, 9101, a rabbit polyclonal against Erk1/2 that is catalytically activated by specfic phosphorylation at threonine 202 and tyrosine 204, and 9102, a rabbit polyclonal generated against p42 and p44 Erk (Erk1/2), were from Cell Signaling Technology (Beverly, MA). 06574, a rabbit polyclonal against TrkA, 06372, a rabbit polyclonal against Ras, 06203, a rabbit polyclonal against Shc, and 4G10, a mouse monoclonal antibody against phospho-tyrosine, were from Upstate Biotechnology (Lake Placid, NY). G158A, a rabbit polyclonal antibody to TrkA, was from Promega (Madison, WI). RTA, a rabbit polyclonal antibody to TrkA, was the kind gift of Louis Reichardt. HRP-conjugated secondary antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA). FITC-conjugated donkey anti-mouse secondary was from Jackson Immunoresearch (West Grove, PA), as was the normal donkey serum used in blocking solutions. Agarose-conjugated goat anti-chicken IgY for immunoprecipitating G158A was from Promega (Madison, WI).

Chemicals and Other Reagents

Mouse NGF was prepared by ion-exchange chromatography as previously described (Mobley, Schenker, and Shooter 1976). Ultralink immobilized protein A/G plus, BCA reagents, ECL reagents, and Iodogen precoated iodination tubes were from Pierce (Rockford, IL). Growth factor reduced Matrigel matrix was from Becton Dickinson (Bedford, MA). Protran nitrocellulose transfer membrane was from Schleicher and Schuell (Keene, NH). X-OMAT x-ray film was from Eastman Kodak Company (Rochester, NY). ^{125}Iodine (IMS-30), PD-10 Sephadex G-25M columns, and ECL-sensitive film were from Amersham Pharmacia Biotech (Piscataway, NJ). The Elk-1 fusion protein, 9184, was from Cell Signaling Technology (Beverly, MA). Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS), penicillin-streptomycin, PBS, and calcium-magnesium-free PBS (CMF-PBS) were from Mediatech (Herndon, VA). Horse serum was from Tissue Culture Biologicals (Tulare, CA). Geneticin was from GIBCO BRL (Gaithersburg, MD). Nonidet P40 (NP40) was from Fluka (Switzerland). All other chemical reagents were from Sigma (St. Louis, MO).

Media and Buffers

Lysis buffer was composed of 20 mM Tris, 137 mM NaCl, 1% NP40, 0.5% deoxycholic acid (DOC), 10% glycerol, 1 mM PMSF, 10 ug/mL aprotinin, 1 ug/mL leupeptin, 500

uM sodium orthovanadate, pH 8.0. Sample buffer was 7 M urea, 125 mM Tris, 100 mM EDTA, 0.1% bromophenol blue, 2% SDS, pH 6.95. Blocking solution for western blots for all antibodies except 100/2 was 5% BSA in TBS-T (20 mM Tris, 137 mM NaCl, 0.2% Tween 20). For 100/2 incubations, blots were blocked in 5% milk in TBS-T. All antibodies except 100/2 were diluted in TBS (20 mM Tris, 137 mM NaCl). 100/2 was prepared in TBS plus 5% milk. Tris iodination buffer was 25 mM Tris, 400 mM NaCl, pH 8.0. Tris/BSA iodination buffer was 25 mM Tris, 400 mM NaCl, 0.25% BSA, 5 mM EDTA, 0.05% sodium azide. The tyrosine scavenging buffer for iodinations was 10 mg/mL tyrosine in PBS, pH 7.4. The acid strip for binding experiments was 0.2 M acetic acid and 0.5 M NaCl. CCV isolation buffer was 100 mM 2-[N-morpholino]ethanesulfonic acid (MES), 1 mM EGTA, 2 mM $MgCl_2$, 0.02% sodium azide, 1 mM beta-mercaptoethanol, 1 mM sodium orthovanadate, 1 mM PMSF, 10 ug/mL aprotinin, 1 ug/mL leupeptin, pH 6.5. CCV isolation D_2O sucrose pad was 100 mM MES, 8% ultrapure sucrose, 1 mM EGTA, 2 mM $MgCl_2$, 1 mM sodium azide, prepared in D_2O (Sigma-Aldrich #15188-2, 99.9% D). The MES-based buffer for membrane fractionation experiments was 25 mM MES, 150 mM NaCl, 10 mM EDTA, 1 mM sodium orthovanadate, 1 mM PMSF, 10 ug/mL aprotinin, 1 ug/mL leupeptin, pH 6.5. The kinase buffer for the Elk phosphorylation assay was 25 mM Tris, 5 mM β-glycerolphosphate, 2 mM DTT, 100 uM sodium orthovanadate, 10 mM $MgCl_2$, 200 uM ATP, pH 7.5). All cell treatments were performed in PGBH, composed of 1 mg/mL

glucose, 1 mg/mL BSA, 10 mM HEPES, in PBS, pH 7.4. Antifade mounting media for immunostaining experiments was 235 mM N-propyl gallate, 90% glycerol, in PBS, pH 7.2. Fixative for immunostaining experiments was 3.7% paraformaldehyde in PBS, pH 7.4. Permeabilization solution for immunostained samples was 0.04% saponin in PBS, pH 7.4, and blocking solution was 10% normal donkey serum, 4% fetal bovine serum, 0.1% Triton X-100, 0.02% SDS in PBS, pH 7.4.

Cell Culture and Cell Treatments

KB PC12 cells (gift of R. Kelly) and nnr5 PC12 cells (gift of L. Reichardt) were maintained at 37°C and 5% CO_2 in DMEM supplemented with 10% horse serum, 5% FBS, 100 U penicillin, and 100 ug/mL streptomycin. 6.24 PC12 cells and M1-nnr5 PC12 cells (both kindly provided by D. Kaplan), were maintained under identical conditions, with the addition of 200 ug/mL geneticin. All cells were grown on plastic, with the exception of PC12 cells used for immunostaining experiments, which were grown on Matrigel coated coverslips for 2 d prior to experimentation. Sterile coverslips were coated by overnight incubation in Matrigel diluted 1:200 in PBS, followed by 2 washes in PBS just prior to cell seeding. In preparation for all experiments, cell growth media was changed to DMEM supplemented with 1% horse serum 16-20 hours prior to experimentation. Immediately prior to every experiment except those involving immunostaining, cells were removed from their plates with 37°C CMF-PBS, resuspended

in 37°C PGBH, distributed into 1 mL aliquots, and rotated for 15 min to equilibrate the cells prior to experimentation. All treatments were performed in suspension, on equivalent numbers of cells, and incubated in a 37°C water bath, with periodic gentle inversion of the tubes to keep the cells suspended. For membrane association studies (3-5 x 10^7 cells per condition), for complex formation experiments (3-5 x 10^7 cells per condition), and for all western blot analyses of CCV fractions (15-50 x 10^7 cells per condition), 50 ng of NGF in 50 uL PGBH was added to each tube at 37°C for the appropriate time. For experiments involving pharmacological inhibitors, the appropriate concentration of the drug was added in a small volume following the equilibration step, and the cell suspensions were then rotated for an additional 15 min at 37°C. For all whole cell internalization (3-5 x 10^7 cells per condition) and CCV generation and internalization experiments (15-50 x 10^7 cells per condition), cell suspensions were chilled to 4°C by rotating in a cold room for 15 min following a 2 min incubation in an ice-water bath. After thoroughly chilling the cells, 50 ng of NGF in 50 uL of 4°C PGBH was added to each tube, and the cell suspensions were rotated at 4°C for a further 1 hr. At the conclusion of this cold-loading step, cell suspensions were warmed in a 37°C water bath for the appropriate time. Following warming, cell suspensions were rapidly chilled in a –5°C salt-ice water bath, then either resuspended in the appropriate cellular subfractionation buffer, or lysed.

Immunofluorescence and Microscopy

Cells grown on Matrigel-coated coverslips were placed in 4°C PGBH contained in 10 cm petri dishes 15 min prior to the start of experimentation. NGF was then added to the media at 50 ng/mL (2 nM), and the cells were incubated at 4°C for 1 hr with very gentle rocking. This cold-loading step was taken to maximize binding of NGF to its receptors without inducing membrane trafficking events. After cold-loading, petri dishes containing the coverslips were placed into a shallow bath of 37°C water for 2 min, 15 min, or were left unwarmed. Following warming, the petri dishes were placed into shallow baths of –5°C salt-ice water, and the media was immediately aspirated and replaced with 4°C PBS. Following this brief wash, cells were fixed for 10 min at RT, rinsed once in PBS, permeabilized for 10 min at RT, rinsed again in PBS, and blocked for 1 hr at RT, utilizing the solutions described above. Cells were then incubated overnight at 4°C in X22 at 6 ug/mL in block. Coverslips were rinsed 3x in PBS at RT, then incubated for 2 hr at RT in FITC-conjugated donkey anti-mouse secondary diluted 1:500 in block. Following 3 final rinses in PBS, coverslips were mounted with the anti-fade media described above on glass slides.

Cells were observed with a Bio-Rad (Hercules, CA) MicroRadience AG-2 laser scanning confocal microscope equipped with argon ion and green HeNe lasers, and attached to a Nikon Eclipse E800 microscope with a 60x Plan-Apo oil immersion objective that had a

numerical aperture of 1.4. Images were collected using an iris aperture of 1.5 mm, a zoom of 4, and a 1024x1024 collection box. After determining the top and bottom optical planes of the cell, an image was collected at the half-height of the cell using 3 passes with a Kalman filter and an average laser power of 30%. Gain and black settings were optimized on unwarmed cell samples using the set-col false coloring filter to get good contrast stretch. These gain and black levels were used for all subsequent image collections. Following collection of the FITC emission for any given cell, the transmitted light image was obtained using a laser power of 10% and optimized gain and black settings. Images were converted from the proprietary Bio-Rad pic format to tiff format using either Confocal Assistant (Bio-Rad, Hercules, CA) or NIH Image 1.62 (developed at the US National Institutes of Health and available on the internet at http://rsb.info.nih.gov/nih-image), and then further processed in Adobe Photoshop 5.5 (Adobe Systems, Mountain View, CA). Electron microscopy of the CCV fraction was performed using standard methods of glutaraldehyde fixation to formar-carbon Ni-grids, osmium tetroxide postfixation, and staining with tannic acid and uranyl acetate.

NGF Iodination

For iodinations, 5 ug of NGF in 100 uL tris iodination buffer was added to 1.0 mCi ^{125}Iodine that had been activated for 9 min at RT in 100 uL of tris iodination buffer in a Pierce Iodo-Gen tube. This mixture was reacted for 9 minutes at RT, then quenched for 5

min at RT by the addition of 50 uL of tyrosine quench solution. The volume of this mixture was then brought to 1 mL with tris/BSA iodination buffer. After removing 2 uL for quantification of total reaction cpm, the remaining 998 uL were loaded onto a PD-10 desalting column that had been pre-equilibrated with 20 mL of tris/BSA buffer. The column was then washed with tris/BSA solution, and five 1 mL fractions were collected, beginning with the first "hot" drop. The 2 uL removed from the reaction mixture, and 2 uL from each of the first 2 fractions, was diluted into 198 uL tris/BSA buffer, then 5 uL of each of these dilutions was further diluted into 445 uL of ddH_2O, while 5 uL of each of fraction 3-5 was diluted straight into 445 uL ddH_2O. To these final water dilutions was added 50 uL of TCA (prepared as 100% TCA with 1 mg/mL DOC), and this precipitation mixture was incubated on ice for 1 hr. These solutions were then centrifuged at 16000g for 10 min at 4°C, the supernatants were separated from the pellets, and the cpm for each was determined. After normalizing for the activity of the ^{125}Iodine and for the dilution factors, the specific activity of the radiolabeled NGF was determined. Typical iodinations produced NGF with a specific activity of 150-200 cpm/pg.

Cell Fractionation

Membrane preparation one: 8000g isolation.

Following treatment as described above, PC12 cells resuspended in 1 mL of 4°C MES buffer were permeabilized in a Balch homogenizer, essentially as described in our

previous work (Grimes et al. 1996; Grimes et al. 1997; Beattie et al. 2000). The suspension of permeabilized cells (cell ghosts), cellular contents, and unpermeabilized cells was centrifuged at 8000g for 35 min at 4°C, generating a pellet equivalent to fractions P1 plus P2 from our earlier work (Grimes et al. 1996). This pellet, enriched in the heaviest cellular membranes and organelles, was washed once with fresh MES buffer, recentrifuged at 8000g for 35 min, and then lysed and immunoprecipitated with X22.

Membrane preparation two: 100000g isolation.

PC12 cells were permeabilized as described above. The permeabilized suspension was centrifuged at 1000g for 10 min to remove the cell ghosts and unpermeabilized cells (fraction P1 from our previous work; Grimes et al. 1996) from the released organelles and cytoplasm. The resulting supernatant was diluted to 6 mL in MES buffer, then centrifuged at 100000g for 1 hr at 4°C, generating a pellet equivalent to fractions P2 plus P3 from our previous work (Grimes et al. 1996). This pellet, highly enriched in released vesicular structures ranging in average size from 63 nm to 180 nm (Grimes et al. 1996), was lysed, immunoprecipitated with X22, and subjected to SDS-PAGE.

Clathrin coated vesicle isolation.

The isolation scheme used to purify clathrin coated vesicles is diagrammed in Figure 4A, and is a modification of an established protocol (Maycox et al. 1992). Following

treatment as described above, cells were resuspended in 1 mL of CCV isolation buffer, triturated 2x with a 22-gauge needle to get a single cell suspension, then permeabilized by 5 complete passes through a Balch homogenizer. The resulting permeabilized cell suspension was then centrifuged at 1000g for 10 min at 4°C in a microfuge. The supernatant from this centrifugation, S1, was diluted to 2 mL with fresh CCV buffer, layered onto a 500 uL 5% glycerol pad made in CCV buffer, and centrifuged at 100000g for 1 hr at 4°C in a Sorvall AH-650 rotor and a Beckman XL-80 ultracentrifuge. The pellet, P2+P3 (equivalent to P2' from Beattie et al. 2000 and Grimes et al. 1997), was resuspended by gentle trituration into 250 uL of CCV buffer. This suspension was mixed with 250 uL of CCV buffer containing 12.5% (wt/vol) ficoll and 12.5% (wt/vol) sucrose, then centrifuged at 40000g for 40 min at 4°C in an ultracentrifuge. S4, the supernatant from this centrifugation, was diluted 1:5 in fresh CCV buffer, and then centrifuged at 100000g for 1 hr at 4°C in an ultracentrifuge. P5, the pellet from this round of centrifugation, was gently resuspended in 1 mL CCV buffer and centrifuged at 16000g for 20 min at 4°C in a microfuge. The supernatant from this spin, S6, was diluted to 2 mL with CCV buffer, layered onto a 500 uL 8% (wt/vol) sucrose pad prepared in CCV buffer that had been made using D_2O, and centrifuged at 112700g for 2 hr at 4°C in a Sorvall AH-650 rotor and a Beckman XL-80 ultracentrifuge. The pellet resulting from this centrifugation, P7, was highly enriched in markers of CCVs.

Elk Phosphorylation Assay

CCVs isolated as described above were resuspended in 25 uL of 4°C Elk phosphorylation assay kinase buffer. After all samples were resuspended, 25 uL of 4°C kinase buffer containing 2 ug of recombinant Elk-GST fusion protein were added to each, and the samples were warmed to 37°C for 30 min in a water bath. The kinase reaction was terminated by addition of 50 uL of sample buffer and boiling, and the entire 100 uL was subjected to SDS-PAGE.

Immunoprecipitation and Western Blotting

All samples were immunoprecipitated in 1 mL lysis buffer plus 100 uL of a 50% slurry of protein A/G-sepharose in lysis buffer, and the following amounts of antibody: 15 ug/mL 06574, 7.5 ug/mL G158A, 30 ug/mL X22, or 0.4 ug/mL 9141. Immunoprecipitations were performed overnight rotating at 4°C. The sepharose beads were then washed twice with lysis buffer, once with water, and then resuspended in 65 uL of sample buffer. Prior to loading 60 uL on 7.5% SDS polyacrylamide gels, samples were boiled for 5 min and then centrifuged for 1 min at 16000g. After transfer to nitrocellulose in a tris-glycine based transfer buffer with 20% methanol, blots were blocked for 1 hr at RT as described above. Blots were probed with the following antibody concentrations: 2.5 ug/mL TD.1, 0.25 ug/mL 4G10, 0.06 ug/mL 100/2, 0.4 ug/mL RTA, 1 ug/mL 06203, 1 ug/mL 06372, 0.05 ug/mL 9101, 0.01 ug/mL 9102, and 0.025 ug/mL 9181. HRP-conjugated

secondaries were used for all blots at 1:20000 dilution. For sequentially probing blots, the membranes were either stripped by incubating for 15 min at RT in 0.2 N NaOH, or by incubating for 30 min at RT in TBS pH 2.0, followed by extensive washing in water and blocking for 1 hr at RT.

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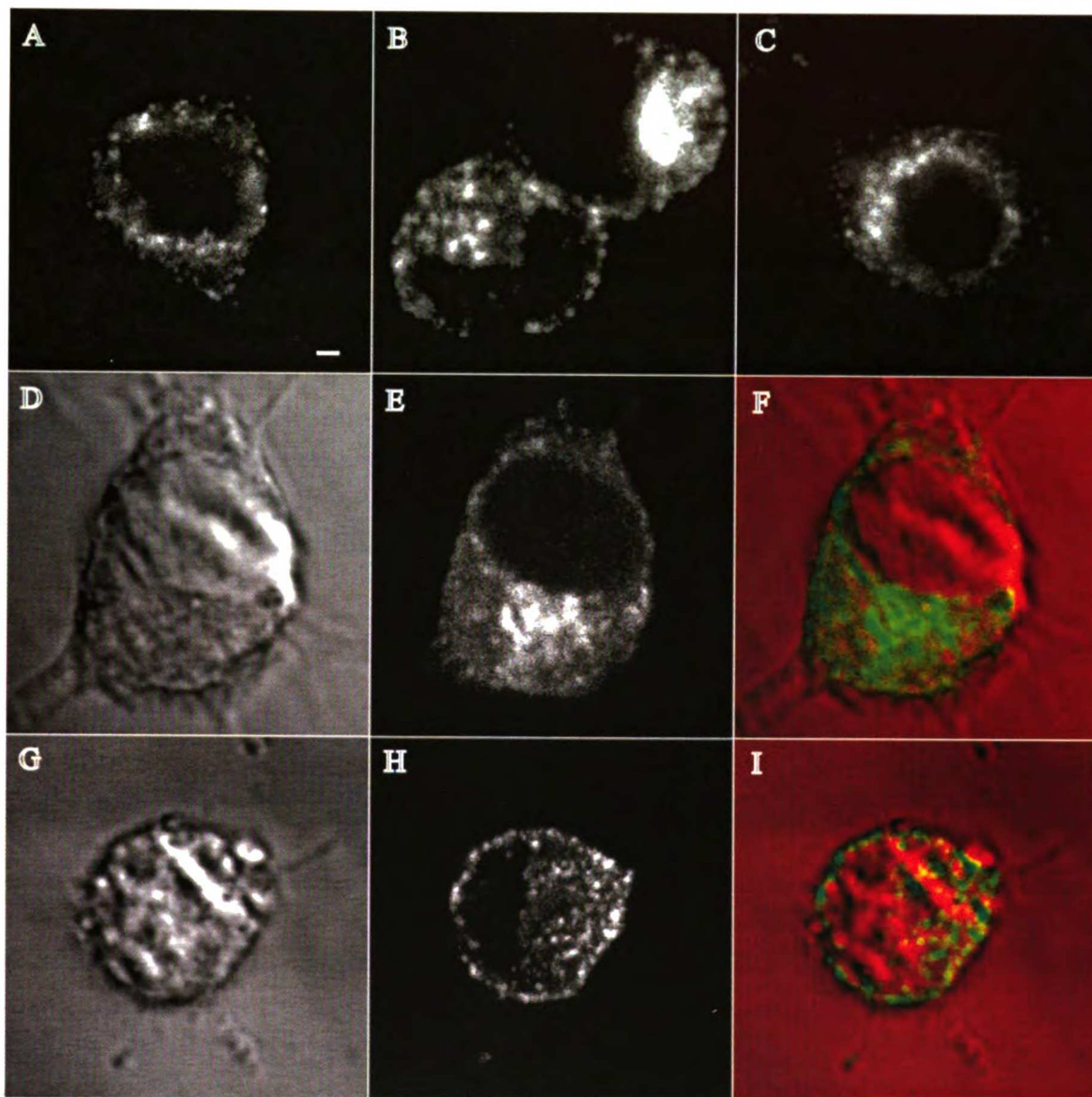
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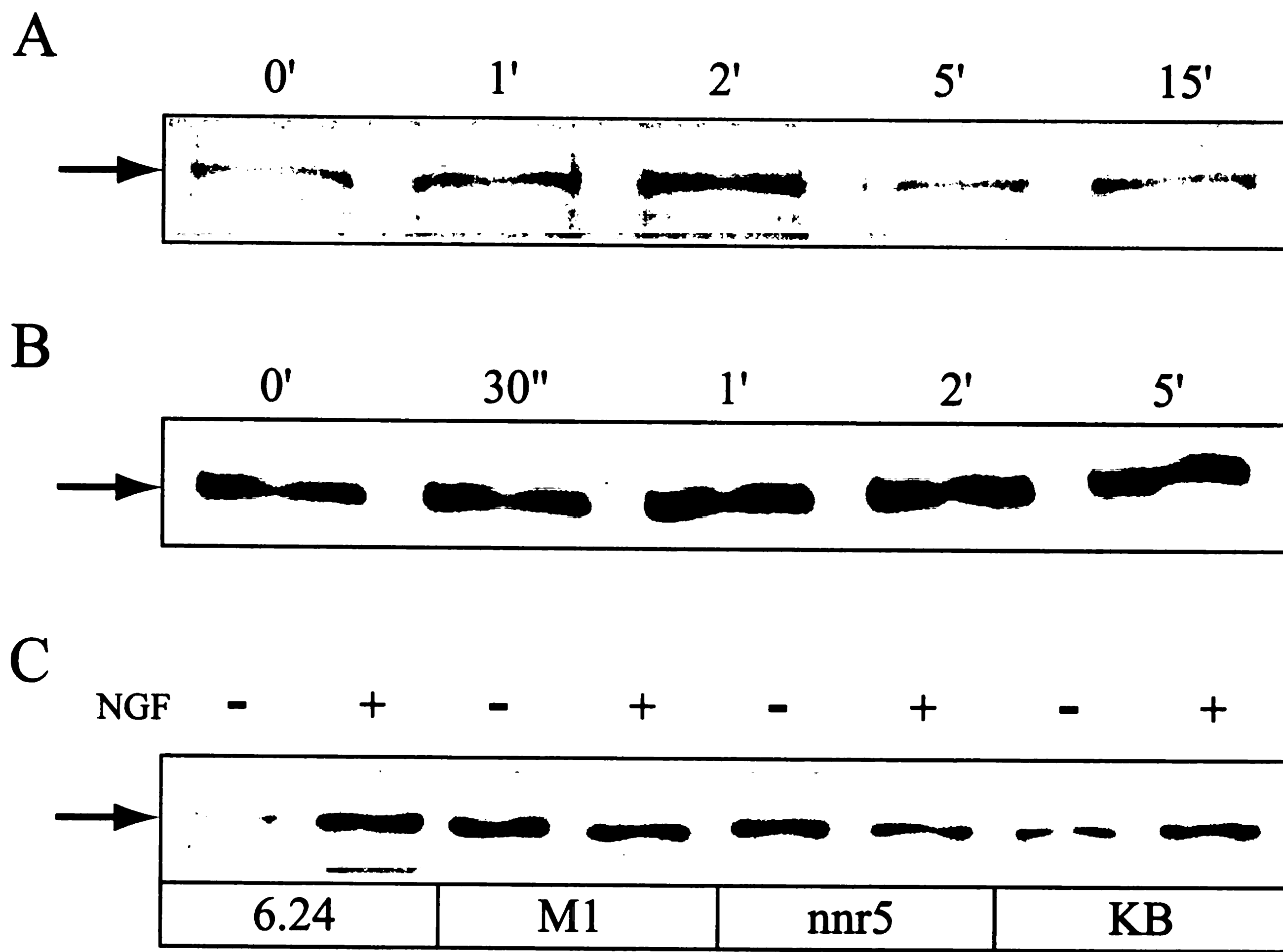
Figure 3.1. NGF Induces a Redistribution of CHC Immunostaining.

PC12 cells cold-loaded for 1 hr at 4°C with 2 nM NGF were warmed to 37°C in the continued presence of NGF for various times. Following warming, cells were chilled, rinsed, fixed, permeabilized, and stained for CHC using X22 and a FITC-conjugated donkey anti-mouse secondary. (A) Unwarmed cell shows diffuse CHC staining primarily localized to the cytosol. (B) Following 2 minutes of warming, the pattern of CHC staining is more punctate and concentrated at the cell periphery. (C) By 15 minutes of warming, the CHC immunostaining is more diffuse and exhibits a perinuclear localization. (D-F) Further evidence of the primarily cytosolic localization of CHC staining in unwarmed cells. Panel (D) shows the transmitted light image of an unwarmed cell, while panel (E) shows the CHC immunofluorescence elicited in the same cell. Panel (F) is the digital overlay of (D) and (E), showing that the CHC signal is largely restricted to the cytosol. (G-I) Evidence that 2 minutes of warming results in redistribution of the CHC staining to the cell periphery. Panel (G) shows the transmitted light image of a cell warmed for 2 minutes in the presence of NGF. Panel (H) is the CHC immunofluorescence pattern produced by the same cell, and panel (I) is the digital colocalization indicating the the punctate CHC signal is predominantly located at the periphery of the cell.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NGF
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+
-
+
-
+
6.24
M1
nnr5
KB

Figure 3.2. NGF, via TrkA, Induces a Redistribution of CHC to Membranes.

Western blot analyses of CHC redistribution in two different membrane preparations, and the effect of altered levels of TrkA or mutated TrkA on such redistribution. (A) Membranes isolated by an 8000g centrifugation of PC12 cell homogenates show an increase in CHC present in the fraction following NGF (2 nM) treatment, peaking at 2 min of NGF. (B) Membranes isolated by a 100000g centrifugation of a supernatant resulting from a 1000g spin of PC12 cell homogenates also show an increase in CHC following treatment with 2 nM NGF, peaking around 1 min of treatment. (C) The redistribution of CHC in the 8000g membrane fraction following 2 minutes of 2 nM NGF is dependent upon a functional kinase domain in TrkA and upon the level of TrkA present in the cell. 6.24 PC12 cells overexpress TrkA, and exhibit an increase in membrane-associated CHC that is even more robust than that seen in KB PC12 cells. In contrast, nnr5 PC12 cells, which express only 6% of the total TrkA found in KB PC12 cells, fail to exhibit an increase in membrane-associated CHC following NGF treatment. Finally, no increase in membrane-associated CHC occurs in nnr5 PC12 cells expressing a mutant TrkA (M1) that has a defective kinase domain.

Figure 3.2. NGF, via TrkA, Induces a Redistribution of CHC to Membranes.

Western blot analyses of CHC redistribution in two different membrane preparations, and the effect of altered levels of TrkA or mutated TrkA on such redistribution. (A) Membranes isolated by an 8000g centrifugation of PC12 cell homogenates show an increase in CHC present in the fraction following NGF (2 nM) treatment, peaking at 2 min of NGF. (B) Membranes isolated by a 100000g centrifugation of a supernatant resulting from a 1000g spin of PC12 cell homogenates also show an increase in CHC following treatment with 2 nM NGF, peaking around 1 min of treatment. (C) The redistribution of CHC in the 8000g membrane fraction following 2 minutes of 2 nM NGF is dependent upon a functional kinase domain in TrkA and upon the level of TrkA present in the cell. 6.24 PC12 cells overexpress TrkA, and exhibit an increase in membrane-associated CHC that is even more robust than that seen in KB PC12 cells. In contrast, nnr5 PC12 cells, which express only 6% of the total TrkA found in KB PC12 cells, fail to exhibit an increase in membrane-associated CHC following NGF treatment. Finally, no increase in membrane-associated CHC occurs in nnr5 PC12 cells expressing a mutant TrkA (M1) that has a defective kinase domain.

Figure 3.3. NGF Induces the Formation of Complexes Containing Activated TrkA, CHC, and AP2.

(A) Following treatment of KB PC12 cells with 2 nM NGF for the indicated times, cell lysates were immunoprecipitated with an anti-TrkA antibody, and the resulting western blot probed for the presence of CHC (upper panel), phospho-tyrosine (middle panel), and AP2 (lower panel). There is a distinct increase in the association of CHC and AP2 with TrkA that is correlated to the level of TrkA activation. (B) Evidence that CHC and AP2 are complexed with activated TrkA. Following treatment of KB PC12 cells with 2 nM NGF for the indicated times, cell lysates were immunoprecipitated with an antibody specifically directed against TrkA that is phosphorylated on Y490. Probing the resulting western blot for CHC, phospho-tyrosine, and AP2 indicated a robust increase in formation of complexes containing activated TrkA, CHC, and AP2.

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Figure 3.4. Isolation and EM Characterization of a Fraction Enriched in CCVs.

Panel (A) shows a schematic representation of the basic protocol used to isolate CCVs from PC12 cells. Following treatment, cells were chilled, rinsed, and resuspended in a MES-based CCV buffer, then "cracked" by repeated passage through a Balch homogenizer. Homogenates were then subjected to several rounds of centrifugation, as indicated, resulting in a fraction highly enriched in markers of CCVs. (B) EM analysis of the CCV fraction isolated as outlined above showing typical CCV profiles. Scale bar in upper micrograph of panel (B) is 65 nm. Scale bar at bottom of panel (B) is 65 nm and refers to the two higher magnification micrographs of individual CCVs.

Figure 3.5. CCV-mediated Internalization of NGF.

(A) Inhibitors of clathrin-mediated endocytosis block ^{125}I-NGF internalization. PC12 cells were treated in suspension with either 100 uM chlorpromazine or 50 uM monodansylcadaverine for 15 min at 37°C, then chilled to 4°C, and cold-loaded for 1 hr at 4°C with ^{125}I-NGF in the continued presence of the inhibitors. To isolate high-affinity binding sites, cells were washed 3 x 15 min by resuspension in 4°C PGBH containing inhibitors as appropriate. After the final resuspension in ice-cold PGBH plus inhibitors, cells were warmed for the indicated times, then chilled, stripped under acidic conditions, and lysed. Lysates were assayed for cpm and values were normalized for nonspecific internalization. Values shown are percent of normalized unwarmed internalization (n=3 for all conditions). (B) NGF induces the formation of CCVs. PC12 cells were cold-loaded with NGF at 4°C for 1 hr, then warmed in the continued presence of NGF for the indicated times. Following treatment, cells were chilled, cracked, and CCVs were isolated. This fraction was lysed and total protein was measured. Values are shown as percent of unwarmed. Following 2 min of warming, CCV production was elevated to 164% ± 6.1% (n=6; $p<0.000001$) of unwarmed. After 5 min, CCV production was 154% ± 5.7% (n=7; $p<0.000001$). And by 15 min, CCV production was 114% ± 2.4% (n=6; $p<0.0001$) of unwarmed. (C) NGF is specifically internalized into CCVs. PC12 cells were cold-loaded with ^{125}I-NGF at 4°C for 1 hr, then warmed in the presence of radiolabeled NGF for the indicated times. Following isolation of CCVs from these cells,

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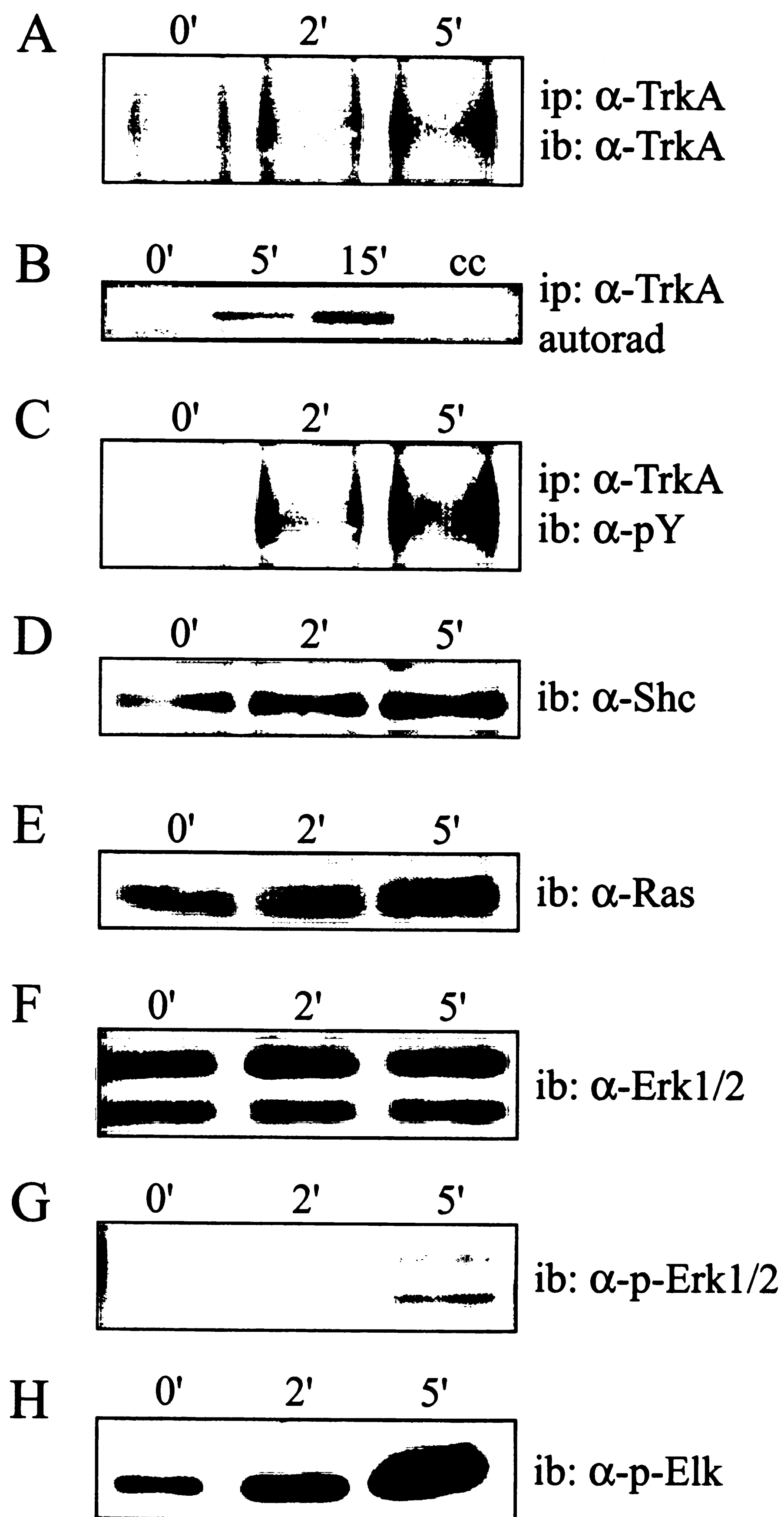
determination of both cpm and total protein in the fractions was made. Specific activity (SA) was calculated as cpm per ug total protein. Values shown are pecent of unwarmed specific activity. Following 2 min of warming, SA was increased to 124% ± 5.7% (n=2; $p<0.001$). By 5 min of warming, SA was increased to 128% ± 7.7% (n=3; $p<0.001$). And after 15 min of warming, SA was elevated to 182% ± 3.9% (n=3; $p<0.00001$) of unwarmed. (D) Inhibitors of clathrin-mediated endocytosis block NGF induction of CCV formation. PC12 cells were treated with either 100 uM chlorpromazine or 50 uM monodansylcadaverine for 15 min at 37°C, then chilled, and cold-loaded for 1 hr at 4°C with 2 nM NGF in the continued presence of the inhibitors. Cells were then either warmed for 5 min at 37°C, or left unwarmed. Following isolation of CCVs, total protein in the fraction was determined. Values shown are as percent of unwarmed (n=3 for all conditions). (E) Inhibitors of clathrin-mediated endocytosis block internalization of NGF into CCVs. Cells were treated as in (D), except ^{125}I-NGF was utilized. Following isolation of CCVs, internalized cpm were determined, and the percent increase in internalized ^{125}I-NGF following 5 min of warming was calculated. Values shown are percent of unwarmed (n=3 for all conditions).

Figure 3.6. NGF Induces the Formation of Signaling Endosomes.

(A) PC12 cells were treated at 37°C with 2 nM NGF for the times indicated, and CCVs were isolated and lysed. Immunoprecipitation of TrkA from these lysates shows the presence of increasing amounts of total TrkA in the CCV fraction induced by NGF. (B) PC12 cells were cold-loaded with 2 nM ^{125}I-NGF for 1 hr at 4°C, then warmed for the indicated times. Following warming, CCVs were isolated, lysed, and the lysates immunoprecipitated with an anti-TrkA antibody (G158A). Immunoprecipitates were subjected to SDS-PAGE, and the gel exposed to x-ray film for 15 days. The presence of a band corresponding to the monomer weight of NGF was found to increase with warming. The specificity of the interaction of ^{125}I-NGF with TrkA in these immunoprecipitates is indicated by the absence of signal in the lane corresponding to cold-competition with 500x unlabeled NGF (cc). (C-G) Same treatment conditions as (A). (C) TrkA immunoprecipitates immunoblotted with anti-phosphotyrosine indicate that TrkA is increasingly activated in the CCV fraction following NGF treatment. (D) Total protein from CCV fractions subjected to SDS-PAGE and immunoblotted with anti-Shc. Indicates that CCVs generated in response to NGF contain increasing amounts of Shc. (E) Total protein from CCV fraction immunoblotted with anti-Ras showing that NGF-induced CCVs contain increasing amounts of Ras. (F) Total protein from CCV fraction immunoblotted with an antibody against Erk1/2, showing no discernable increase in total Erk1/2 in the CCV fraction following NGF treatment. (G) However, the same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blot reprobed with anti-phospho-Erk1/2 shows a dramatic increase in the amount of activated Erk present in the CCVs generated in response to NGF. (H) NGF-induced CCVs are competent to transmit a signal in a cell-free assay. CCVs isolated from untreated cells and cells treated for 2 or 5 min with 2 nM NGF were incubated in kinase buffer with a Elk-GST fusion protein that can serve as a substrate for phosphorylation by activated Erk1/2. Following 2 and 5 min of NGF treatment, there was a robust increase in the ability of isolated CCVs to phosphorylate the Elk-GST substrate, indicating that CCVs are signaling endosomes.

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Figure 3.7. A Model of the "Signaling Endosome" Cascade.

The classical signaling cascade elicited by NGF binding to TrkA is depicted on the left side of the figure. In general, this model does not take into account the spatial organization of the signaling elements. Our data suggest that many of the signaling cascade elements are in fact packaged into a discrete signaling platform, or signaling endosome, which can be used to transmit the neurotrophic signal from the axon tip or the periphery of the cell to the nucleus.

Chapter 4

The Role of a Cbl:Clathrin Complex in TrkA Signaling and Endocytosis

Introduction

The Cbl Family and the "Cbl Homology" Domain.

Cbl (Casitas B-lineage lymphoma; pronounced "sibyl") was initially identified as the cellular homolog of the transforming gene of the Cas NS-1 murine retrovirus, isolated from a wild mouse found near Lake Casitas, CA (Hartley and Row 1976; Langdon and others 1989a). v-Cbl is formed as the result of a recombination between the Gag-encoding sequence of the Cas-Br-M virus and the cellular c-Cbl gene, and acts to acutely induce pro-B-cell lymphomas, T-cell lymphomas, erythroleukemias, and myeloid leukemias in mice (Frederickson and others 1984). Introduction of v-Cbl into fibroblasts results in transformation, consistent with its role as an oncogene (Langdon and others 1989a). v-Cbl encodes the amino-terminal 355 residues of the 906 residue full-length c-Cbl. Cbl is a 120 kDa cytoplasmic protein that is ubiquitously expressed, and is especially abundant in thymus, testis, and cells of haemopoietic lineage (Langdon and others 1989b). Sequence analysis of the Cbl protein predicts a number of adaptor regions, including several proline-rich SH3-interacting domains, a novel phosphotyrosine-binding domain (PTB domain), a 14-3-3 protein binding domain, a Ring-finger domain, a ubiquitin-binding domain, and 22 tyrosines that may participate in SH2 interactions (Fukazawa and others 1995; Liu and others 1997; Lupher and others 1996; Meisner and others 1995). Subsequent analysis of Cbl function has identified it is a gregarious adaptor protein that is found in complex with a host of proteins, ranging from the epidermal

growth factor receptor (EGFR), the T-cell receptor (TCR), and the insulin receptor, to Src, PI3 kinase, ZAP-70, and Vav (Lupher and others 1998).

When first cloned, Cbl showed no homology to any known mammalian protein, but showed greater than 93% homology between murine and human sequences (Blake and others 1991). Since that time, several Cbl family members have been identified, including Cbl-b and Cbl-3 (Keane and others 1999; Keane and others 1995). In addition, homologs of mammalian Cbl have been identified in *Caenorhabditis elegans* (Sli-1) and *Drosophila* (D-Cbl) (Hime and others 1997; Meisner and others 1997; Yoon and others 1995). The *C. elegans* Sli-1 gene encodes a protein of 582 amino acids that exhibits an overall homology of 43% to mammalian Cbl, and a 55% homology within the amino-terminal Ring-finger motif (Yoon and others 1995). D-Cbl is a protein of 448 residues, weighing 53 kDa. It shows 63% similarity within its amino terminus to mammalian Cbl and Sli-1, and exhibits 93% identity within a region around the Ring-finger domain (Hime and others 1997; Meisner and others 1997), suggesting that this region is critical to Cbl function. On the basis of the high degree of homology exhibited by the amino-termini of all Cbl family members identified to date, Bustelo and others (1997) have coined the term "Cbl homology" domain, or CH domain, to describe the region of Cbl that contains the PTB domain and the Ring-finger motif. In fact, while Cbl-b and Sli-1 retain several of the proline-rich SH3 binding domains found in Cbl, D-Cbl only contains

the CH domain, suggesting that this region is sufficient to confer the majority of Cbl function to the protein.

Analysis of the v-Cbl sequence indicates that the truncation occurs just upstream to the start of the Ring-finger motif. This finding suggests that the Ring-finger may serve the role of controlling Cbl function, and that in the absence of this component of the CH domain, Cbl exhibits transforming properties. Two other naturally occuring Cbl mutants that differ from v-Cbl have been used to further characterize the function of Cbl, especially as regards oncogenicity. The first mutant, HUT 78, exhibits a smaller carboxy-terminal truncation than v-Cbl, producing a protein of 655 amino acids that contains an intact Ring-finger. This protein is unable to induce transformation in fibroblasts (Blake and Langdon 1992), suggesting that a domain between residues 355 (end of v-Cbl) and 655 participates in controlling the activity of Cbl. The second mutant form of Cbl was isolated from a 70Z/3 pre-B lymphoma tumor. 70Z-Cbl exhibits a 17 residue deletion mutation that occurs just upstream of the Ring-finger motif (Andoniou and others 1994). Other than this mutation, which confers transformational capability to the mutant that exceeds that found in v-Cbl, the protein is intact and identical to Cbl. In fact, while v-Cbl is not detectably phosphorylated, 70Z-Cbl exhibits enhanced tyrosine phosphorylation relative to Cbl (Andoniou and others 1996). Hence, v-Cbl is generally considered to function in a dominant-negative manner by competing for Cbl binding sites and partners

without consequent activation, while 70Z-Cbl functions in a positive manner by interacting with Cbl partners and becoming hyperactive. That both of these mutant proteins are oncogenic suggests that both must lead to the same loss of function. It also suggests that an intact Ring-finger is critical to this function, as the only common feature shared by these two mutations is loss of Ring-finger integrity.

Cbl as an Element of Receptor Tyrosine Kinase Signaling.

Subsequent to its initial identification, Cbl was found to be equivalent to the 120 kDa major phosphorylated species in activated T cells (Donovan and others 1994). Following that discovery, Cbl was shown to be rapidly, prominently, and transiently tyrosine phosphorylated in response to activation of a large number of cell surface receptors. These include the TCR and B-cell receptor (Buday and others 1996; Cory and others 1995; Donovan and others 1994; Kim and others 1995; Panchamoorthy and others 1996), the CD19, CD38, CD16, and CD5 lymphocyte receptors (Cerboni and others 1998; Dennehy and others 1998; Kitanaka and others 1997; Kitanaka and others 1996; Kontani and others 1996), the FcεRI and FcγR Fc receptors (Marcilla and others 1995; Ota and others 1996; Tanaka and others 1995), the EGF, PDGF, FGF, NGF, CSF-1, c-Kit/Steel factor, and prolactin receptors (Bonita and others 1997; Bowtell and Langdon 1995; Galisteo and others 1995; Hunter and others 1997; Meisner and Czech 1995; Wang and others 1996; Wisniewski and others 1996), the GM-CSF, erythropoietin, thrombopoietin,

IL-2, IL-3, IL-4, and interferon-α receptors (Anderson and others 1997; Barber and others 1997; Brizzi and others 1996; Gesbert and others 1998; Odai and others 1995; Sattler and others 1997a; Uddin and others 1996; Ueno and others 1998), and the integrin receptors (Ojaniemi and others 1997; Sattler and others 1997b). Cbl is also phosphorylated downstream from insulin receptor activation, but apparently only within differentiated adipocytes (Ojaniemi and others 1997; Sattler and others 1997b). Finally, Cbl is constituitively phosphorylated in cells that have been transformed with activated Src, suggesting that this non-receptor tyrosine kinase may play a significant role in the phosphorylation of Cbl (Andoniou and others 1996).

Evidence that Cbl interacts with several members of the Src family of non-receptor tyrosine kinases suggests that these kinases may generally mediate the phosphorylation of Cbl downstream from a variety of RTKs. Cbl appears to be constituitively associated with Fyn and Lck in unstimulated T-cells, and Cbl can be isolated from cellular lysates in association with Fyn and Lck SH3-domain fusion proteins (Donovan and others 1994; Fukazawa and others 1995; Reedquist and others 1994; Tsygankov and others 1996). Similarly, in B-cells, Cbl was found in constituitive complexes with Lyn, Fyn, Blk, and Lck (Marcilla and others 1995; Panchamoorthy and others 1996; Tezuka and others 1996). The functional significance of these findings is suggested by experiments showing that overexpression of Fyn in T-cell hybridomas led to increased phosphorylation of Cbl

in response to TCR stimulation. In contrast, TCR stimulation in T-cells isolated from Fyn-deficient mice does not elicit Cbl phosphorylation (Tezuka and others 1996). Furthermore, overexpression of Lyn in mast cells enhanced Fcε receptor-induced phosphorylation of Cbl (Ota and others 1996), while BCR stimulation in Lyn-deficient chicken B-cells was unable to induce Cbl phosphorylation (Tezuka and others 1996). Hence, Src family kinases appear to mediate Cbl phosphorylation downstream from many receptors, and may do so by holding Cbl in a constituitive complex that permits rapid phosphorylation following activation of the kinase.

Cbl also associates directly with RTKs, including EGFR, PDGFR, and the CSF-1 receptor (Bonita and others 1997; Bowtell and Langdon 1995; Fukazawa and others 1996; Galisteo and others 1995; Meisner and others 1995; Tanaka and others 1995; Thien and Langdon 1997a). Cbl utilizes two mechanisms for binding to these receptors. In the first, a proline-rich region of Cbl interacts with the SH3 domain of Grb2, which then adapts Cbl to the RTK (Meisner and others 1995; Thien and Langdon 1997a). The second mechanism utilizes direct contact of Cbl with the RTK mediated via the PTB domain within the amino-terminus of Cbl (Thien and Langdon 1997a). Both of these mechanisms appear to be utilized under different cellular contexts. Interestingly, while EGFR tyrosine kinase activity is required for EGF-induced Cbl phosphorylation, association of Cbl with EGFR is not required (Galisteo and others 1995; Thien and Langdon 1997a). This finding

strengthens the argument that a non-receptor tyrosine kinase such as Src is involved in translating EGFR kinase activity into Cbl phosphorylation.

A number of potential phosphorylation sites exist within Cbl. There are 22 tyrosines within the entire protein, 13 of which are located in the CH domain. Moreover, 8 of these amino-terminal tyrosines are absolutely conserved in all Cbl homologs, suggesting that they are critical to Cbl function. In addition, 3 tyrosines are conserved within the carboxy-terminal region of Cbl and Cbl-b, and these tyrosines are phosphorylated downstream from Abl, EGFR, and FcεR activation (Andoniou and others 1996). It is interesting to note that tyrosine 307, which is conserved in all Cbl homologs, is directly adjacent to glycine 306, the site of a G306E mutation that renders Cbl non-functional (Thien and Langdon 1997a).

Cbl is induced to interact with several SH2 domain-containing proteins following phosphorylation. One of the primary partners is the p85 regulatory subunit of PI3 kinase, and following TCR, BCR, and EGFR activation there is an increase in Cbl-associated PI3 kinase activity (Donovan and others 1996; Fukazawa and others 1995; Hartley and others 1995; Kim and others 1995; Meisner and others 1995; Odai and others 1995; Ojaniemi and others 1997; Panchamoorthy and others 1996; Reedquist and others 1996; Soltoff and Cantley 1996). The consensus site within Cbl for binding of PI3 kinase is a YEAM

sequence that begins with tyrosine 731 (Liu and others 1997b). Interestingly, Cbl appears to be the major phospho-protein associated with PI3 kinase in T-cells, and this complex is particularly enriched in the membrane fraction of homogenized T-cells. In fact, membrane-associated Cbl was shown to contain 20 times more PI3 kinase activity than cytosolic Cbl, suggesting that Cbl may act to recruit PI3 kinase to the plasma membrane following TCR activation (Hartley and Corvera 1996).

Another group of phospho-Cbl binding partners are the Crk family members. Crk is composed of one SH2 domain and one SH3 domain, while Crk-II and Crk-L each have one SH2 and two SH3 domains (ten Hoeve and others 1993). The SH2 domain within these proteins mediates an inducible interaction with Cbl that occurs following activation of the TCR, BCR, and cytokine receptors (Barber and others 1997; Buday and others 1996; Reedquist and others 1996; Sawasdikosol and others 1996; Smit and others 1996a; Smit and others 1996b). The implications of such interactions are unclear, but they may play a role in coupling specific receptors to activation of the Rap1 pathway. This is supported by the finding that increased Fyn activity leads to the formation of a (phospho-Cbl)-CrkL-C3G complex that is associated with heightened activity of Rap1 (Boussiotis and others 1997). It is important to note that following TCR stimulation, essentially all of the phosphorylated Cbl within the cell is co-immunoprecipitated with CrkL (Reedquist

and others 1996; Sawasdikosol and others 1996), suggesting that this interaction is crucial to TCR signaling.

Cbl as a Negative Regulator of Receptor Tyrosine Kinase Activity.

The first evidence that Cbl might play a role in negative regulation of receptor tyrosine kinase (RTK) signaling came from genetic screening studies in *C. elegans*. Analysis of the Sli-1 gene showed that deletion or mutation of Sli-1 restored signaling through a weakly active mutant Let-23, the *C. elegans* EGFR homolog (Jongeward and others 1995; Yoon and others 1995). Moreover, overexpression of Sli-1 suppressed normal Let-23 signaling (Jongeward and others 1995), suggesting that Sli-1 functions as a negative regulator of Let-23 signaling. Interestingly, mutation of Sli-1 within the context of normal Let-23 did not exert any effect on Let-23 signaling within the pathway for vulval induction. However, in combination with a mutation in Unc-101, the *C. elegans* homolog of the medium chain (μ) clathrin adaptin protein, mutant Sli-1 abrogated Let-23-mediated vulval induction (Jongeward and others 1995; Lee and others 1994). As Unc-101 is also defined as a negative regulator of Let-23 function on the basis of restoration of mutant Let-23 signaling, this finding suggests that Cbl and the clathrin pathway may intersect in the negative regulation of RTK signaling.

Further evidence for the role of Cbl as a negative regulator of RTK signaling comes from studies showing that overexpression of D-Cbl in *Drosophila* compromises the development of the R7 photoreceptor neuron, apparently by inhibiting EGFR signaling (Meisner and others 1997). Other investigators have shown that overexpression of Cbl in mast cells suppresses FcεRI-induced Syk kinase activity and blocks histamine and serotonin release (Ota and Samelson 1997). Likewise, overexpression of Cbl in T-cells decreases Ras-dependent activation of Erk and AP-1 downstream from TCR ligation (Rellahan and others 1997). Cbl is further implicated in the Ras pathway by studies showing that 70Z-Cbl induces transcriptional activation of NFAT, the nuclear factor of activated T-cells, and that this activation is blocked by expression of dominant-negative Ras (Liu and others 1997a). Finally, 70Z-Cbl and v-Cbl induce hyperphosphorylation of the PDGF and EGF receptors, and induce hyperactivation of signaling downstream from these receptors (Bonita and others 1997; Thien and Langdon 1997b), suggesting that Cbl loss-of-function and oncogenicity correlate with increased RTK signaling.

The phenotype of Cbl-null ($Cbl^{-/-}$) mutant mice also supports a negative regulatory role for this adaptor protein. These mice are viable and fertile, but exhibit increased mammary growth, and increased levels of TCR expression and signaling. Moreover, $Cbl^{-/-}$ mice exhibit lymphoid hyperplasia (in which the lymph nodes are enlarged and filled with increased numbers of T- and B-cells) and primary splenic medullary hematopoiesis

(marked by enlargement of the spleen and the presence of large numbers of megakaryocytes and normoblasts within the parenchyma of the spleen), consistent with Cbl-mediated negative regulation of hematopoietic proliferation (Murphy and others 1998). In particular, the increase in megakaryocytes within the spleen likely represents failure or dysregulation of an autocrine loop controlling megakaryocyte differentiation. Cbl is a prominently phosphorylated target in thrombopoietin-stimulated cells, and is implicated in the sustained activation of Erk that prompts myeloid cells to secrete a megakaryocyte differentiation factor (Racke and others 1997; Sasaki and others 1995). Likewise, the mammary hyperplasia that occurs in $Cbl^{-/-}$ mice may be explained by enhanced EGF signaling in the control of mammary branching and development (Coleman and others 1988; Curtis and others 1996; Fowler and others 1995; Snedeker and others 1991; Vonderhaar 1987). Finally, enhanced TCR signaling within the $Cbl^{-/-}$ mice may explain the proliferation of thymocytes occurring in these animals. While development of a functional T-cell repertoire occurs normally in these mice, surface expression of CD4, CD8, and the TCR is dramatically increased on thymocytes. Also, there is dramatic hyperphosphorylation of many intracellular proteins following surface receptor crosslinking, and $Cbl^{-/-}$ thymocytes respond to TCR-mediated signaling in the absence of normally obligate coreceptor aggregation, suggesting that the increased expression of TCR on the surface of these cells leads to hyper-responsiveness (Murphy and others 1998).

Additional cellular evidence for a negative regulatory function of Cbl comes from studies showing that antisense repression of Cbl expression in osteoclasts leads to inhibition of bone resorption (Tanaka and others 1996). Interestingly, antisense repression of Src expression elicits an identical phenotype, and in osteoclasts derived from Src-deficient mice Cbl phosphorylation is greatly reduced and bone resorption and remodelling is compromised, leading to an osteopetrotic phenotype (Soriano and others 1991). Hence, Cbl appears to function downstream of Src in controlling bone resorption by osteoclasts, a process that requires active endocytosis and vesicular transport of the degradation products (Blair 1998).

The mechanism by which Cbl acts to negatively regulate RTK and immune receptor signaling remains unclear. However, several lines of evidence suggest that Cbl may enhance ligand-induced ubiquitination, internalization, and degradation of cell surface receptors (Lee and others 1999; Levkowitz and others 1998; Miyake and others 1998). For example, the increased surface expression of CD4, CD8, and the TCR in $Cbl^{-/-}$ mice suggests that Cbl plays a role in removing these receptors from the plasma membrane. More directly, evidence from CSF-1 receptor (CSFR) signaling in macrophages indicates that Cbl directly controls the polyubiquitination of CSFR and mediates an increase in the rate of CSFR endocytosis (Lee and others 1999). Specifically, in $Cbl^{-/-}$ macrophages,

CSFR was not polyubiquitinated in response to CSF, endocytosis of CSFR was 2-3 times slower than in wildtype macrophages treated with CSF, and proliferation was markedly increased in response to CSF, a phenomenon which was directly correlated with persistance of CSFR signaling at the plasma membrane (Lee and others 1999). Cbl was also recently identified as a key mediator of surface downregulation of EGFR (Levkowitz and others 1998). Within the context of EGFR overexpression in CHO cells, overexpression of Cbl led to an acceleration of EGFR internalization and degradation. Moreover, expression of v-Cbl caused internalized EGFR to be shunted to a recycling compartment which trafficked the receptor back to the plasma mebrane, resulting in a failure to downregulate the receptor in response to ligand (Levkowitz and others 1998). In this same study, Cbl was localized to a vesicular compartment, and was fractionated within an endosomal fraction following EGF treatment. Furthermore, Cbl was rapidly recruited to EGFR-containing vesicles following EGFR internalization, suggesting that it is involved in a sorting event that occurs mesial to endocytosis of the receptor (Levkowitz and others 1998). Hence, Cbl is implicated in the ligand-induced internalization and downregulation of several surface receptors, via a mechanism that is reversed by mutations that render Cbl oncogenic. The data presented within this chapter suggest that Cbl may participate in a Src-dependent mechanism that controls RTK internalization via the clathrin pathway, and that Cbl, within the context of differentiative signaling downstream from TrkA, acts to positively regulate neuritogenesis. They also suggest that

Cbl may be an important integration point for convergence of neurotrophin signaling and depolarization, a convergence that certainly has tremendous significance for the study of learning- and memory-related plasticity. The remainder of this introduction will touch briefly on the role of depolarization in the control of neurite outgrowth, and the reader is refered to chapter 1 of this manuscript for a more detailed account of Src signaling and neuritogenic signaling in PC12 cells, and to chapter 5 for a discussion of the role of neurotrophins in neural plasticity.

Depolarization Enhances Neurite Outgrowth in Response to Suboptimal Levels of NGF.

PC12 cells that overexpress TrkA exhibit a low level of spontaneous neurite outgrowth, and respond to NGF by elongating neuritic processes much more quickly than normal PC12 cells treated with NGF (Hempstead and others 1992). This effect is recapitulated in the response that NGF-primed PC12 cells exhibit following neurite stripping and subsequent replating – essentially, the cells are poised to rapidly respond to NGF by virtue of an increased complement of surface TrkA. Increased expression of TrkA at the surface leads to spontaneous dimerization of the receptor in the absence of ligand, and therefore to the spontaneous autophosphorylation of tyrosines within the intracellular domain. A low level of such spontaneous receptor activation appears to be sufficient to prime PC12 cells for further neuritogenic signaling in response to NGF. Interestingly,

Messing and colleagues discovered that K^+-mediated depolarization of PC12 cells overexpressing TrkA led to robust neurite outgrowth that exceeded the outgrowth induced in normal PC12 cells in response to 2 nM NGF (Solem and others 1995). This effect was maximal at 45 mM K^+, and was dependent upon Ca^{2+} influx through voltage-gated Ca^{2+} channels. On the other hand, depolarization of normal PC12 cells, even for extended times, resulted in only a small percentage of cells elaborating only very short processes. However, normal PC12 cells pretreated for 15 days with 20 pM NGF, a level that by itself is insufficient to induce neurite outgrowth, were induced to grow extensive neurites in response to depolarization with 45 mM K^+ (Solem and others 1995). These findings are consistent with other work showing that K^+-mediated depolarization enhanced NGF-induced neurite outgrowth and increased NGF binding to surface receptors (Koike 1987a; Koike 1987b), and with work showing that depolarization maintains neurites after NGF withdrawal (Teng and Greene 1993). While Messing and his colleagues suggest a model in which activation of TrkA or TrkA-induced signaling cascades are not involved in depolarization-induced neurite outgrowth under conditions of increased TrkA expression, other findings argue against this. For example, Greenberg and colleagues have shown that depolarization leads to transient activation of Erk1/2 in PC12 cells (Rosen and others 1994), while evidence from Koike that depolarization induces more binding of NGF to surface receptors suggests that TrkA signaling would be increased by depolarization (Koike 1987a; Koike 1987b). Finally, the results presented

within this chapter support a model in which depolarization leads to increased TrkA internalization, increased TrkA activation, and enhancement of neuritogenic signaling cascades engaged by TrkA. However, the effects presented in this chapter occur within minutes, while the effects examined by Messing and colleagues took place over many days, suggesting that temporal considerations may be important in defining a complete mechanistic basis for the effect of depolarization on neurite outgrowth.

Results

Depolarization Enhances NGF Internalization and TrkA Activation.

As discussed above, previous work has suggested that potassium-mediated depolarization of the rat PC12 pheochromacytoma cell line modulates NGF-induced differentiative signaling via TrkA (Solem and others 1995). To investigate the mechanism responsible for this modulation, I asked whether depolarization enhanced the endocytosis of NGF. PC12 cells on collagen-coated plates were treated with ^{125}I-NGF (100 pM) for 1 hr at 37°C in depolarization buffer that contained either 5 mM K^+ (equivalent to normal saline, hence undepolarized) or 50 mM K^+. Figure 4.1A shows that depolarization induced an increase in the internalization of radiolabeled NGF to 192% of the the undepolarized value (±10%; n=3; $p<0.005$). This effect was dependent upon calcium-influx, as depolarization in the absence of calcium and magnesium (Figure 4.1A "50 mM-Ca^{2+}")

did not induce a statistically significant increase in ^{125}I-NGF internalization (107% ± 15%; n=2; p=0.63).

NGF internalization may occur predominantly via TrkA (CL Howe and AP Kruttgen, unpublished observations). Hence, increased internalization of NGF may be correlated with increased TrkA activation. To determine whether depolarization modulated NGF-induced tyrosine phosphorylation of TrkA, I treated PC12 cells with NGF (2 nM) for 2 min at 37°C in depolarization buffer that contained 5, 10, 25, 50, or 100 mM K^+ (Figure 4.1B). In the presence of physiologically normal K^+ levels (5 mM), NGF induced a typical increase in TrkA phosphorylation. Increasing concentrations of K^+ enhanced the phosphorylation of TrkA in response to NGF, with the peak enhancement occurring at 50 mM K^+. 100 mM K^+ was not as efficacious as 50 mM K^+ at increasing TrkA phosphorylation. Because 50 mM K^+ induces a depolarization that is more physiological relevant than that induced by 100 mM K^+, this finding suggests that the effect of depolarization on TrkA activation may have biological significance.

Using 50 mM K^+ as the optimal potassium concentration for depolarization, I next sought to determine the time course over which depolarization enhanced TrkA activation. Under normal conditions, NGF (2 nM) treatment elicits TrkA phosphorylation that is apparent by 1 min, peaks at 2 min, and begins to return to baseline by 5 min. Figure 4.1C shows

that treatment with NGF in the presence of 50 mM K^+ substantially increased TrkA phosphorylation as early as 1 min. The effect was maximal at 2 min, coinciding with the normal peak in TrkA activation. By 5 min of NGF under depolarizing conditions, the effect of K^+ was negligible. This finding indicates that depolarization acts to increase the efficacy of TrkA activation at early timepoints without substantially altering the kinetics of activation. This is important, as the initial finding that depolarization enhanced TrkA activation at 2 min could have been due to a shift in the timing of peak TrkA activation. The finding that depolarization enhances TrkA activation at both 1 min and 2 min, and that at 5 min there is no substantial difference with depolarization, suggests that K^+ may be acting to increase the absolute number of TrkA receptors that become phosphorylated in response to NGF. Verification of this suggestion will require further analysis of the effect of depolarization on surface levels of TrkA.

Finally, the effect of depolarization on TrkA activation was dependent upon extracellular calcium. In the absence of both calcium and magnesium, 2 min of NGF (2 nM) elicited TrkA phosphorylation that was essentially identical to that elicited by NGF in the presence of calcium and magnesium (Figure 4.1D, lane 3 vs lane 1). However, NGF plus depolarization under calcium- and magnesium-free conditions did not elicit the enhanced TrkA phosphorylation that was seen in the presence of calcium and magnesium (Figure 4.1D, lane 4 vs lane 2).

Depolarization Modulates the Phosphorylation of Cbl Downstream From TrkA Activation.

TrkA activation elicits several well-characterized signaling cascades that are necessary for NGF-induced differentiation of PC12 cells. I wondered whether the effect of depolarization on TrkA activation translated into a similar enhancement or modulation of downstream signaling elements. To test this, PC12 cells were treated for 5 min at 37°C with 5 mM K^+ or 50 mM K^+ in depolarization buffer, or with NGF (2 nM) in depolarization buffer containing either 5 mM K^+ or 50 mM K^+. Following treatment, cells were lysed in sample buffer, and equivalent amounts of total protein were loaded onto 7.5% SDS-polyacrylamide gels. After transfer, all tyrosine phosphorylated species were detected. Figure 4.2A shows a protein of 120 kDa that was uniquely phosphorylated under these conditions. In particular, this protein was shown to exhibit a modest level of tyrosine phosphorylation in response to depolarization alone (Figure 4.2A, lane 3), a large response to NGF alone (Figure 4.2A, lane 5), and a dramatically reduced response to the combination of NGF and depolarization (Figure 4.2A, lane 6). This unique pattern of activation prompted me to investigate this protein more thoroughly. I subsequently identified the protein as Cbl, a 120 kDa adaptor protein that is generally considered a negative regulator of receptor tyrosine kinase activity.

To verify the effect of NGF on the phosphorylation state of Cbl and to ascertain the temporal dynamics of the effect, I treated PC12 cells with NGF (2 nM) at 37°C for various times, then immunoprecipitated Cbl and probed for phospho-tyrosine. Figure 4.2B shows the result of an extended timecourse of NGF, while Figure 4.2C shows a higher-resolution timecourse. Both figures indicate that NGF induces tyrosine phosphorylation of Cbl that is detectable by 1 min of NGF, maximal by 5 min, and decreased by 15 min. The timing of this phosphorylation corresponds to that seen for the 120 kDa band detected in lane 5 of Figure 4.2A.

I wondered whether the phosphorylation of Cbl in response to NGF treatment was mediated by TrkA. To test this, I analyzed Cbl phosphorylation following 5 min of NGF (2 nM) at 37°C in a variety of PC12 derivatives. P8 and 6.24 cells are PC12 cells that overexpress human TrkA, while M1-nnr5 and 22.7-nnr5 cells express kinase-defective TrkA within the context of very little endogenous wildtype TrkA. The 490/785-nnr5 PC12 mutant expresses TrkA that lacks tyrosines 490 and 785, again, within the context of very little endogenous TrkA. Figure 4.2D (lanes 9 and 10) shows that in KB PC12 cells NGF induces the same tyrosine phosphorylation of Cbl that was seen in Figures 4.2B and 4.2C. Moreover, in the TrkA overexpressors P8 and 6.24, Cbl is robustly phosphorylated in response to NGF (Figure 4.2D, lanes 7 and 8, and lanes 1 and 2, respectively). Note that in P8 cells a higher level of background Cbl phosphorylation

exists in the absence of NGF treatment, suggesting that spontaneous TrkA activation in the absence of ligand is inducing Cbl phosphorylation. Interestingly, Cbl phosphorylation is not increased in response to NGF in any of the TrkA mutants, including the M1-nnr5, 22.7-nnr5, and the 490/785-nnr5 cell lines. The higher level of Cbl phosphorylation that is seen in the untreated 490/785-nnr5 cells is likely caused by a background level of tyrosine phosphorylation that TrkA exhibits in these cells (data not shown). Nonetheless, Cbl phosphorylation is not increased in these cells in response to NGF, suggesting that one of these sites may mediate an interaction between Cbl and TrkA. Hence, NGF-induced Cbl tyrosine phosphorylation appears to be mediated by TrkA.

Based on the results of Figure 4.2A, I next sought to determine the effect of K^+-mediated depolarization on Cbl phosphorylation. I first asked whether depolarization alone led to Cbl phosphorylation. In fact, I found that Cbl was tyrosine phosphorylated very rapidly in response to 50 mM K^+ (Figure 4.3A). 30 sec of depolarization was sufficient to induce tyrosine phosphorylation of Cbl, with the peak of phosphorylation occuring by 1 min. By 5 min the level of Cbl phosphorylation was decreasing, and had returned almost to baseline by 15 min. This finding explains the low level of Cbl phosphorylation that was seen in lane 3 of Figure 4.2A – by 5 min of depolarization, the peak of Cbl phosphorylation was already past.

I next asked whether the effect of depolarization of Cbl phosphorylation was calcium-dependent. Figure 4.3B shows that in the absence of calcium and magnesium, 50 mM K^+ was not able to elicit substantial phosphorylation of Cbl. While some phosphorylation may be evident following 5 min of depolarization in the absence of divalent cations, the amount is low compared to that seen in Figure 4.3A, and certainly does not fit the timecourse of activation that occurs in the presence of calcium.

Figure 4.2A suggested that at 5 min of treatment, the amount of Cbl phosphorylated in response to NGF was much greater than that which occurred in response to depolarization. To ascertain the relative phosphorylation amplitude that occurs throughout the timecourse, I compared depolarization to NGF (2 nM) treatment at 30 sec, 1, 2, and 5 min. Figure 4.3C shows that 30 sec of depolarization induced phosphorylation of Cbl that was many-fold greater than the phosphorylation induced by 5 min of NGF treatment. However, in keeping with the findings of Figure 4.2A, by 5 min of depolarization, the relative phosphorylation of Cbl was several-fold less than that elicited by 5 min of NGF. Hence, while both depolarization and NGF induce the tyrosine phosphorylation of Cbl, they do so with dramatically different efficacies and temporal kinetics.

Finally, lane 6 of Figure 4.2A suggested that the combination of NGF and depolarization had a dramatic effect on the phosphorylation state of Cbl, as compared to NGF alone. This was confirmed by experiments in which PC12 cells were treated simultaneously with 50 mM K^+ and NGF (2 nM) at 37°C for various times. Figure 4.3D shows that, like depolarization only, depolarization plus NGF elicited Cbl phosphorylation that was maximal at 1 min, returning nearly to baseline by 5 min. Hence, the combination of NGF and depolarization induces Cbl phosphorylation that exhibits the temporal dynamics of depolarization alone.

Qualitative examination of the Cbl phosphorylation that was elicited by simultaneous depolarization and NGF treatment suggested that even the maximal phosphorylation state elicited by these two agents was less than that elicited by either alone. Figure 4.3E shows that, in fact, NGF plus depolarization evoked only a modest phosphorylation of Cbl at 1 min, as compared to that evoked by either NGF or 50 mM K^+. Thus, the combination of depolarization and NGF exhibits the timecourse of depolarization only, but exhibits an amplitude of Cbl phosphorylation that is significantly blunted in comparison to either NGF or depolarization alone.

The Effect of Depolarization and NGF on Cbl Phosphorylation may be Mediated by Src.

Previous work in other cell types suggested that the non-receptor tyrosine kinase Src was responsible for Cbl phosphorylation downstream from the activation of several different receptor tyrosine kinases. To determine whether this was true for NGF-induced Cbl phosphorylation in PC12 cells, I first asked whether Src was activated in response to NGF. Figure 4.4A shows that Src was tyrosine phosphorylated in response to NGF, with activation peaking between 2 and 5 min of NGF. It is interesting to note that the peak of TrkA phosphorylation precedes the peak in Src activation, and that both precede the peak in Cbl phosphorylation. This strongly suggests that TrkA activates Src which then phosphorylates Cbl.

Next, I asked whether pharmacological inhibition of Src could disrupt Cbl phosphorylation in response to either NGF or depolarization. Cells were pretreated in suspension for 15 min with PP1 (100 nM), a potent and selective inhibitor of Src family kinases. Following pretreatment, cells were treated with either NGF (2 nM) for 5 min or 50 mM K^+ for 1 min, or were left untreated. Figure 4.4B shows that PP1 preincubation blocked the background Cbl phosphorylation that existed in untreated cells (lanes 1 and 2). Moreover, PP1 completely inhibited the phosphorylation of Cbl that was elicited by either 5 min of NGF (lanes 3 and 4) or 1 min of depolarization (lanes 5 and 6).

Based on my earlier observation that depolarization enhanced NGF internalization, and that depolarization modified NGF-induced signaling through TrkA and Cbl, I wondered if inhibition of Src could reduce NGF internalization. To test this, I utilized srcDN2-PC12 cells, a cell line that expresses a dominant-negative mutant of Src. Src activity and Cbl phosphorylation in response to NGF is significantly blunted in these cells (data not shown). Figure 4.4C shows that the specific activity (cpm per ug of cellular protein) of ^{125}I-NGF internalization was significantly reduced in the srcDN2 cells compared to normal KB PC12 cells. This inhibition was seen even at early timepoints, but was most dramatic following 1 hr of incubation at 37°C. In control experiments I determined that the effect was also seen when normalized to a per cell basis, suggesting that the reduction in internalization of radiolabeled NGF in the srcDN2 cells was in fact due to a defect in uptake, not an artifact of cellular size or protein content differences elicited by the mutation (data not shown).

Cbl is Trafficked to Intracellular Membranes Distinct from Clathrin-Coated Vesicles in Response to NGF Treatment.

Previous work has suggested that Src may play a role in the endocytosis of receptor tyrosine kinases (Wilde and others 1999). Moreover, the experiments described above indicate that NGF internalization is sensitive to the effects of dominant-interfering Src,

and that Cbl phosphorylation in response to either NGF or depolarization is blocked by inhibition of Src. Therefore, I hypothesized that Cbl may play a role in trafficking events associated with TrkA internalization. To test this hypothesis, I analyzed the association of Cbl with various membrane fractions following NGF treatment.

Figure 4.5A shows that NGF induced the association of Cbl with a membrane-enriched fraction generated by a 100000g centrifugation of PC12 cell homogenates. Briefly, PC12 cells were treated with NGF (2 nM) for various times at 37°C, then chilled, and homogenized by repeated passage through a Balch homogenizer. This generated a homogenate that contained cytosol, released intracellular membranes and organelles, large sheets of plasma membrane, and largely intact cells with ruptured plasma membranes (Grimes and others 1996). In control experiments I showed that this homogenization procedure resulted in essentially 100% of cells failing to exclude trypan blue (data not shown). Hence, following homogenization, all cells were ruptured, with many releasing intracellular membranes. Homogenates were then centrifuged at 100000g for 1 hr at 4°C through a glycerol pad to separate membranes from cytosol. This procedure effectively collects all membranes and organelles (Grimes and others 1996). The membrane-enriched fraction was then lysed and immunoprecipitated for Cbl. Probing the resulting western blot with an anti-Cbl antibody indicated that NGF induced the association of Cbl with membranes over an extended timecourse. The membrane-

association continued to increase out to 15 min, the longest timepoint examined. This suggests that NGF induces a long-lasting recruitment of Cbl to membranes.

I next asked whether Cbl on these membranes was phosphorylated. Figure 4.5B shows that there was a peak in tyrosine phosphorylation of Cbl in the membrane fraction following 2 min of NGF treatment. Interestingly, the absolute level of phosphorylated Cbl in this fraction was low compared to the level of phosphorylation elicited on cytosolic Cbl (Figure 4.5B). Moreover, cytosolic Cbl exhibited a timecourse of phosphorylation that peaked very early, following 1 min of NGF. This is in clear contradiction to the timing of Cbl phosphorylation in the whole cell. It suggests that Cbl may be differentially phosphorylated in the cytosol versus on membranes. In fact, these data would suggest that Cbl may be phosphorylated very quickly in the cytosol, and may then associate with membranes in this phosphorylated state. The summation of all phosphorylated Cbl following NGF treatment may show peak activation between 2 and 5 min, but the phosphorylation of Cbl within specific cellular fractions may exhibit more rapid kinetics. In addition, these findings are consistent with the idea that TrkA activation in response to NGF leads to the activation of cytoplasmic Src, which then phosphorylates Cbl. This phosphorylation may then target Cbl for trafficking to membranes, including intracellular membranes and plasma membrane.

To more fully explore the trafficking of Cbl in response to NGF, I asked whether phosphorylated Cbl moved into specific intracellular membrane fractions. Previous work has defined 2 fractions of intracellular membranes gathered from cellular homogenates by differential centrifugation (Grimes and others 1996). Briefly, cells were homogenized as described above. These homogenates were then centrifuged at 1000g for 10 min to remove large sheets of plasma membrane and whole cells. The supernatant from this centrifugation was then centrifuged at 8000g for 35 min to collect a fraction, refered to as P2, enriched in large released intracellular membranes and organelles. Finally, the supernatant from this centrifugation was spun at 100000g for 1 hr to collect small released intracellular membranes. This fraction is refered to as P3. Fractions P2 and P3 were then lysed and immunoprecipitated for Cbl. The resulting western blots were probed for phospho-tyrosine. Figure 4.5C shows that 2 min of NGF (2 nM) induced the association of phosphorylated Cbl with both P2 and P3. Interestingly, inhibition of Src by pretreatment with PP1 did not alter the association of phospho-Cbl with the P2 fraction, but completely inhibited its association with P3. This suggests that NGF-induced activation of Src is required for the association of phosphorylated Cbl with small intracellular membranes and organelles.

Finally, as discussed in chapter 3 of this manuscript, NGF and TrkA are internalized via clathrin-coated vesicles (CCVs), and many downstream signaling partners are induced to

associate with CCVs following NGF treatment. The findings presented above led me to wonder whether Cbl was also trafficked to CCVs in response to NGF. Surprisingly, I found that quite the opposite occurred (Figure 4.5D). Following 2 min of NGF there was a dramatic decrease in the amount of Cbl that was associated with a highly-enriched CCV fraction. This decrease is made all the more robust by the fact that following 2 min of NGF there is nearly a doubling in the amount of CCVs that are recovered from cells (Chapter 3). The amount of Cbl in this fraction showed a small rise by 5 min of NGF, but was certainly much less than the amount found in untreated cells. The association of Cbl with CCVs in untreated cells and the subsequent dramatic redistribution away from such vesicles in response to NGF treatment is hard to explain. However, these findings point to a surprisingly complex pattern of trafficking exhibited by Cbl in response to NGF, and suggest that more work is necessary to understand the role such trafficking may play in the cellular function of Cbl.

Clathrin Heavy Chain is Found in Complex With Cbl in Response to NGF and Depolarization.

My earlier observations of Cbl immunoprecipitates isolated from NGF treated cells suggested that a 180-190 kDa protein was induced to form a complex with Cbl. Based on the trafficking results discussed above, I wondered whether this protein might be the clathrin heavy chain (CHC). In fact, Figure 4.6A shows that CHC was found in Cbl

immunoprecipitates, and that the amount of CHC increased following NGF treatment. The peak in association at 5 min coincided with the peak in Cbl phosphorylation evoked in response to NGF treatment. Likewise, depolarization with 50 mM K^+ was found to enhance the amount of CHC found in Cbl immunoprecipitates, with the peak in association coinciding with the peak in Cbl phosphorylation elicited by this treatment (Figure 4.6B). Hence, Cbl phosphorylation is correlated with increased association of CHC.

I next asked whether Cbl within specific membrane fractions was associated with CHC. Figure 4.6C shows that Cbl within the membrane-enriched fraction characterized in Figure 4.5A is associated with CHC in untreated cells. Following 1 min of NGF treatment the amount of CHC complexed with Cbl decreases dramatically. Interestingly, this decrease persists through 5 min of NGF, only beginning to increase at 15 min of treatment. Comparing Figure 4.6C to Figure 4.5A shows that the decrease in CHC associated with Cbl parallels the increase in Cbl association with the membrane-enriched fraction. Thus, as Cbl is recruited to membranes in response to NGF, it ceases to be in complex with CHC. Moreover, comparing Figure 4.6C to Figure 4.5B shows that the decrease in CHC found in complex with Cbl exactly parallels the increase in membrane-associated phospho-Cbl, suggesting that the phosphorylation state of Cbl is controlling its association with CHC. To summarize, unphosphorylated Cbl that is associated with

membranes in untreated cells is in a complex with CHC. Following NGF treatment, Cbl increases its association with membranes in parallel with an early increase in Cbl phosphorylation. Phosphorylated Cbl associated with membranes is no longer in complex with CHC, suggesting that Cbl phosphorylation state may control complex formation, at least in terms of membrane-associated Cbl. As there is a correlation between Cbl phosphorylation and CHC association at the level of the whole cell, the findings for membranes must represent a specific mechanism of control over complex formation.

As indicated in Figure 4.5C, NGF induces the recruitment of phospho-Cbl to the P2 and P3 fractions. I wondered whether CHC was associated with Cbl in these fractions. Figure 4.6D shows that in untreated cells Cbl in the P2 fraction is associated with CHC, while Cbl in the P3 fraction is not. Moreover, 2 min of NGF (2 nM) led to a loss of CHC in complex with Cbl within the P2 fraction, but an increase in CHC complexed with Cbl within the P3 fraction. Comparing Figure 4.6D to Figure 4.5C shows that there is a complete disconnect between the extent of Cbl phosphorylation and the amount of CHC found in complex with Cbl. Cbl within the P2 fraction behaves in a manner akin to Cbl within the total membrane fraction – as it becomes more phosphorylated, it ceases to be associated with CHC. On the other hand, Cbl within the P3 fraction behaves in exactly the opposite manner. As it becomes more phosphorylated, it increases its association with CHC.

The situation becomes even more complex when the role of Src is analyzed. Figure 4.6D shows that inhibition of Src by preincubation with PP1 does not block the increase in CHC found in complex with Cbl within the P2 fraction. This is consistent with the the finding shown in Figure 4.5C that PP1 does not inhibit recruitment of phospho-Cbl to the P2 fraction. Surprisingly, preincubation of cells with PP1 – a treatment which blocks the recruitment of phospho-Cbl to P3 – has no effect on the amount of Cbl in complex with CHC within the P3 fraction. Whether this means that PP1 does not block the recruitment of total Cbl to the P3 fraction in response to NGF, and that therefore Cbl phosphorylation state within the P3 fraction is unrelated to the extent of Cbl:CHC complex formation, remains to be determined. Further work is necessary to clarify the nature of Cbl recruitment to various membrane fractions, the role that phosphorylation plays in formation of a Cbl:CHC complex, and the functional role of such a complex within the cell.

There are two possible explanations for the observation that CHC is found in Cbl immunoprecipitates. The first is that Cbl is associated with an adaptor protein that binds to CHC. The second is that Cbl itself exhibits a novel clathrin adaptor function, binding directly to CHC. To test the first possibility, I looked for the presence of the plasma membrane specific adaptor complex AP2 in Cbl immunoprecipitates following NGF

treatment. Figure 4.6E shows that very little AP2 was found in complex with Cbl. However, the AP2 that was detectable appeared to peak following 5 min of NGF treatment – the same time that CHC peaks in association with Cbl. Hence, further analysis is required to determine whether AP2, or the Golgi-specific adaptor AP1, may mediate the association of Cbl with CHC. In addition, experiments in which Cbl is mutated at specific sites in an attempt to block CHC association are needed to determine whether Cbl can bind directly to clathrin.

Overexpression of Cbl Enhances Neurite Outgrowth in Response to NGF.

The finding that Cbl was a common and uniquely controlled substrate of NGF and depolarization suggested that it might play a role in K^+-mediated enhancement of neurite outgrowth. Also, the finding that depolarization and Src are both involved in control of NGF internalization, and that Src is downstream of both NGF and depolarization in the phosphorylation of Cbl, suggested that Cbl was more generally involved in differentiative signaling and neurite outgrowth. To begin to address the role that Cbl might play in neuritogenic signaling, I transfected PC12 cells with a Cbl expression vector. Several stably transfected clones were isolated, and Figure 4.7A shows the level of Cbl expression in one such clone. Figure 4.7B shows that the overexpressed Cbl was robustly phosphorylated in response to 5 min of NGF treatment. Finally, these cells were tested for their ability to grow neurites in response to NGF treatment. KB PC12 cells or Cbl

overexpressing PC12 cells (Cbl-OX) were treated with NGF at a concentration of either 0.1 nM or 1 nM for 1 or 2 days. Figure 4.7C shows untreated PC12 cells, and Figure 4.7F shows untreated Cbl-OX cells. Note the presence of small neuritic projections in the untreated overexpressors. Following treatment for 1 day with 0.1 nM NGF – an NGF concentration that is normally ineffective in eliciting neurites – the Cbl-OX cells exhibited increased neurite outgrowth (Figure 4.7G), while normal PC12 cells were unaffected (Figure 4.7D). Likewise, 1.0 nM NGF had little effect on normal PC12 cells after one day (Figure 4.7E), but induced the formation of neurites in Cbl-OX cells. By 2 days of 1.0 nM NGF, both cell types exhibited neurites, though the Cbl-OX cells (Figure 4.7J) showed much more robust and complex neurite outgrowth than the KB PC12 cells (Figure 4.7I). Thus, it appears that overexpression of Cbl enhanced neuritogenic signaling induced by even very low levels of NGF. Further experiments addressing the effect of Cbl overexpression on NGF internalization and TrkA signaling are needed to determine the mechanism of this effect, but the preliminary evidence presented herein suggests that Cbl functions to control the internalization of TrkA, thereby promoting neuritogenic signaling by enhancing TrkA signaling.

Discussion

The relationship between a presynaptic terminal and its postsynaptic target is dynamically regulated by a host of anterograde and retrograde factors. In the anterograde

direction, the presynaptic terminal controls ionic flux within the postsynaptic element by releasing neurotransmitters that bind to specific postsynaptic receptors. Likewise, in the retrograde direction, postsynaptic targets release neurotrophins that bind to presynaptic neurotrophin receptors in order to control the morphological and biochemical complexion of the presynaptic terminal. Obviously, within the brain, at any given synapse, there is a complex interplay of these anterograde and retrograde signals. An interesting hypothesis is that convergence of these signals at specific synapses may induce a synergism in the connection between the presynaptic and postsynaptic elements that leads to an overall strengthening in the connection. Such a strengthening may modify more widespread network responses within the nervous system, leading to a global change in neuronal activity that reflects a specific learning event. In other words, the strengthening (and conversely, the weakening) of a synaptic connection may serve as the essential element of memory. The convergence of anterograde and retrograde signals is therefore an exciting arena to enter if we wish to better understand the mechanistic basis of memory. In the work presented herein, I have shown that the convergence between depolarization and neurotrophin signaling that occurs within the presynaptic terminal may be mediated by specific intracellular proteins that are sensitive to regulation by ion flux and receptor tyrosine kinase activity.

Using PC12 cells as a model of the presynaptic terminal, I have shown that Cbl may serve as a key integrator of calcium- and RTK-dependent signaling, and that this integration is manifested within the control of neurotrophin receptor trafficking. I first showed that depolarization enhanced the internalization of NGF and the activation of TrkA in response to NGF (Figure 4.1). This finding supports previous work showing that depolarization enhances neuritogenesis in response to low levels of NGF (Solem and others 1995). It suggests that enhanced internalization of TrkA may promote neurite outgrowth, a suggestion that is supported by a recent study showing that TrkA internalization was involved in differentiative signaling induced by NGF (Zhang and others 2000). Further support for this idea comes from work showing that MAP kinase signaling downstream from EGFR activation requires receptor internalization for maximal efficacy (Vieira and others 1996), and from my own unpublished observation that inhibition of clathrin-mediated endocytosis of TrkA prevents NGF induction of Erk1/2 phosphorylation within PC12 cells. Hence, receptor trafficking appears to be a potential convergence point for depolarization and neurotrophin signaling.

I was intrigued by the possibility that depolarization and NGF might act through a common intracellular agent that could signal to control TrkA trafficking. To screen for such a protein, I asked about the tyrosine phosphorylation of total cellular proteins in response to K^+, NGF, or K^+ plus NGF. I discovered a prominently phosphorylated 120

kDa protein that exhibited a unique pattern of phosphorylation in response to these different treatments (Figure 4.2). Further analysis indicated that this protein was the multi-adaptor Cbl. I showed that Cbl was phosphorylated by both NGF and K^+, but that these two treatments resulted in very different phosphorylation kinetics (Figures 4.2 and 4.3). NGF induced a slower, more prolonged phosphorylation of Cbl, while depolarization led to a very rapid phosphorylation of Cbl that peaked between 30 seconds and 1 minute of treatment. Interestingly, depolarization plus NGF led to a phosphorylation timecourse that was identical to that induced by depolarization only. Moreover, the combination of depolarization and NGF dramatically reduced the amplitude of Cbl phosphorylation. Hence, Cbl is a convergence point for signaling initiated by both depolarization and NGF, and in combination these two factors may uniquely control Cbl function. Further experimentation led to the discovery that Src may mediate Cbl phosphorylation in response to depolarization and TrkA signaling. I found that pharmacological inhibition of Src activity abrogated the tyrosine phosphorylation of Cbl that occurs in response to either K^+ or NGF (Figure 4.4). It is very interesting to note that NGF internalization was defective in cells that express a dominant-interfering form of Src. This finding links Src activity to TrkA internalization, and supports previous work showing that EGF endocytosis is delayed or decreased in response to Src inhibition (Wilde and others 1999). Furthermore, combining the finding that depolarization increased NGF internalization and Cbl phosphorylation with evidence that Src inhibition

decreased NGF internalization and blocked K^+-induced Cbl phosphorylation, suggests a model in which Src signals downstream from depolarization to elicit Cbl phosphorylation and Cbl involvement in the endocytic pathway.

Further evidence for a role of Cbl in endocytosis comes from my finding that Cbl is recruited to membranes in response to NGF treatment. The data provided in Figure 4.5 suggest that Cbl is phosphorylated in the cytosol following TrkA-mediated NGF-induced activation of Src, and that it is subsequently recruited to membranes, including the plasma membrane. Subfractionation of these membranes showed that phospho-Cbl is recruited to a variety of intracellular membranes. Surprisingly, phospho-Cbl was still found in the P2 fraction when Src was inactivated, even though on a whole cell lysate basis, Cbl phosphorylation appears to be completely blocked. On the other hand, inhibition of Src did block the recruitment of phospho-Cbl to the small intracellular membranes of the P3 fraction. These findings suggest either that there is Src-independent phosphorylation of Cbl within a highly specific membrane fraction, or that a very low level of Src activation that persists following treatment with inhibitor is sufficient to specifically phosphorylate Cbl within an endosomal compartment. Further analysis of the trafficking of total Cbl versus phosphorylated Cbl is needed to resolve the complexities of Cbl trafficking.

The trafficking issue is further complicated by the finding that while Cbl is induced to rapidly move out of a clathrin-coated vesicle fraction (Figure 4.5), it is also induced to associate with the clathrin heavy chain in response to both NGF and depolarization (Figure 4.6). It is unclear as to why at the whole cell level there is a correlation between Cbl phosphorylation and CHC association, while at the level of specific membrane fractions the relationship is inverted. Without further analysis of the association of CHC with unphosphorylated Cbl in specific cellular compartments it is hard to interpret these data. However, a few points are indicated: 1) phosphorylated Cbl in the whole cell is associated with CHC; 2) phosphorylated Cbl in association with total membranes is not complexed with CHC; 3) unphosphorylated Cbl associated with large intracellular membranes is in complex with CHC, and this complex is broken upon phosphorylation of Cbl, recapitulating the total membane fraction finding; 4) unphosphorylated Cbl associated with small intracellular membranes is not in complex with CHC, but is induced to form a complex with CHC following phosphorylation, reflecting the whole cell situation; 5)Src has no effect on the loss of CHC in complex with Cbl in the P2 fraction, consistent with the finding that Cbl is still phosphorylated in this fraction after inhibition of Src; and 6) inhibition of Src, while blocking phosphorylation of Cbl within the P3 fraction, has no effect on the Cbl:CHC complex within this fraction. These findings are very interesting, but also incredibly confusing. They suggest that the relationship between Cbl trafficking, Cbl phosphorylation, and formation of a Cbl:CHC

complex is very dynamic and very tightly regulated within highly specific cellular compartments. Further work is obviously needed to clarify these relationships, but a simplistic model of the data is offered in Figure 4.8. This figure shows that K^+ and NGF may converge at the level of Src activation and the phosphorylation of Cbl, possibly via an increase in intracellular calcium-levels. Phosphorylation of Cbl may then lead to the formation of a complex that contains Src, Cbl, and CHC. This complex may then be recruited to the plasma membrane, permitting CHC to interact with TrkA and increase endocytosis of the receptor. Downstream from this event, Cbl may be trafficked to specific endocytic fractions. The model suggests that Cbl and Src are key mediators of a depolarization-induced increase in TrkA endocytosis, and that such endocytosis may allow TrkA to signal more efficiently for the induction of neurite outgrowth.

In support of this model, I found that overexpression of Cbl primed PC12 cells for neurite outgrowth in response to low levels of NGF (Figure 4.7). Importantly, PC12 cells overexpressing Cbl exhibited small neuritic projections even in the absence of NGF, a finding that is reminiscent of the effect of TrkA overexpression. Remarkably, cells overexpressing Cbl elaborated very robust and highly branched neurites in response to NGF treatment for 2 days. These neurites were qualitatively similar to the neurites elicited in normal PC12 cells only after 1 week of NGF (data not shown). Hence, overexpression of Cbl recapitulates the effects of TrkA overexpression discussed in the

introduction. Moreover, previous studies have shown that overexpression of Src led to increased neurite outgrowth in response to NGF, while inhibition of Src blocked neurite outgrowth or caused neurite retraction in cells that already had neurites (D'Arcangelo and Halegoua 1993; Rusanescu and others 1995; Thomas and others 1991). These findings support the model presented above, and suggest that Src and Cbl are critically involved in neuritogenic signaling. It is tempting to draw a parallel between the effects of Src and Cbl on osteoclast bone resorption and the effects presented herein for TrkA endocytosis and neuritogenic signaling. Much of the data I have shown suggests that this parallel is valid, but further work is necessary to characterize the role of Cbl and Src in endocytosis and differentiation. In particular, the internalization of NGF in cells overexpressing Cbl and in cells expressing mutants of Cbl needs to be addressed, as does the correlation between TrkA endocytosis and Cbl:CHC complex formation.

Perhaps the strongest piece of data presented in this chapter is the formation of a complex that contains Cbl and CHC in response to both NGF and depolarization (Figure 4.6). The formation of such a complex suggests the exciting possibility that Cbl is a novel clathrin adaptor protein that serves the same function for RTKs that β-arrestin serves for G-protein coupled receptors (Kirchhausen 2000) (also see chapter 1). β-arrestin participates in the clathrin-mediated downregulation of G-protein coupled receptors that is part of desensitization, and does so by adapting both CHC and AP2 to the receptor. Several lines

of evidence suggest that Cbl may also bind CHC and AP2. First, as indicated in Figure 4.6, CHC and AP2 are both found in Cbl immunoprecipitates. Second, a tertiary-structure-based search of the Brookhaven protein data bank suggested that Cbl shares structural homology with several CHC binding proteins, including AP2 and the novel clathrin adaptor protein Nef (VAST Search; data not shown). Third, primary sequence analysis of Cbl indicates that it has a number a potential clathrin and AP2 binding sites, including 4 dileucine motifs and several potential YxxΦ motifs (for example, YMAF, starting at tyrosine 268 in human Cbl). Interestingly, the Cbl loss-of-function mutation G306E disrupts a glycine that is adjacent to tyrosine 307. Such a glycine-tyrosine dipeptide motif has been implicated in the trafficking of several different proteins, including lgp and lamp glycoproteins, the HIV-1 envelope glycoprotein, and the β-amyloid precursor protein (Boge and others 1998; Harter and Mellman 1989; Kornfeld and Mellman 1989; Lai and others 1995). This suggests that the G306E loss-of-function phenotype may involve a defect in Cbl-CHC interaction. Finally, as shown in Figures 4.9 and 4.10, Cbl contains a potential "clathrin box" (Kirchhausen 2000). This motif, which is LIELD in β-arrestin and LLNLD in AP2, is found in a number of clathrin interacting proteins. In Cbl, the sequence LRPLD (residues 708 to 712 in human Cbl) fits the mold of an LxxLD clathrin box consensus sequence, suggesting that this region within Cbl may mediate direct binding to CHC. Further work is necessary to determine whether Cbl is, in

fact, a novel clathrin adaptor protein, but the evidence presented herein makes a strong case for such a role.

Conclusions.

The role of neurotrophin signaling in the genesis, maintenance, and plasticity of neuronal connectivity has been recognized in recent years as a critical element of learning and memory. However, while Hebb's concept of "cells that fire together wire together" has helped shape our understanding of the role neuronal activity plays in learning and memory (Hebb 1949), no coherent guiding principle has been offered concerning the role of neurotrophin signaling in these events. Much of the work in the field is still stuck in the "pharmacological" phase, in which neurotrophins are given or taken away, and changes in plasticity are cataloged. While such a Linnaean undertaking is certainly useful, it does not address the root mechanistic question: how does neurotrophin signaling control, shape, and modify activity-dependent plasticity within the central nervous system? If neurotrophins are to play more than just a cameo role in plasticity – if they are to be instructive and determinantal – then a new paradigm is needed. Such a paradigm will require at least two critical elements: 1) that neurotrophin availability reflect synaptic activity, most likely through a mechanism of activity-dependent release from the postsynaptic cell; and 2) that neurotrophin responsiveness in the presynaptic terminal be activity-dependent and synapse-specific. These criteria will intimately link

neurotrophin signaling to the "fire together, wire together" conceptual framework. The work described in this chapter is an attempt to address the second element of the new neurotrophin paradigm – that neurotrophin responsiveness occur in an activity-dependent manner. I have shown that depolarization controls NGF signaling, at least in part by enhancing TrkA trafficking. Moreover, I have shown that Cbl may serve as a key integration point for signaling that occurs downstream of both depolarization and TrkA activation, and that it may tune neurotrophin responsiveness to the level of activity by controlling neurotrophin receptor trafficking. These studies are still in their infancy, but they point to a new, more mechanistic model to explain the role of neurotrophins in neuronal plasticity.

Methods

Antibodies

X22, a mouse monoclonal against CHC, was from Affinity Bioreagents (Golden, CO). 100/2, a mouse monoclonal against the α-subunit of AP2, was from Sigma (St. Louis, MO). 06574, a rabbit polyclonal against TrkA, and 4G10, a mouse monoclonal antibody against phospho-tyrosine, were from Upstate Biotechnology (Lake Placid, NY). The anti-Cbl rabbit polyclonal antibody SC170 and HRP-conjugated secondary antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA). 327, an anti-Src monoclonal antibody, was from Calbiochem (La Jolla, CA).

Chemicals and Other Reagents

Mouse NGF was prepared by ion-exchange chromatography as previously described (Mobley and others 1976). Ultralink immobilized protein A/G plus, BCA reagents, ECL reagents, and Iodogen precoated iodination tubes were from Pierce (Rockford, IL). Protran nitrocellulose transfer membrane was from Schleicher and Schuell (Keene, NH). X-OMAT x-ray film was from Eastman Kodak Company (Rochester, NY). ^{125}Iodine (IMS-30), PD-10 Sephadex G-25M columns, and ECL-sensitive film were from Amersham Pharmacia Biotech (Piscataway, NJ). Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS), penicillin-streptomycin, PBS, and calcium-magnesium-free PBS (CMF-PBS) were from Mediatech (Herndon, VA). Horse serum was from Tissue Culture Biologicals (Tulare, CA). Geneticin was from GIBCO BRL (Gaithersburg, MD). Nonidet P40 (NP40) was from Fluka (Switzerland). PP1 was from Calbiochem (La Jolla, CA). All other chemical reagents were from Sigma (St. Louis, MO).

Media and Buffers

Lysis buffer was composed of 20 mM Tris, 137 mM NaCl, 1% NP40, 0.5% deoxycholic acid (DOC), 10% glycerol, 1 mM PMSF, 10 ug/mL aprotinin, 1 ug/mL leupeptin, 500 uM sodium orthovanadate, pH 8.0. Sample buffer was 7 M urea, 125 mM Tris, 100 mM EDTA, 0.1% bromophenol blue, 2% SDS, pH 6.95. Blocking solution for western blots

for all antibodies except 100/2 was 5% BSA in TBS-T (20 mM Tris, 137 mM NaCl, 0.2% Tween 20). For 100/2 incubations, blots were blocked in 5% milk in TBS-T. All antibodies except 100/2 were diluted in TBS (20 mM Tris, 137 mM NaCl). 100/2 was prepared in TBS plus 5% milk. Tris iodination buffer was 25 mM Tris, 400 mM NaCl, pH 8.0. Tris/BSA iodination buffer was 25 mM Tris, 400 mM NaCl, 0.25% BSA, 5 mM EDTA, 0.05% sodium azide. The tyrosine scavenging buffer for iodinations was 10 mg/mL tyrosine in PBS, pH 7.4. The acid strip for internalization experiments was 0.2 M acetic acid and 0.5 M NaCl. CCV isolation buffer was 100 mM 2-[N-morpholino]ethanesulfonic acid (MES), 1 mM EGTA, 2 mM $MgCl_2$, 0.02% sodium azide, 1 mM beta-mercaptoethanol, 1 mM sodium orthovanadate, 1 mM PMSF, 10 ug/mL aprotinin, 1 ug/mL leupeptin, pH 6.5. CCV isolation D_2O sucrose pad was 100 mM MES, 8% ultrapure sucrose, 1 mM EGTA, 2 mM $MgCl_2$, 1 mM sodium azide, prepared in D_2O (Sigma-Aldrich #15188-2, 99.9% D). The MES-based buffer for membrane fractionation experiments was 25 mM MES, 150 mM NaCl, 10 mM EDTA, 1 mM sodium orthovanadate, 1 mM PMSF, 10 ug/mL aprotinin, 1 ug/mL leupeptin, pH 6.5. All cell treatments not involving depolarization were performed in PGBH, composed of 1 mg/mL glucose, 1 mg/mL BSA, 10 mM HEPES, in PBS, pH 7.4. Depolarization buffer was 200 mg/L $CaCl_2$, 97.7 mg/L $MgSO_4$, 3.7 g/L $NaHCO_3$, 4.5 g/L glucose, 584 mg/L L-glutamine, 400 mg/L KCl (5.4 mM), 3.477 g/L NaCl (59.5 mM; normal saline is 109.5 mM), pH 7.6. For depolarization experiments, non-depolarized samples (that is 5

mM K^+) were brought up to 109.5 mM NaCl by the addition of a concentrated NaCl solution. For depolarized samples, an additional 45 umol of KCl was added to 1 mL of depolarization buffer, bringing the final concentration of the solution to 50 mM K^+, 59.5 mM Na^+.

Cell Culture and Cell Treatments

KB PC12 cells (gift of R. Kelly) and srcDN2 PC12 cells (gift of S. Halegoua) were maintained at 37°C and 5% CO_2 in DMEM supplemented with 10% horse serum, 5% FBS, 100 U penicillin, and 100 ug/mL streptomycin. 6.24 PC12 cells, M1-nnr5, 22.7-nnr5, P8-nnr5, and 490/785-nnr5 PC12 cells (gift of D. Kaplan) were maintained under identical conditions, with the addition of 200 ug/mL geneticin. All cells were grown on plastic. In preparation for all experiments, cell growth media was changed to DMEM supplemented with 1% horse serum 16-20 hours prior to experimentation. Immediately prior to every experiment cells were removed from their plates with 37°C CMF-PBS, resuspended in 37°C PGBH, distributed into 1 mL aliquots, and rotated for 15 min to equilibrate the cells prior to experimentation. All treatments were performed in suspension, on equivalent numbers of cells, and incubated in a 37°C water bath, with periodic gentle inversion of the tubes to keep the cells suspended. For all experiments not involving depolarization (3-5 x 10^7 cells per condition), 50 ng of NGF in 50 uL PGBH was added to each tube at 37°C for the appropriate time. For depolarization experiments,

cells (3-5 x 10^7 cells per condition) were resuspended in depolarization buffer, equilibrated, and then the appropriate volume of a balanced K^+/Na^+ solution was addded to bring the buffer to the final K^+ concentration while maintaining osmolarity, essentially as previously described (Koike 1987a; Koike 1987b). For experiments involving pharmacological inhibitors, the appropriate concentration of the drug was added in a small volume following the equilibration step, and the cell suspensions were then rotated for an additional 15 min at 37°C. For all whole cell internalization experiments (1 x 10^7 cells per condition), cells on collagen-coated plates were treated with ^{125}I-NGF (2 nM) at 37°C for the appropriate time. Following this incubation, cells were rapidly chilled by floating the plates on a –5°C salt-ice water bath, then stripped in acid-stripping buffer and lysed.

NGF Iodination

For iodinations, 5 ug of NGF in 100 uL tris iodination buffer was added to 1.0 mCi ^{125}Iodine that had been activated for 9 min at RT in 100 uL of tris iodination buffer in a Pierce Iodo-Gen tube. This mixture was reacted for 9 minutes at RT, then quenched for 5 min at RT by the addition of 50 uL of tyrosine quench solution. The volume of this mixture was then brought to 1 mL with tris/BSA iodination buffer. After removing 2 uL for quantification of total reaction cpm, the remaining 998 uL were loaded onto a PD-10 desalting column that had been pre-equilibrated with 20 mL of tris/BSA buffer. The

column was then washed with tris/BSA solution, and five 1 mL fractions were collected, beginning with the first "hot" drop. The 2 uL removed from the reaction mixture, and 2 uL from each of the first 2 fractions, was diluted into 198 uL tris/BSA buffer, then 5 uL of each of these dilutions was further diluted into 445 uL of ddH_2O, while 5 uL of each of fraction 3-5 was diluted straight into 445 uL ddH_2O. To these final water dilutions was added 50 uL of TCA (prepared as 100% TCA with 1 mg/mL DOC), and this precipitation mixture was incubated on ice for 1 hr. These solutions were then centrifuged at 16000g for 10 min at 4°C, the supernatants were separated from the pellets, and the cpm for each was determined. After normalizing for the activity of the ^{125}Iodine and for the dilution factors, the specific activity of the radiolabeled NGF was determined. Typical iodinations produced NGF with a specific activity of 150-200 cpm/pg.

Cell Fractionation

Membrane preparation one: total membranes.

Following treatment as described above, PC12 cells resuspended in 1 mL of 4°C MES buffer were permeabilized in a Balch homogenizer, essentially as described in our previous work (Grimes and others 1997; Grimes and others 1996). The suspension of permeabilized cells (cell ghosts), cellular contents, and unpermeabilized cells was centrifuged at 100000g for 1 hr at 4°C to separate all membranes from cytosol.

Membrane preparation two: 8000g isolation (P2).

PC12 cells were permeabilized as described above. The permeabilized suspension was centrifuged at 1000g for 10 min to remove the cell ghosts and unpermeabilized cells, and the supernatant was recentrifuged at 8000g for 35 min at 4°C, generating a pellet equivalent to fractions P1 plus P2 from our earlier work (Grimes and others 1997; Grimes and others 1996). This pellet, enriched in the heaviest cellular membranes and organelles, was washed once with fresh MES buffer, recentrifuged at 8000g for 35 min, and then lysed and immunoprecipitated.

Membrane preparation three: 100000g isolation (P3).

PC12 cells were permeabilized as described above. The permeabilized suspension was centrifuged at 1000g for 10 min to remove the cell ghosts and unpermeabilized cells (fraction P1 from our previous work) (Grimes and others 1997; Grimes and others 1996) from the released organelles and cytoplasm. The supernatant was then centrifuged at 8000g for 35 min to remove the P2 fraction. The resulting supernatant was diluted to 6 mL in MES buffer, then centrifuged at 100000g for 1 hr at 4°C, generating a pellet equivalent to fraction P3 from our previous work (Grimes and others 1997; Grimes and others 1996). This pellet, highly enriched in released vesicular structures ranging in average size from 63 nm to 180 nm (Grimes and others 1997; Grimes and others 1996), was lysed, immunoprecipitated, and subjected to SDS-PAGE.

Clathrin coated vesicle isolation.

The isolation scheme used to purify clathrin coated vesicles is diagrammed in Figure 4A, and is a modification of an established protocol (Maycox and others 1992). Following treatment as described above, cells were resuspended in 1 mL of CCV isolation buffer, triturated 2x with a 22-gauge needle to get a single cell suspension, then permeabilized by 5 complete passes through a Balch homogenizer. The resulting permeabilized cell suspension was then centrifuged at 1000g for 10 min at 4°C in a microfuge. The supernatant from this centrifugation, S1, was diluted to 2 mL with fresh CCV buffer, layered onto a 500 uL 5% glycerol pad made in CCV buffer, and centrifuged at 100000g for 1 hr at 4°C in a Sorvall AH-650 rotor and a Beckman XL-80 ultracentrifuge. The pellet, P2+P3 (equivalent to P2' from (Grimes and others 1997), was resuspended by gentle trituration into 250 uL of CCV buffer. This suspension was mixed with 250 uL of CCV buffer containing 12.5% (wt/vol) ficoll and 12.5% (wt/vol) sucrose, then centrifuged at 40000g for 40 min at 4°C in an ultracentrifuge. S4, the supernatant from this centrifugation, was diluted 1:5 in fresh CCV buffer, and then centrifuged at 100000g for 1 hr at 4°C in an ultracentrifuge. P5, the pellet from this round of centrifugation, was gently resuspended in 1 mL CCV buffer and centrifuged at 16000g for 20 min at 4°C in a microfuge. The supernatant from this spin, S6, was diluted to 2 mL with CCV buffer, layered onto a 500 uL 8% (wt/vol) sucrose pad prepared in CCV buffer that had been

made using D_2O, and centrifuged at 112700g for 2 hr at 4°C in a Sorvall AH-650 rotor and a Beckman XL-80 ultracentrifuge. The pellet resulting from this centrifugation, P7, was highly enriched in markers of CCVs.

Immunoprecipitation and Western Blotting

All samples were immunoprecipitated in 1 mL lysis buffer plus 100 uL of a 50% slurry of protein A/G-sepharose in lysis buffer, and the following amounts of antibody: 15 ug/mL 06574, 2 ug/mL SC170, or 1 ug/mL 327. Immunoprecipitations were performed overnight rotating at 4°C. The sepharose beads were then washed twice with lysis buffer, once with water, and then resuspended in 65 uL of sample buffer. Prior to loading 60 uL on 7.5% SDS polyacrylamide gels, samples were boiled for 5 min and then centrifuged for 1 min at 16000g. After transfer to nitrocellulose in a tris-glycine based transfer buffer with 20% methanol, blots were blocked for 1 hr at RT as described above. Blots were probed with the following antibody concentrations: 2.5 ug/mL TD.1, 0.25 ug/mL 4G10, 0.06 ug/mL 100/2, or 0.2 ug/mL SC170. HRP-conjugated secondaries were used for all blots at 1:20000 dilution. For sequentially probing blots, the membranes were either stripped by incubating for 15 min at RT in 0.2 N NaOH, or by incubating for 30 min at RT in TBS pH 2.0, followed by extensive washing in water and blocking for 1 hr at RT.

Transfection

The Cbl-pGEM4Z construct was kindly provided by Wallace Langdon (Nedlands, Western Australia). The Cbl insert was removed from the pGEM4Z backbone by XbaI and SalI restriction cuts, and then inserted into the pALTER-MAX expression vector (Promega, Madison, WI). For transfections, 15 ug of Cbl-pALT plasmid were incubated at RT for 30 min with 75 uL of LipoFectamine (Gibco BRL) in 1.6 mL OptiMem media (Gibco BRL). Then 1×10^7 PC12 cells on collagen-coated 10 cm plates were washed 2x with serum-free DMEM at 37°C, and the LipoFectamine/DNA complex in 6.4 mL OptiPrep was added to the cells. These cells were incubated for 3 hr at 37°C, and then 50 mL of DMEM plus serum were added. After 2 d transfectants were selected with 400 ug/mL geneticin.

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Figure 4.1 Depolarization Enhances NGF Internalization and TrkA Activation.

A) PC12 cells were incubated with ^{125}I-NGF for 1 hr at 37°C under depolarizing (50 mM K^+) or non-depolarizing conditions (5 mM K^+). Depolarization increased the amount of internalized radiolableled NGF by nearly 2-fold (192%±10%; n=3; *=statistically significant, p<0.005). The effect was dependent upon extracellular calcium, as depolarization in the absence of calcium and magnesium did not elicit an increase in NGF internalization.

B) Depolarization enhances TrkA phosphorylation in response to NGF. Cells were treated for 2 min with NGF (2 nM) in the presence of various concentrations of K^+. 50 mM K^+ elicited the maximal increase in TrkA phosphorylation.

C) Depolarization enhances TrkA phosphorylation at early times, but has no effect at later timepoints. 50 mM K^+ increased TrkA phosphorylation in response to NGF (2 nM) at 1 and 2 minutes, but did not alter the response to 5 min of NGF.

D) The effect of depolarization on TrkA phosphorylation in response to NGF was dependent upon extracellular calcium. 50 mM K^+ increased TrkA phosphorylation in response to 2 min of NGF (2 nM). The effect was abrogated when cells were treated in the absence of calcium and magnesium.

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Figure 4.2 Cbl is Phosphorylated in Response to NGF Treatment.

A) PC12 cells were treated with 5 or 50 mM K^+ for 5 min, or were treated with NGF (2 nM) in the presence of 5 or 50 mM K^+ for 5 min. Total cellular lysates were resolved by SDS-PAGE, and the resulting blot was probed for the presence of tyrosine phosphorylated species. A 120 kDa band was phosphorylated in response to depolarization and in response to NGF, but was less phosphorylated when NGF was applied together with 50 mM K^+.

B and C) This protein was identified as Cbl. NGF (2 nM) elicited tyrosine phosphorylation of Cbl that peaked at 5 min.

D) NGF-induced phosphorylation of Cbl was dependent upon TrkA kinase activity. Cells were treated for 5 min with NGF (2 nM). KB PC12 cells show an increase in Cbl phosphorylation in response to NGF, as indicated above. 6.24 and P8-nnr5 cells overexpressing TrkA exhibit increased phosphorylation of Cbl in response to NGF. M1-nnr5 and 22.7-nnr5 cells expressing kinase-defective TrkA do not show an increase in Cbl phosphorylation in response to NGF. Likewise, 490/785-nnr5 cells, which express TrkA lacking 2 critical non-kinase domain tyrosines, also do not show an increase in Cbl phosphorylation in response to NGF.

Figure 4.3

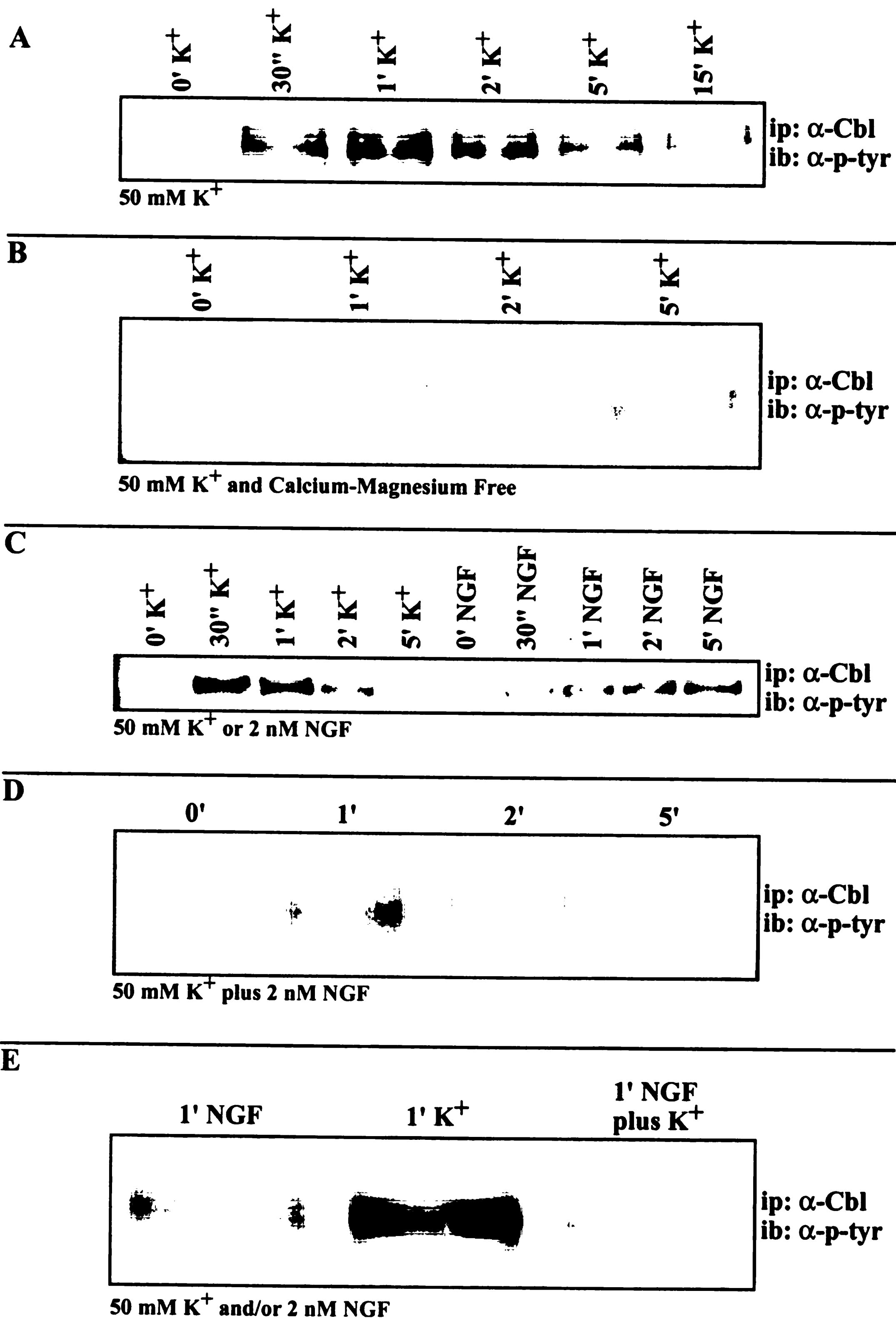


Figure 4.3 Cbl is Phosphorylated in Response to Depolarization.

A) PC12 cells were treated for various times with 50 mM K^+. Depolarization elicited maximal tyrosine phosphorylation of Cbl at 1 min.

B) The effect of depolarization on Cbl phosphorylation was dependent upon the presence of extracellular calcium.

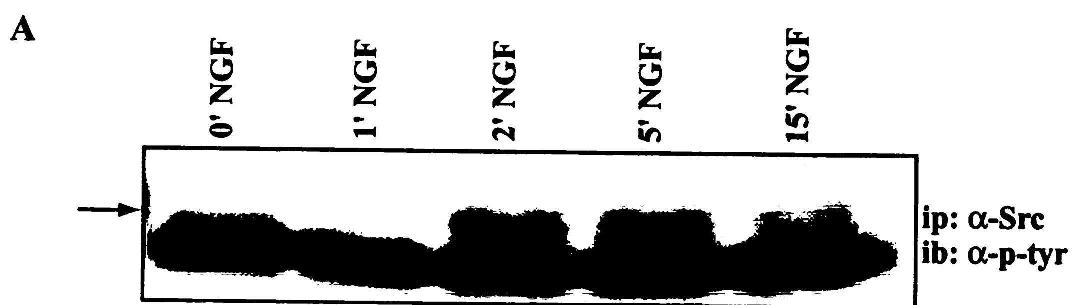
C) Depolarization (50 mM K^+) elicited phosphorylation of Cbl that was several-fold greater than that elicited by NGF (2 nM) and occurred over an accelerated timecourse.

D) NGF (2 nM) plus depolarization led to Cbl phosphorylation that peaked by 1 min, recapitulating the timecourse that is elicited in response to K^+ only.

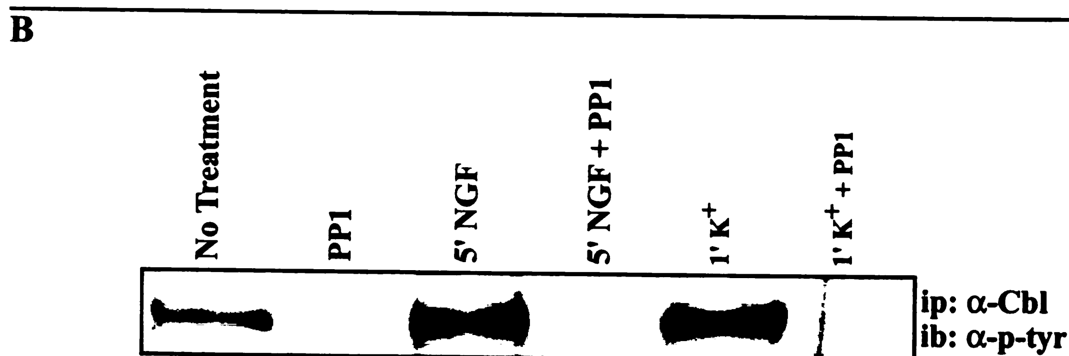
E) NGF (2 nM) plus depolarization led to maximal Cbl phosphorylation that was much less intense than that elicited by either NGF or depolarization only.

Figure 4.4

A



B



C

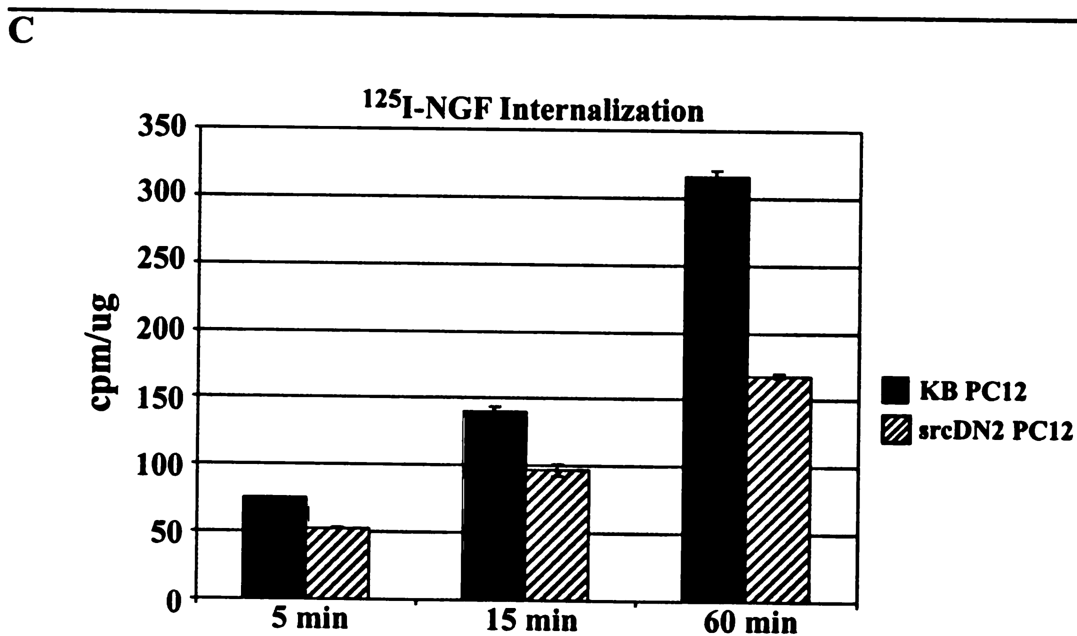


Figure 4.4 Src Mediates Cbl Phosphorylation in Response to NGF and Depolarization and is Involved in NGF Internalization.

A) PC12 cells were treated for various times with NGF (2 nM). Src tyrosine phosphorylation, a marker of Src activation, was maximal between 2 and 5 min of NGF treatment.

B) Inhibition of Src activity with 100 nM PP1 blocked Cbl phosphorylation in response to NGF (2 nM) and depolarization (50 mM K^+), and also blocked all background Cbl phosphorylation.

C) Internalization of ^{125}I-NGF was reduced in srcDN2-PC12 cells expressing a dominant-negative form of Src. NGF internalization was reduced at all timepoints considered. By 60 min, the amount of NGF internalized by srcDN2-PC12 cells was half that internalized by normal KB PC12 cells.

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Figure 4.5 Cbl is Trafficked to Specific Subcellular Membrane Fractions in Response to NGF Treatment.

A) NGF (2 nM) induces the association of Cbl with a total cellular membrane fraction that includes plasma membrane and most intracellular membranes.

B) Cbl is rapidly and maximally phosphorylated in the cytosol, and is dephosphorylated as it associates with total cellular membranes.

C) Phosphorylated Cbl is recruited to the P2 and P3 fractions in response to 2 min of NGF (2 nM). Such recruitment to P3 is blocked by inhibition of Src activity with 100 nM PP1, but is unaffected in the P2 fraction.

D) Cbl is induced to leave a highly enriched clathrin coated vesicle fraction following NGF treatment. Given that the absolute amount of clathrin coated vesicles isolated following 2 and 5 min of NGF is 2-fold greater than that isolated from untreated cells, the amount of Cbl within this fraction following NGF treatment is actually even less in terms of specific activity.

Figure 4.6

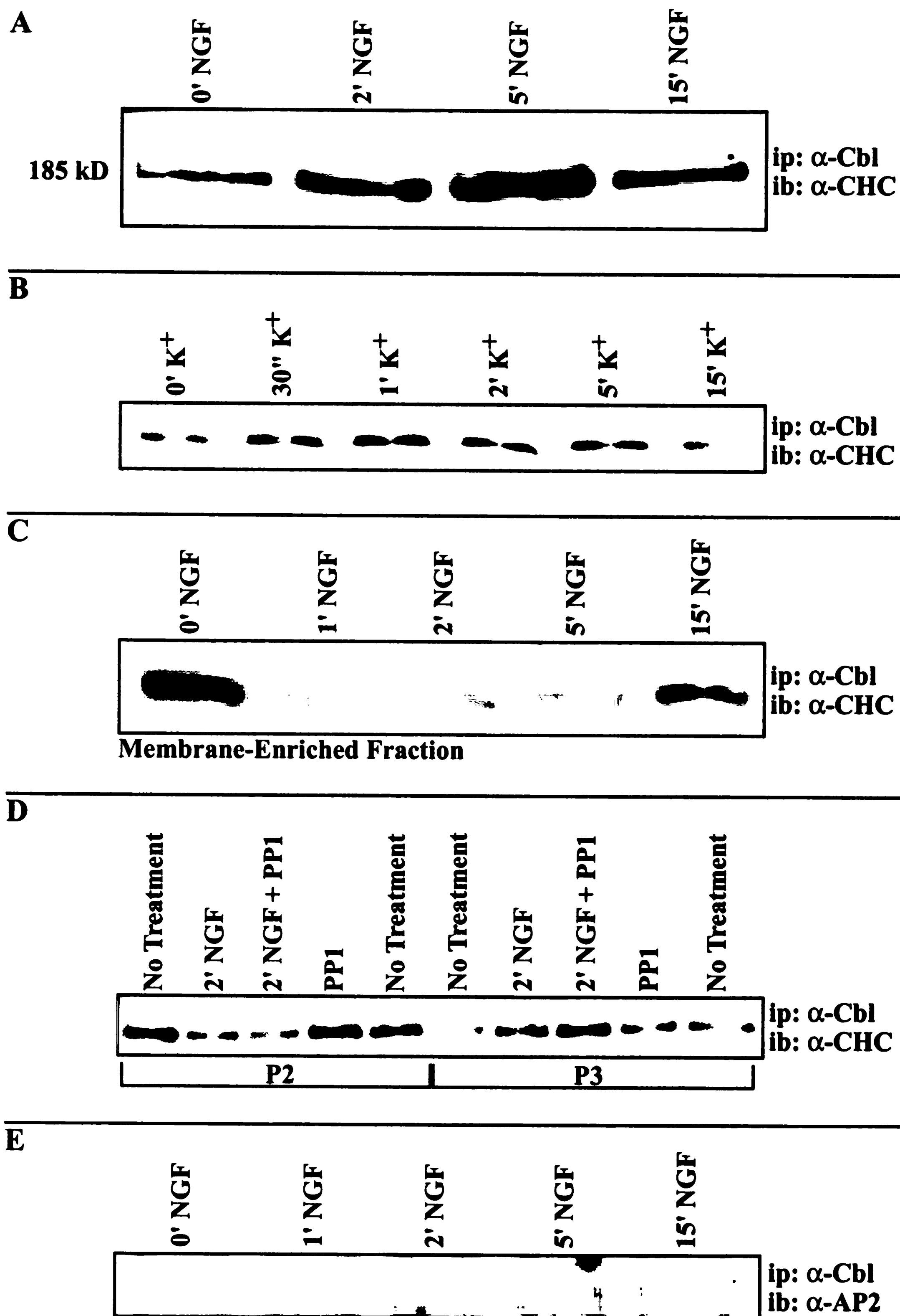

Figure 4.6 Clathrin Heavy Chain is Found in Complex with Cbl Following NGF Treatment and Depolarization.

A) NGF (2 nM) induces the association of CHC with Cbl. The association is maximal at 5 min, the same time that Cbl phosphorylation is maximal in response to NGF treatment.

B) Depolarization also leads to the formation of a Cbl:CHC complex, and the peak in association matches the peak of Cbl phosphorylation induced by 50 mM K^+.

C) CHC is associated with Cbl within a total membrane fraction in untreated cells. However, following NGF (2 nM) treatment, as Cbl is becoming more associated with membranes and is becoming less phosphorylated, the Cbl:CHC complex is broken.

D) As with total cellular membranes, Cbl within the P2 fraction is preassociated with CHC. This association is broken by treatment with NGF (2 nM). This effect is blocked by Src inhibition with PP1. In contrast, a Cbl:CHC complex increases in the P3 fraction following NGF treatment for 2 min. This increase is not blocked by inhibition of Src by PP1, even though Cbl phosphorylation is blocked.

E) AP2 associates with Cbl in response to NGF treatment along a timecourse that matches the increase in CHC found in Cbl immunoprecipitates.

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Figure 4.7 Overexpression of Cbl Enhances Neurite Outgrowth in Response to NGF Treatment.

A) PC12 cells were transfected with a full-length Cbl expression vector.

B) This Cbl is phosphorylated in response to NGF. Hence, transfectants respond to NGF with dramatically enhanced Cbl phosphorylation.

C-J) PC12 cells or cells overexpressing Cbl (Cbl-OX) were treated with NGF for 1 or 2 days. C) Untreated PC12 cells. D) PC12 cells treated with 0.1 nM NGF for 1 d do not show neurite outgrowth. E) PC12 cells treated with 1.0 nM NGF for 1 d also do not exhibit neurites. F) Untreated Cbl-OX cells show small neuritic processes. G) In response to 0.1 nM NGF for 1 d Cbl-OX cells exhibited increased neurite outgrowth. H) 1.0 nM NGF for 1 d elicits even greater neurite outgrowth in Cbl-OX cells. I) PC12 cells treated with 1.0 nM NGF for 2 d show a mixture of cells that exhibit neurites and cells that do not. J) In contrast, 2 d of 1.0 nM NGF causes all Cbl-OX cells to exhibit robust and highly branched neurites.

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Figure 4.8 Model of the Role Cbl and Src Play in TrkA Internalization.

Both depolarization and NGF may lead to increases in intracellular calcium levels. Such increases may result in activation of Src. Alternatively, depolarization and TrkA activation may lead to Src activation via separate mechanisms. Once activated, Src phosphorylates Cbl within the cytosol, inducing the formation of a complex that contains Cbl and CHC, and may contain Src. This complex is recruited to the plasma membrane, bringing CHC into proximity of TrkA. Cbl may be dephosphorylated following association with TrkA or the plasma membrane. Increased association of CHC with TrkA may lead to internalization of the receptor into a variety of intracellular vesicles, including clathrin-coated vesicles and larger endosomal organelles. The trafficking of Cbl and the Cbl:CHC complex to these membranes is not yet resolved.

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Figure 4.9 Sequence Alignment of the Clathrin Binding Domain of β-Arrestin and a Small Fragment of Cbl.

The region of β-arrestin that mediates binding to clathrin contains an LIELD motif that is critical to the binding interaction. This region aligns with an LRPLD sequence within Cbl, suggesting that this region may share functional homology.

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Figure 4.10 Clathrin-Box Homology.

A number of proteins that bind clathrin contain a consensus sequence that mediates this binding. Cbl contains an LRPLD sequence that may conform to an LxxLD clathrin-box consensus motif.

Chapter 5

Nerve Growth Factor Effects on Cholinergic Modulation of Hippocampal and Cortical Plasticity

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I. Introduction

The idea that diffusible trophic factors are important for the development of the nervous system was first postulated around the turn of the century by such authors as Shorey (292, 293) and Ramon y Cajal (262, 263, 264). It was later extended by Levi-Montalcini and Hamburger, who showed that diffusible neurotrophic factors released by targets of innervation act to enhance the survival and differentiation of developing neurons (130, 190, 192) . In the years since, a role for neurotrophic factors in cell death, cell proliferation, cell maturation, cell differentiation, and synapse elimination has been detailed and described by a host of authors (187, 188, 232, 233, 234, 259, 260, 261, 315), and can be distilled into three basic principles described by the "Neurotrophic Factor Hypothesis" (259). These three principles are: 1) during development an excess of connections are initially formed between neuronal populations and their target tissue; 2) these projection neurons depend upon and compete with their neighbors for limiting amounts of a neurotrophic factor (NTF) produced by the cells of the target tissue; 3) those neurons which most successfully compete for the NTF survive and thrive, while those that are unsuccessful are eliminated (Figure 5.1). The Neurotrophic Factor Hypothesis was initially developed in the context of the peripheral nervous system (PNS), and especially with regard to the pre-eminent growth factor – nerve growth factor (NGF).

More Recently, the Neurotrophic Factor Hypothesis has been expanded to include a role for growth factors in other aspects of nervous system development and maintenance, including synaptic plasticity – the dynamic functional and morphological modification of synaptic connections within the developing and mature central and peripheral nervous systems. Neurotrophins have been shown to play key roles in the generation and maintenance of synaptic events that are crucial to learning and memory. Largely due to the use of only a few experimental paradigms and to a concentration on the function of glutamatergic synapses, most research on the role of NTFs in synaptic plasticity has focused on members of the neurotrophin family other than NGF. Furthermore, NGF has proven relatively ineffective in current paradigms of learning and memory plasticity (e.g. long-term potentiation in hippocampal slice preparations). We think that this is the result of failing to effectively test the relationship between NGF and primary synapses within the hippocampus and cortex. TrkA, the receptor tyrosine kinase for NGF, is not present on primary synaptic elements (e.g. Schaffer collaterals and dendrites of CA1 hippocampal pyramidal neurons; thalamocortical afferent terminals and cortical postsynaptic neurons). Rather, it is present on a highly specific population of cholinergic afferents which play a critical role in modulating activity at primary hippocampal and cortical synapses. It is interesting to speculate as to how NGF may influence the biology of these synapses through its actions on cholinergic afferents. We will offer a model in which NGF serves as a metamodulator of synaptic plasticity. In

other words, while NGF does not directly modulate synaptic activity at primary hippocampal and cortical synapses, it does powerfully and dynamically modulate the efficacy of cholinergic innervation of these primary synapses, thereby controlling the degree and nature of their synaptic plasticity (Figure 5.2). In fact, we propose that NGF signaling through the TrkA receptor within the hippocampus and cortex serves to create a plasticity space – a structural and functional domain in which the number or efficacy of synapses can be changed (Figure 5.3). The size of this space, dynamically regulated by NGF and NGF signaling, is envisioned as determining the extent to which learning and memory can occur, and controlling the efficacy of experience-dependent plasticity. In what follows, we will describe the history of NGF and the NTF family, the dynamic relationship between NGF actions and the basal forebrain cholinergic system, the role of this cholinergic system in learning, memory, and experience-dependent plasticity, and finally, the role NGF plays in these same events. We will make the case that NGF plays a central, critical role in the plasticity of cortical and hippocampal synapses.

II. The History of NGF and the Neurotrophin Family

NGF was first identified in landmark experiments performed by Rita Levi-Montalcini and Victor Hamburger in the late 1940's and early 1950's. In concert with earlier observations, they identified the target of projection neurons as key to the

development of those same neurons, and suggested that the target determined the size of its neural ganglia, perhaps via "metabolic exchange between the neurite and the substrate in which it grows" (130). This idea of "metabolic exchange" was first proposed by Marian Shorey in her work on motor neuron differentiation and survival (292, 293), and by Santiago Ramón y Cajal in his work on neuroblast differentiation (262), but wasn't applied to a system which would permit identification of a specific factor(s) responsible for the exchange until Bueker implanted a fragment of a mouse sarcoma into the body wall of a 3-day old chick embryo (35). He discovered that the sarcoma induced a 20-40% enlargement of the dorsal root ganglia (DRG) which innervated the graft, with no apparent effect on motor neuron growth. Levi-Montalcini and Hamburger repeated these experiments and discovered that, in addition to DRG enlargement, the sarcoma induced extensive innervation of the graft by sympathetic neurons, and that these sympathetic ganglia were also enlarged (190). They hypothesized that the sarcoma released a soluble, diffusible factor which promoted the growth and differentiation of sensory and sympathetic neurons, and called the agent the nerve growth factor (NGF). With the finding in 1954 that the mouse sarcoma could induce a growing halo of neurites from an explanted sympathetic ganglion (192), an *in vitro* bioassay was developed that made it possible to purify and characterize NGF from snake venom (52) and from mouse submandibular gland (51).

Due largely to the availability of significant amounts of purified NGF and of NGF antibodies, the role of NGF in the development of sympathetic neurons and neural crest-derived sensory neurons was studied extensively. Indeed, for many years NGF was the only well-defined NTF. Receptors for NGF are present on sensory and sympathetic axon terminals at the time of their innervation of target tissues (61, 340), and there is a strong correlation between NGF levels in the target and the extent of innervation (178, 291). Furthermore, delivery of anti-NGF serum during development leads to the death of sympathetic neurons and elimination of sympathetic ganglia (51). Thus, for sympathetic and sensory neurons in the PNS, NGF appears to satisfy the requirements for a target-derived neurotrophic factor as predicted by the neurotrophic factor hypothesis.

Since the initial discovery of NGF, many other NTFs have been identified and isolated (Table 1). NGF is now considered to be the first identified member of the neurotrophin (NT) gene family. This family also includes brain-derived neurotrophic factor (BDNF), neurotrophin-3 (NT-3), and neurotrophin-4/5 (NT-4/5). BDNF was discovered as the result of experiments which showed that NGF supports only a subset of neural crest-derived sensory neurons and does not support cranial ganglia-derived sensory neurons, even though these neurons are completely dependent upon target-derived factors for survival. BDNF was originally isolated as a neurotrophic factor for nodose ganglion neurons (13, 89, 184), and its function was later extended to the central nervous system (205, 250, 251, 284, 315, 344). The discovery of BDNF catalyzed the

cloning efforts that eventually identified NT-3 and NT-4/5. NT-3 was identified as a specific factor required for survival of the large dorsal root ganglia sensory neurons that innervate muscle and skin (91, 146, 208), while NT-4/5 was found to play a role in the same systems as BDNF (151, 204). Gene disruption studies have shown that each of the neurotrophins, as well as other neurotrophic factors such as ciliary neurotrophic factor (CNTF) and glial-cell line-derived neurotrophic factor (GDNF), do fulfill the role defined by the original formulation of the neurotrophic factor hypothesis for specific neuronal populations (193) (Table 1). Interestingly, however, their original role as survival-mediating target-derived factors has been extended to include a role in the functional modification of synaptic connections within the central and peripheral nervous systems (23, 28, 205, 307). This is particularly well documented for BDNF, NT-3, NT-4/5, and CNTF.

BDNF, NT-3, and CNTF have been shown to exert a potentiating effect on the frequency of spontaneous synaptic currents when applied to developing *Xenopus* neuromuscular junctions (206, 306). This effect was caused by enhancement of neurotransmitter release, either by increasing the number of available synaptic vesicles, or by potentiating the probability of vesicular fusion and consequent transmitter release. Furthermore, BDNF and CNTF were able to synergistically potentiate transmitter release (307), suggesting that coincident availability of multiple neurotrophic factors at presynaptic terminals may generate complex effects on synaptic potentiation.

A number of recent studies link the actions of NTFs to synaptic plasticity. NTF secretion is increased by depolarization or synaptic activation (27, 126, 322), and neurotrophic factor expression within the central nervous system is regulated by electrical activity (10, 109, 128, 153, 165, 172, 251, 316, 325, 343, 344). Moreover, there is widespread cortical and hippocampal expression of neurotrophins and their cognate receptors (11, 29, 143, 168, 252, 324). These findings suggest that neurotrophins may play a role in synaptic plasticity within the central nervous system. Indeed, BDNF and NT-3 were shown to potentiate synaptic transmission at the synapse formed between Schaffer collaterals and CA1 pyramidal neurons within the hippocampus (158, 159, 160, 161, 180, 181, 195, 196). This neurotrophin-induced potentiation led to a decrease in paired-pulse facilitation, an electrophysiological correlate of short-term plasticity (286), indicating a presynaptic mechanism, and only partially occluded another form of synaptic potentiation, termed long-term potentiation (LTP), suggesting at least partial divergence in potentiation mechanisms utilized by the neurotrophins and LTP. Interestingly, the stimulation paradigm used to elicit LTP induces significant increases in BDNF and NT-3 mRNA, suggesting that increased neurotrophin expression may play a role in LTP (251). This is supported by a significant impairment of LTP in mice with targeted disruption of the BDNF gene (179, 250) – an impairment which was rescued by acute treatment with exogenous BDNF (250) or by adenovirus-mediated BDNF expression (179). Furthermore, inhibition of the BDNF receptor tyrosine kinase, TrkB, by application of a

TrkB-IgG fusion protein, reduced the magnitude of LTP induced in hippocampal slices (97). Finally, Erin Schuman and her colleagues have shown that different temporal patterns of synaptic activity yielded forms of LTP that displayed differential sensitivity to TrkB activation (161), suggesting that the role of neurotrophins in synaptic plasticity is dynamically regulated by the nature of synaptic activity. As will be discussed, while NGF was shown to be ineffective in modifying LTP in these experiments, it does play a significant role in modifying and modulating potentiation within the context of a highly specific synaptic system involving cholinergic innervation arising from the basal forebrain.

III. The Relationship Between NGF and Basal Forebrain Cholinergic Neurons

Basal forebrain cholinergic neurons (BFCNs) provide the best studied model for the application of the neurotrophic factor hypothesis to the central nervous system (CNS). The cells of the basal forebrain project to specific targets within the hippocampus and neocortex (Figure 5.4), and these targets produce NGF. BFCNs express both NGF receptors: p75NTR, a single transmembrane glycoprotein that is homologous to members of the tumor necrosis factor receptor family, and TrkA, a receptor tyrosine kinase that is coupled to several critical signaling cascades (123). A significant amount

of data indicate that NGF is a trophic factor for BFCNs. During development there is tight correlation between NGF levels in the hippocampus and neocortex and elaboration of choline acetyltransferase (ChAT) activity in the basal forebrain (7, 183, 327). Furthermore, intraventricular administration of NGF to postnatal and adult rats elicits a robust and selective enhancement of ChAT activity in the basal forebrain and in the targets of BFCN innervation (116, 155, 230, 231), and leads to increases in BFCN cell size, and increased basal forebrain expression of ChAT, p75NTR, and TrkA mRNA (47, 194, 346). Likewise, acetylcholine dynamically regulates expression of NGF mRNA in hippocampal neurons (60). Finally, intraventricular administration of anti-NGF antibodies reduces BFCN ChAT activity, and reduces TrkA and ChAT gene expression (194, 321), while NGF heterozygous gene deletion leads to a reduction in BFCN cell number and size (50). Taken together, these data implicate NGF as a neurotrophic molecule that is present in limiting amounts and for which BFCNs compete during development.

Is there a role for NGF in adult BFCNs? There are high levels of NGF mRNA and protein in adult hippocampus (117, 177, 291, 327), and TrkA and p75NTR continue to be expressed in adult BFCNs and to bind NGF. Furthermore, adult BFCNs respond to intraventricular delivery of NGF by becoming hypertrophic and exhibiting increased gene expression (144; see also 33). Also, the axon terminals of adult BFCNs retrogradely transport NGF from the hippocampus and cortex to the cell body (287, 288). Transection

of the fimbria-fornix, which severs BFCN projection axons, results in elevation of hippocampal NGF levels (113, 323) without concomitant changes in NGF mRNA, suggesting that NGF is accumulating as a result of decreased retrograde transport. BFCNs die following fimbria-fornix transection, but can be rescued by intraventricular administration of NGF (136, 176, 182, 333). On the other hand, ablation of target neurons in the hippocampus of adult animals with sparing of BFCN axons does not lead to BFCN death, but only to cell shrinkage and decreased ChAT immunostaining (299, 300, 312), suggesting that intact adult BFCNs are dependent upon target-derived NGF for maintenance of cholinergic phenotype, but not for survival. This is further supported by recent evidence which shows that intracortical infusion of anti-NGF antibodies or a TrkA antagonist quickly causes a reduction in the number and size of vesicular acetylcholine transporter-immunoreactive sites, again suggesting a role for NGF in maintenance of cholinergic phenotype (66). These data indicate that a developmental switch occurs, whereupon BFCNs change from a dependence on NGF for survival and differentiation, to a dependence on NGF for maintenance of phenotypic differentiation only. Axotomy may reverse the switch, recreating in the axotomized neuron a dependency upon NGF for survival. The NGF-dependent maintenance of mature CNS neurons was not anticipated by the original neurotrophic factor hypothesis, but is a finding that is consistent with continuing NGF actions in neurons of the PNS (118).

Why NGF is required to maintain mature BFCNs is unknown. However, we note that these neurons play a critical role in modulating realtime cortical and hippocampal processing involved in learning and memory. We propose that NGF, supplied by target neurons, acts to modulate the activity of BFCN axon terminals. NGF may serve not only to maintain the cholinergic phenotype of BFCNs, but also to influence synaptic biology by dynamically modifying and modulating cholinergic synaptic efficacy and connectivity. This property of NGF actions may exist during development and into adulthood, creating a "plasticity space" in which cholinergic innervation dynamically shapes, modifies, modulates, and facilitates learning and memory. In support of this model, we will first analyze the role of cholinergic innervation in learning and memory, and then in experience-dependent plasticity. Then we will examine the role of NGF in these same forms of plasticity, finally detailing a model which places NGF in the unique position of regulating cholinergic modulation of hippocampal and cortical plasticity.

IV. Anatomy and Connectivity of Basal Forebrain Cholinergic Neurons

The anatomy of BFCN connectivity is both widespread and complex. BFCNs project in a topographically organized manner to virtually all regions of the hippocampus, limbic system, and neocortex (Figure 5.4) (228, 229), and form synaptic connections with

virtually all classes of neurons. Cholinergic axons synapse onto dendrites and cell bodies of target neurons, and onto presynaptic elements of intrinsic hippocampal and cortical connections. The widespread nature of cholinergic innervation is further complicated by the presence of a host of muscarinic and nicotinic acetylcholine receptor subtypes (53, 70, 186) which mediate specific and sometimes disparate actions. However, BFCNs can be grossly categorized on the basis of their anatomical location within the basal forebrain. Four principle structures comprise the basal forebrain cholinergic system: the medial septal nucleus (MSN), the vertical nucleus of the diagonal band of Broca (VDB), the horizontal nucleus of the diagonal band of Broca (HDB), and the nucleus basalis of Meynert (NBM), also known respectively as Ch1, Ch2, Ch3, and Ch4 in the nomenclature of Mesulam (228, 229).

The MSN is comprised of small cholinergic neurons which lie medially within the medial septum. These neurons project primarily to the hippocampus, and exhibit a topography which maps mediolateral position within the MSN to dorsoventral (septal-temporal) position within the hippocampus (114, 246, 295, 301). Cholinergic neurons within the VDB also project to the hippocampus, but also to cingulate cortex, the preoptic area, and the hypothalamus (25). The HDB contains cholinergic neurons which are larger than those of the MSN and VDB, and which project to the entorhinal cortex, the pyriform cortex, and the olfactory bulb (342). Finally, the NBM contains magnocellular cholinergic neurons which provide the principle cholinergic innervation of all neocortical

regions, as well as of the basolateral nucleus of the amygdala (227, 228, 229, 273, 337). These four nuclei, MSN, VDB, HDB, and NBM, form a contiguous group of cholinergic neurons which together serve to innervate the entire allo- and neocortex, and which provide global modulation of intrinsic synaptic connections within virtually every region involved in learning, memory, and adaptation.

V. The Role of Cholinergic Neurotransmission in Learning- and Memory-related Plasticity

Theta rhythm, a physiologically relevant marker of cholinergic modulation, is an oscillatory pattern generated in hippocampal and cortical neurons by cholinergic innervation during periods of learning. Cholinergic-agonist induced theta oscillations create a state of enhanced hippocampal plasticity during which synapses can be modified by normally ineffective stimulation. This enhancement of plasticity is sensitive to the temporal correlation between stimulation and oscillation, in that stimulation in-phase with the theta oscillation is amplified, while out-of-phase inputs are depressed (149). The effects of cholinergically-induced theta rhythm on physiological measures of potentiation are recapitulated in the relationship between muscarinic acetylcholine receptor (mAChR) antagonism, memory impairment, and hippocampal theta rhythm. For example,

scopolamine, a mAChR antagonist, induces memory impairment as assessed by T-maze alternation, and is accompanied by increased theta rhythm. This effect could be reversed by carbachol, a muscarinic agonist, suggesting that loss of correlation between the cholinergic rhythmic oscillations and behaviorally meaningful environmental stimuli led to an inability to modify working memory (115).

Cortical population potentials are also enhanced by cholinergic afferents. This enhancement is correlated with acetylcholine (ACh)- or muscarinic agonist-induced modification of long-term potentiation (LTP), a form of synaptic potentiation which is regarded as critical to learning and memory (16, 42, 73, 152, 255, 305). In neocortex, the muscarinic agonist oxotremorine induces a short-term depression of evoked sensorimotor cortical responses (200), followed by a slow-onset, progressively developing, long-lasting potentiation of glutamate-evoked potentials. This potentiation is inhibited by pre-application of muscarinic antagonists such as atropine, pirenzepine, or gallamine (199). Furthermore, low concentrations of carbachol (0.01-0.1 uM) attenuate tetanus-induced LTP in the hippocampus without affecting pre-stimulus field excitatory post-synaptic potential (EPSP) amplitude. This inhibitory effect is reduced by the M2 mAChR subtype-specific antagonist AF-DX-116, but not by pirenzepine, an M1 mAChR specific antagonist. In contrast, higher concentrations of carbachol (10 uM) decrease the amplitude of pre-stimulation field EPSPs, but significantly potentiate the magnitude of LTP. This facilitation is sensitive to M1 mAChR antagonism, but not to AF-DX-116

antagonism of M2 mAChR. Hence, hippocampal mossy fiber-CA3 LTP is sensitive in a concentration-dependent manner to either facilitation or inhibition by ACh acting through M1 or M2 mAChR subtypes, respectively (186, 210). These data suggest that ACh plays a significant role in modulating LTP, and that this modulation has behaviorally-relevant consequences.

A role of ACh in memory formation is further supported by studies of ACh synthesis. Increased hippocampal and cortical levels of choline acetyltransferase (ChAT), the key synthetic enzyme for ACh, are correlated with accelerated Morris water maze learning in rats (249). In these experiments, rats were exposed to enriched environments during the course of development. This exposure led to long-lasting increases in ChAT activity and ACh synthesis, changes which may have accelerated learning and memory formation in the hippocampus and cortex. This speculation is supported by data showing that hippocampal ChAT activity levels in aged rats are correlated with behavioral performance in the Morris water maze (79). Aged rats with elevated hippocampal ChAT activity performed better in a spatial learning task than age-matched rats with lower ChAT activity levels. Finally, Rylett and Schmidt have shown that increased production of ChAT generates a long-lasting increase in ACh synthesis and release. They suggest that this change enhances plasticity by priming the hippocampus and cortex for memory formation (275).

How does enhanced ACh release lead to potentiation of hippocampal and cortical plasticity? Synchronization of ACh release from cholinergic terminals and glutamate release at primary hippocampal or cortical synapses may facilitate signaling through glutamate receptors in three ways. First, cholinergic stimulation may help relieve the Mg^{2+} block of the NMDA glutamate receptor subtype (NMDAR) by inducing Ca^{2+} influx or Ca^{2+} release from internal stores (Figure 5.5). Cholinergic facilitation of NMDAR activation by Mg^{2+} relief may bring NMDAR-mediated coincidence detection mechanisms into play, thereby enhancing learning and memory related potentiation events (73). A second potential mechanism of cholinergic facilitation may involve interactions between downstream signaling cascades initiated by glutamate receptors and mAChR (Figure 5.5). NMDAR and metabotropic glutamate receptors (mGluR) activate several protein kinase signaling cascades in common with mAChR, including calcium/calmodulin kinase II (CamKII) (107, 140, 242, 313), protein kinase C (PKC) (5, 30, 106, 319, 339), protein kinase A (PKA) (157, 219, 270, 311), phosphoinositide 3-kinase (PI3-K) (207, 345), src (96, 150, 175, 209, 245, 279), and mitogen-activated protein kinase (MAPK) (85, 86, 131, 164, 245, 338). Each of these kinases have been implicated in the induction of LTP, and facilitation or modulation of any or all of them by coincident mAChR and glutamate receptor activation could enhance or accelerate synaptic potentiation. Furthermore, several of these kinases appear to be involved in feedback phosphorylation of NMDAR subunits, an event which has been implicated in

modulation of NMDAR activity (19, 140, 310, 326). For example, carbachol microinjection has been shown to induce tyrosine phosphorylation of the NMDAR 2B subunit in a manner comparable to physiological stimulation (271). Such cholinergically-mediated phosphorylation of NMDAR may be involved in potentiating synaptic plasticity by facilitating NMDAR-dependent signaling or by modifying NMDAR and mGluR trafficking (202, 203).

Finally, cholinergic afferents may also facilitate synaptic potentiation by enhancing morphological modification of synapses in the hippocampus and cortex. It has been suggested that mAChR-mediated stimulation of protein kinase pathways leading to phosphorylation of microtubule-associated protein 2 (MAP-2) might destabilize the postsynaptic cytoskeleton and permit spine elaboration and elongation (336). If so, potentiation could occur during a cycle of brief, permissive degradation of pre-existing dendritic MAP-2 networks, followed by a round of new MAP-2 synthesis and elaboration. The selective enrichment of mAChR and MAP-2 in specific populations of hippocampal and cortical neurons suggests that these cells could be primed for cholinergically-mediated morphological plasticity (336). Furthermore, it has been shown that passive avoidance training enhances mAChR immunoreactivity, and that the neurons enriched in mAChR are also enriched in PKC and MAP-2 (319). Moreover, mAChR-mediated PKA activation (311) leads to phosphorylation of MAP-2 at sites which protect it from calpain-mediated proteolysis (154), an event that could promote the formation of

new dendritic branches and spines, or restructure pre-existing synaptic structures. The evidence discussed so far supports a role for ACh in learning and memory and in potentiation of synaptic plasticity. If ACh is in fact a central element in synaptic plasticity, then cholinergic innervation should play a key role in learning and memory.

We will now focus our attention on the role of cholinergic innervation in spatial learning and experience-dependent plasticity of the visual cortex. Although it will not be reviewed, there is also evidence for involvement of cholinergic innervation in other systems such as auditory and somatosensory cortex plasticity (1, 15, 82, 166, 276, 347). The majority of data regarding the role of cholinergic innervation in learning and memory are culled from lesion studies. Lesions of the basal forebrain with ibotenic acid, quisqualic acid, AMPA, or IgG-saporin result in impairment of Morris water maze acquisition and radial maze performance (54, 74, 185, 213, 214, 278, 281). Furthermore, these impairments are alleviated by treatment with cholinergic agonists or by transplantation of grafts that release ACh (80, 98, 141, 142, 335).

BFCNs have also been shown to be involved in attentional processes which are critical to learning and memory. One example is found in studies of visual attention. In these studies, rats were trained to detect brief flashes of light presented randomly in a five-choice serial reaction apparatus that consisted of five spatially discrete lights located at one end of a chamber. The rats were trained to respond at the location of the most recently presented light flash, run to the opposite end of the chamber to receive their

reward, and then turn and attend to the light array for the next stimulus. This task was shown to be impaired by ibotenic acid-, quisqualate-, and AMPA-induced lesions of the NBM (237, 238, 239, 268, 269), by direct infusion into basal forebrain of muscimol, a GABA receptor agonist, (241) or by intraventricular infusion of hemicholinium (238). These defects appear to be specifically mediated by a cholinergic deficit, since physostigmine, nicotine, and transplantation of ACh releasing fetal neurons all reversed the impairments (238, 240).

VI. The Role of Cholinergic Neurotransmission in Experience-dependent Visual Cortical Plasticity

ACh has been shown to play a role in experience-dependent modifications of the visual cortex (18). Experience-dependent plasticity of visual cortex is an extremely useful system for analysis of factors putatively involved in learning and memory-related synaptic modifications. This form of plasticity differs temporally from that of spatial learning and memory-dependent attentional processes, in that the modifications occur over days and weeks and within tightly defined windows of visual cortical development.

Monocular deprivation (MD) is the hallmark experimental manipulation in the study of visual cortical experience-dependent plasticity. Hubel and Wiesel demonstrated

nearly forty years ago that the visual cortex of kittens is highly susceptible to use-dependent modification of both structural and functional architecture (147, 148, 329, 330, 331). At birth, the kitten visual cortex is still in a plastic, immature state in which most neurons respond equally well to visual stimulation through either eye (Figure 5.6A). Over the course of the first two postnatal months, the functional connectivity of the visual cortex gradually solidifies into a highly ordered state wherein eye-specific afferents from the lateral geniculate nuclei (LGN) innervate alternating patches of the visual cortex called ocular dominance columns (ODCs) (Figure 5.6B). Neurons within any given ODC are heavily biased to respond preferentially to stimulation through one eye or the other (Figure 5.7A). As elegantly proven by Hubel and Wiesel, formation of ODCs in kitten visual cortex is highly labile, and is malleable between approximately the third and eighth weeks of postnatal development. MD via monocular lid suture within this so-called critical period is sufficient to generate nearly complete reorganization of ODCs such that the majority of visual cortical neurons lose their ability to respond to the deprived eye (DE) (Figure 5.7B). This effect is marked anatomically by a reduction in cortical territory occupied by terminals of DE LGN afferents, and a concomitant expansion in territory occupied by non-deprived eye (NDE) afferent terminals (Figure 5.6C). Such reorganization, or ODC plasticity, is thought to be the result of competition between inputs from ipsilateral and contralateral LGN laminae for synaptic connectivity onto binocular visual cortical neurons. This competition normally results in segregation into

ODCs, but as a consequence of MD, the NDE comes to dominate cortical activity and thereby cortical connectivity (58, 119, 289, 308).

The presumed mechanism for ODC reorganization is Hebbian plasticity (135, 304), in which synapses that receive appropriately correlated activity in the presynaptic and postsynaptic elements are functionally and structurally strengthened, while synapses which are inactive or inappropriately activated are weakened or disconnected (58). The current working hypothesis for mediating this Hebbian mechanism within visual cortex utilizes the same cellular mechanisms of LTP and long-term depression (LTD) considered to be responsible for hippocampal forms of learning and memory (26, 57, 65, 105, 167). Potentiation and expansion of NDE synapses may be the result of increased associativity, a key element of LTP. In other words, a decrease or loss of patterned activity in DE pathways may make it more likely that NDE synaptic activity will be correlated with activity of postsynaptic neurons, such that initially weak connections between NDE terminals and cortical neurons will be strengthened. Alternatively or simultaneously, loss of correlated activity between DE synaptic terminals and postsynaptic neurons may promote LTD. This form of LTD is called homosynaptic LTD, in that the lack of patterned activity at a given DE synapse can lead to depotentiation or depression of that same synapse. It is also possible that potentiation of NDE synapses leads in some way to a concomitant depotentiation or depression of nearby inactive DE synapses, a process termed heterosynaptic LTD (38).

What role does cortical cholinergic innervation play in modifying ODC plasticity? Utilizing the anti-cholinergic and anti-noradrenergic drug 6-hydroxydopamine, Kasamatsu and Pettigrew (162, 163) showed that cholinergic and noradrenergic neuromodulatory innervation was necessary for ODC plasticity. Simultaneous destruction or inhibition of these two modulatory inputs reduced ODC plasticity. It has also been shown that lesions of basal forebrain in adult cats massively reduces visual cortical responsivity to visual stimuli (282). Bear and Singer (18) further elucidated the role of ACh in ODC plasticity, by showing that a lesion of either the noradrenergic or the cholinergic projection alone was insufficient to block ODC plasticity, as loss of one neuromodulator could apparently be compensated for by the other. However, Gu and Singer (127), in an effort to more precisely determine the role of cholinergic neuromodulation in ODC plasticity, found that muscarinic antagonism via scopolamine or pirenzepine was sufficient to significantly inhibit MD-induced ODC reorganization, while the nicotinic antagonists hexamethonium and mecamylamine were ineffective. Furthermore, muscarinic receptor involvement appeared to be specific for M1 mAChR, as pirenzepine effectively reduced ODC plasticity while the M2 specific antagonist gallamine was less effective. This is consistent with the idea that postsynaptic neuron activation is necessary for ODC plasticity (266), as M1 mAChR are predominantly expressed on cortical neurons (257, 290, 320), while M2 mAChR and nAChR are located predominantly presynaptically on thalamocortical afferents (222, 257, 258).

As indicated earlier, MD-induced ODC plasticity involves not only the cortical expansion of NDE inputs, but also loss of DE occupied territory. Hence, two mechanisms must be operating simultaneously – one which inactivates or invalidates DE synapses, and one which strengthens and expands NDE connections. It is possible that in order to inactivate DE synapses on a given postsynaptic neuron, NDE synapses upon the same neuron must first reach a critical threshold for NMDAR activation (121, 122). In other words, loss of DE synaptic connectivity occurring via heterosynaptic depression or depotentiation may require that NDE synapses on the same cells be activated to a critical level that can only be attained if NDE afferents activate NMDAR-gated Ca^{2+} conductances (17, 171). This idea is consistent with *in vitro* visual cortical slice data indicating that LTD induction requires a transient elevation of postsynaptic intracellular Ca^{2+} (31, 32). mAChR activation of cortical neurons reduces both voltage- and Ca^{2+}-dependent K^+ conductances (224, 225, 226), which in turn increases the amplitude of excitatory postsynaptic responses. Both carbachol and muscarine have been shown to increase depolarizing responses of cortical pyramidal neurons upon high-frequency stimulation of excitatory afferents in slices of rat visual cortex (31). Moreover, this effect is associated with activation of NMDAR-gated Ca^{2+} conductances (32). Hence, it is possible that antagonism of M1 mAChR in the Gu and Singer (127) experiments reduced the probability that NDE activity could generate sufficient postsynaptic depolarization to

reach the threshold required to initiate heterosynaptic depression of DE synapses, and therefore suppressed MD-induced ODC reorganization.

An effect of mAChR activation on NMDAR activation threshold could also be involved in facilitation of NDE cortical expansion. Just as M1 mAChR activation would increase the probability of NDE-induced heterosynaptic depression of DE inputs, so too might mAChR activation increase the probability of LTP at NDE synapses. Simultaneous loss of DE inputs via heterosynaptic LTD and facilitation of NDE inputs via LTP would result in the characteristic pattern of MD-induced ODC reorganization. Finally, ACh might facilitate ODC reorganization by directly modulating intracellular Ca^{2+} levels via the inositol pathway. ACh raises intracellular Ca^{2+} via IP_3-induced release of Ca^{2+} from intracellular stores, and this Ca^{2+} may increase the amplitude of NMDAR-dependent synaptic activity (217), thereby facilitating both heterosynaptic depression of DE inputs and potentiation of NDE inputs.

VII. The Role of NGF in Experience-dependent Visual Cortical Plasticity

If one places the concept of synaptic competition in the context of the role of cholinergic modulation in visual cortical plasticity and the clear relationship between NGF and BFCN function, one is inevitably drawn to apply the neurotrophic factor hypothesis to ODC plasticity. While there is compelling evidence that LGN afferents

compete for limiting amounts of the TrkB ligands BDNF or NT-4/5 (39, 40 41), we wish to concentrate on the role NGF may play in defining cholinergic facilitation of plasticity at LGN synapses on visual cortical postsynaptic neurons.

Lamberto Maffei and his colleagues pioneered the analysis of NGF actions in visual cortical plasticity. They asked whether supplying exogenous NGF to the developing visual cortex of young rats or kittens could block MD-induced ODC plasticity. They showed in rats that intraventricular injection of NGF every other day throughout the critical period (272), beginning on PD14, just prior to eye opening, and ending on PD44, blocked the MD-induced ocular dominance shift toward the NDE (21, 211). Similarly, chronic intraventricular infusion of NGF for two weeks during the critical-period in kittens partially blocked ODC plasticity and maintained the number of binocularly responsive neurons (46). Furthermore, kittens that were initially monocularly deprived, then exposed to binocular vision or binocular vision plus NGF, were shown to recover more functional binocular connections when treated with NGF (46). In another set of experiments, strabismus was surgically induced in rats. Under these conditions, visual cortical neurons would normally completely lack binocular responsiveness. However, in rats treated with NGF, loss of binocular response in visual cortex was inhibited, and the ocular dominance distribution appeared very similar to unoperated rats (78). Complementing these findings, NGF appeared to preserve cortical responsiveness, orientation selectivity, spontaneous discharge, and DE visual acuity and contrast

sensitivity (21, 46, 75, 78, 99, 211, 256). Furthermore, NGF-treatment prevented the reduction of visual cortical parvalbumin immunoreactivity normally associated with MD in rats (21, 48), and prevented the shrinkage of LGN cells in DE laminae following MD (46, 77). Thus, it appears that exogenous NGF is able to reduce or inhibit virtually all of the changes that result from synaptic plasticity in response to MD in both rats and kittens. In effect, NGF appears to substitute for visual experience (46, 78).

That endogenous NGF plays a role in MD-induced visual cortical plasticity was suggested by the results of transplanting anti-NGF secreting hybridoma cells into the lateral ventricle of rats. Such treatment induced dramatic shrinkage of LGN neurons, decreased the overall number of binocularly responsive cortical neurons, and impaired visual acuity (20). Significantly, transplanting anti-NGF-secreting cells into young rats extended the critical period for MD (76), suggesting that endogenous NGF regulates maturation and synaptic plasticity in the visual cortex.

MD following the critical period in normal cats has no effect on ODC organization, suggesting that maturational processes occur during development that lead to the eventual solidification of synaptic connections. However, based on data that suggest a role for ACh in normal adult visual cortical function (282, 283), it is interesting to ask what effect NGF might have on visual cortical plasticity in the adult. Gu *et al.* (126) found that exogenous supply of NGF via minipump to the primary visual cortex of adult cats for two weeks, paired with simultaneous monocular lid suture, induced an

ODC shift toward the deprived eye – completely opposite to the ODC shift elicited by MD during the critical period. While several interpretations are possible, the most attractive mechanism to explain this result relies on the tight regulation of BFCNs by NGF levels (194, 300). NGF treatment of adult cat visual cortex induced an increase in phosphorylated GAP-43 and synaptophysin (125). These two proteins mark axonal sprouting and new synapse formation (71, 328). Furthermore, it has been shown that NGF facilitates cholinergic sprouting in the adult CNS (112, 129, 134, 277). As mentioned earlier, cholinergic innervation potentiates NMDA-mediated glutamatergic synaptic transmission, and increased cholinergic input induced by sprouting may raise the overall level of visual cortical responsiveness. In effect, NGF-induced cholinergic sprouting may make postsynaptic visual cortical neurons "twitchier", allowing them to respond to suboptimal DE inputs as though they were normal. We believe that a heightened response to suboptimal DE inputs plays a role in NGF effects on critical period visual cortex, and we will return to this idea in more detail when we discuss our model of NGF-mediated cholinergically-induced creation of a plasticity space within hippocampus and cortex. We further speculate that this hypercholinergic state may cause a hyper-reactive response to NDE inputs that results in synaptic regression. Decreased NDE synaptic efficacy could be mediated by downregulating postsynaptic glutamate receptors or by causing a long-lasting decrease in NDE terminal neurotransmitter release. This model is supported by evidence that LTP-inducing tetanic stimulation, if followed

by a continuous stimulus train, will actually induce LTD (14, 303). One prediction of this model is that coinfusion of a cholinergic antagonist with NGF during MD in adult cats would block the cholinergically-mediated change in postsynaptic neuronal responsiveness, and thereby prevent selection of DE inputs over NDE inputs.

VIII. The Role of NGF in Learning and Memory-related Plasticity

Given the evidence presented earlier for a role of cholinergic innervation in modulating learning and memory plasticity, it is not surprising that NGF is also involved in these events. In the normal, uninjured adult rat CNS, biochemical data indicates that chronic intraventricular infusion of NGF elicits increased hippocampal ChAT activity, high-affinity choline uptake activity, ACh synthesis, ACh release, and nitric oxide synthase activity (108, 274). On the other hand, chronic administration of anti-NGF antibodies decreased these parameters (317). Electrophysiological data further support these biochemical changes, indicating that chronic intraventricular NGF enhanced markers of cholinergic function in the NBM-amygdala axis. Essentially, chronic NGF increased the likelihood of generating a slow cholinergic EPSP in amygdalar pyramidal neurons upon NBM stimulation, and increased cholinergically-mediated inhibition of a Ca^{2+}-activated K^{+}-mediated inhibitory afterhyperpolarization (I_{AHP})(235). In contrast, intraventricular infusion of anti-NGF antibodies impaired Morris water maze

performance and induced hyperlocomotion. While water maze acquisition was unimpaired, extinction and reversal performance were significantly reduced (317), and habituation was greatly attenuated (244). These behavioral data indicate that decreased NGF in the normal adult rat CNS induces learning inflexibility, a hallmark of reduced cholinergic function, and a situation which is mimicked by cholinergic antagonists (69, 132). Finally, long-term intraventricular infusion of NGF in normal rats improved performance in a conditioned taste aversion test. NGF treatment facilitated acquisition and increased the resistance to extinction of a lithium chloride-induced aversion to saccharin. This effect was directly correlated with NGF-induced elevation of BFCN ChAT levels, indicating that NGF plays a role in cholinergically-mediated non-spatial, affective memory systems (201). However, several studies have shown that exogenous NGF administered to young rats or to unimpaired aged rats results in disruption of spatial learning (9, 49, 215), suggesting that NGF-induced cholinergic hypertrophy may, in some behavioral contexts, lead to an inability to learn.

Data from NGF heterozygous knockout mice further support a role for NGF in learning and memory plasticity. Heidi Phillips and her colleagues (50) tested spatial learning and memory function in mice heterozygous for NGF gene disruption (*ngf* +/-). These mice exhibited decreased NGF mRNA and protein within the hippocampus, and they showed a concomitant loss of approximately one-third of ChAT(+) or p75(+) septal neurons, as well as shrinkage of the remaining cholinergic neurons and loss of ChAT

activity and cholinergic fiber density within the hippocampus (50). In these same animals, Morris water maze performance was significantly impaired in terms of acquisition and retention. The *ngf* +/- mice performed as well as wildtype littermates on the visible platform test, indicating that the acquisition and retention deficits observed in the hidden platform task were specific to a learning and memory function. Furthermore, performance in a hidden platform task that did not require spatial memory was identical to wildtype littermate performance, again supporting the argument that performance deficits are the result of spatial memory problems, and not due to sensory, motor, or motivational defects. Finally, deficiency of NGF within the CNS was directly implicated in the spatial memory defect by the ability of chronic (5 week) NGF infusion to rescue the behavioral abnormality. As acute (3 day) infusions of NGF were ineffective in rescuing water maze performance, it is likely that the behavioral amelioration of long-term NGF treatment was mediated by NGF-induced recovery of BFCN function. This is further supported by the fact that *ngf* +/- mice showed nearly complete recovery of AChE(+) fiber density to wildtype levels within the hippocampal formation following NGF treatment (50).

Additional data for a role of NGF in learning and memory plasticity come from studies that used NGF to ameliorate age-related cognitive dysfunction. Aged rats, as with aged humans, display age-dependent decreases in learning and memory parameters which correlate with loss and dysfunction of BFCNs (4, 68, 101, 110, 145, 173, 174, 218). NGF

infusion has been shown to reverse both acquisition and retention deficits in spatial memory in aged rats, and these improvements are accompanied by reversal of BFCN atrophy and dysfunction (9, 100, 101, 103, 104, 215, 216, 220, 221). Moreover, chronic intraventricular infusion of NGF in aged rats improves deficits in LTP, ameliorating potentiation impairments to levels equivalent to young rats (22). These data suggest that NGF is able to elicit a functional recovery within the septohippocampal axis, an effect that may be due to NGF-induced sprouting of cholinergic terminals within the hippocampus, or to an NGF-induced reconnection of dysfunctional synapses.

Finally, NGF appears to play a role in controlling the maturation of behavioral responses that are modulated by BFCN function. Single intraventricular injections of NGF in suckling mice have been shown to enhance scopolamine-induced locomotor hyperactivity (3), and a single intrahippocampal NGF injection in young rat pups accelerated the development of cholinergically-mediated spontaneous alternation by five days (139). Furthermore, intraventricular injections of NGF on postnatal days 2 and 4 were able to accelerate the appearance of scopolamine-induced hyperactivity by approximately 15 days, from postnatal day 20 to postnatal day 5 (43). Thus, it appears that NGF not only regulates mature BFCN function, but also that it can accelerate the developmental profile for cholinergic maturation.

IX. The Metamodulatory Function of NGF in Plasticity

We propose that NGF serves a metamodulatory role within the hippocampus and cortex. That is to say, while NGF itself does not directly modulate or modify glutamatergic synaptic transmission within the hippocampus or cortex, it does modify the efficacy of cholinergic modulation at hippocampal and cortical glutamatergic synapses. By acting on cholinergic synapses, NGF indirectly controls the set-point for learning and memory plasticity. Figure 5.3 illustrates the basic idea: plasticity occurs at the intersection of cortical/hippocampal connectivity, cholinergic modulation, and NGF control of cholinergic efficacy.

Three principle ideas lead to the metamodulatory role we propose for NGF. First, from the data presented, it is clear that NGF does not directly alter the synaptic efficacy of hippocampal or cortical connections – i.e. NGF does not "pharmacologically" modify plasticity. Second, NGF does modify cholinergic function. It directly and dynamically controls the cholinergic phenotype of BFCNs, modulating both the extent of cholinergic innervation and the efficacy of ACh release from cholinergic terminals. Third, ACh and cholinergic innervation directly and specifically modulate potentiation and depotentiation of cortical and hippocampal synapses. Thus, on the basis of these observations, we conclude that NGF is in a unique position to indirectly control plasticity by directly controlling cholinergic function.

Our model fits the available evidence, and provides a framework for better understanding learning and memory-related plasticity. Figure 5.8 describes in more detail how we think NGF creates a plasticity space. Figure 5.8A shows the relationship between NGF concentration in target regions and the degree of plasticity which is available within hippocampus and cortex. There is a median, optimal concentration of NGF within hippocampus and cortex that leads to a level of cholinergic innervation and ACh release which is optimal for keeping hippocampal and cortical neurons primed to respond to inputs – i.e. a level of ACh release which keeps postsynaptic neurons primed for plasticity (Figure 5.8A, 5.8B). Decreasing the available NGF by injecting anti-NGF antibodies, or decreasing the ability of cholinergic terminals to respond to NGF (perhaps by disrupting retrograde transport of the NGF signal (124) or by inhibiting TrkA availability) leads to a decrease in ACh release and a concomitant loss of plasticity (Figure 5.8B). In such a state, the cortex or hippocampus is "frozen" in place, meaning that there is a marked reduction in the ability to modify the number or efficacy of glutamatergic synapses. This is evidenced by poor learning and perseveration of behavior. On the other hand, increasing NGF levels also leads to a "frozen" state (Figure 5.8B). Excess NGF leads to increased ACh release, which then makes postsynaptic neurons hyper-reactive and responsive to any input, even uncorrelated input. This effectively results in a loss of plasticity and a disruption of learning and memory (9, 49, 215, 277). Finally, our model also offers two possible explanations for the ability of

NGF to prevent the plasticity associated with monocular deprivation. Under conditions of monocular lid suture, DE afferents in the visual cortex do not fire in a correlated manner or with the robustness of NDE afferents, but they do fire spontaneously. Our model suggests that exogenous NGF, by enhancing ACh release, potentiates DE inputs and prevents the postsynaptic neuron from discriminating between inputs from either eye on the basis of impotent or uncorrelated input. NGF effectively improves the probability that DE inputs will be able to compete with NDE inputs for cortical territory. Another possible explanation is that exogenous NGF accelerates the maturation of the visual cortex by freezing plasticity and locking pre-existent input relationships in place. The Gu results, in which excess NGF paired with MD in an adult cat led to cortical reorganization in favor of the DE, suggests that the first explanation is more likely, and that a developmental change occurs in the sensitivity of cortical plasticity to NGF levels, as indicated in figure 5.8C. In other words, during development, cortical and hippocampal plasticity is able to withstand higher NGF levels or a broader optimal concentration peak, and hence able to withstand greater cholinergic modulation than adult hippocampus and cortex. Therefore, when excess NGF is given to a monocularly deprived juvenile it potentiates DE inputs, but not at the expense of NDE inputs. Conversely, when excess NGF is given to a deprived adult, it potentiates DE inputs while punishing NDE inputs. The mechanistic basis for this developmental switch in sensitivity is unexplained, but it is interesting to note that adult levels of cortical and hippocampal NGF are significantly

lower than juvenile levels (7), and this decrease occurs within the critical period. It is conceivable that decreased NGF levels in cortex and hippocampus result from the switch to a new setpoint for cholinergic modulation of plasticity. Finally, we offer a possible experiment to determine whether NGF given during juvenile MD is potentiating weak DE inputs or freezing visual cortical connectivity in place. We suggest that NGF infusion be paired with MD generated by intraocular injection of TTX. Under these conditions, DE afferents will be essentially completely silent, not even exhibiting spontaneous activity. If NGF blocks ODC plasticity by potentiating suboptimal DE inputs, then in this experiment NGF will fail to block ODC reorganization because there will be no suboptimal activity to potentiate. If, on the other hand, NGF acts to accelerate maturation of a pre-existing pattern of input, then activity in the DE pathway will be irrelevant and NGF will successfully block ODC reorganization in the presence of silenced DE inputs.

X. Nerve Growth Factor and Cholinergic Metamodulation in Alzheimer's Disease

Our model focuses attention on NGF actions at cholinergic synapses and the role that cholinergic neurotransmission plays in attention, learning, and memory. Alzheimer's Disease (AD) is characterized by devastating changes in cholinergic function, and by

deficits in memory and other cognitive functions (56, 248). Among the most important questions regarding AD biology, is whether it is possible to restore cholinergic neurotransmission and improve cognition in AD patients. NGF acts to enhance cholinergic markers in aged and lesioned BFCNs. Our model of NGF as a metamodulator of hippocampal and cortical plasticity offers new hope for therapeutic intervention. If NGF control of cholinergic function is a critical element in maintenance of learning and memory plasticity, and if failed cholinergic neurotransmission is a critical determinant of cognitive dysfunction in AD, then effective delivery of NGF to cholinergic synapses in AD patients may induce reinvigoration of cholinergic function and have beneficial effects on cognition. Though NGF has been given to AD patients using intraventricular administration (88), the trials conducted to date do not allow conclusions to be drawn regarding its efficacy, or lack thereof. Additional trials assessing the effects of human NGF in a large number of patients using standardized protocols will be required. Important problems to be solved before such trials are initiated are the pain experienced at even relatively small doses of NGF, and the hypertrophy of Schwann cells and sprouting of sympathetic and sensory neurites in or near the spaces bathed by CSF containing NGF (334). The development of more effective delivery methods may obviate these problems (198, 212). Finally, our model strongly suggests that the focus of innovative therapeutics in AD should highlight interventions that enhance ACh release and cholinergic potentiation of intrinsic

hippocampal and cortical synapses, without overstimulating cholinergic neurotransmission.

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Figure 5.1.

Representation of the Neurotrophic Factor Hypothesis. Initially exuberant connections between projection neurons and target tissue are lost during development as the result of competition for limiting amounts of a neurotrophic factor (red circles) released by the target.

Neurotrophic Factor Hypothesis

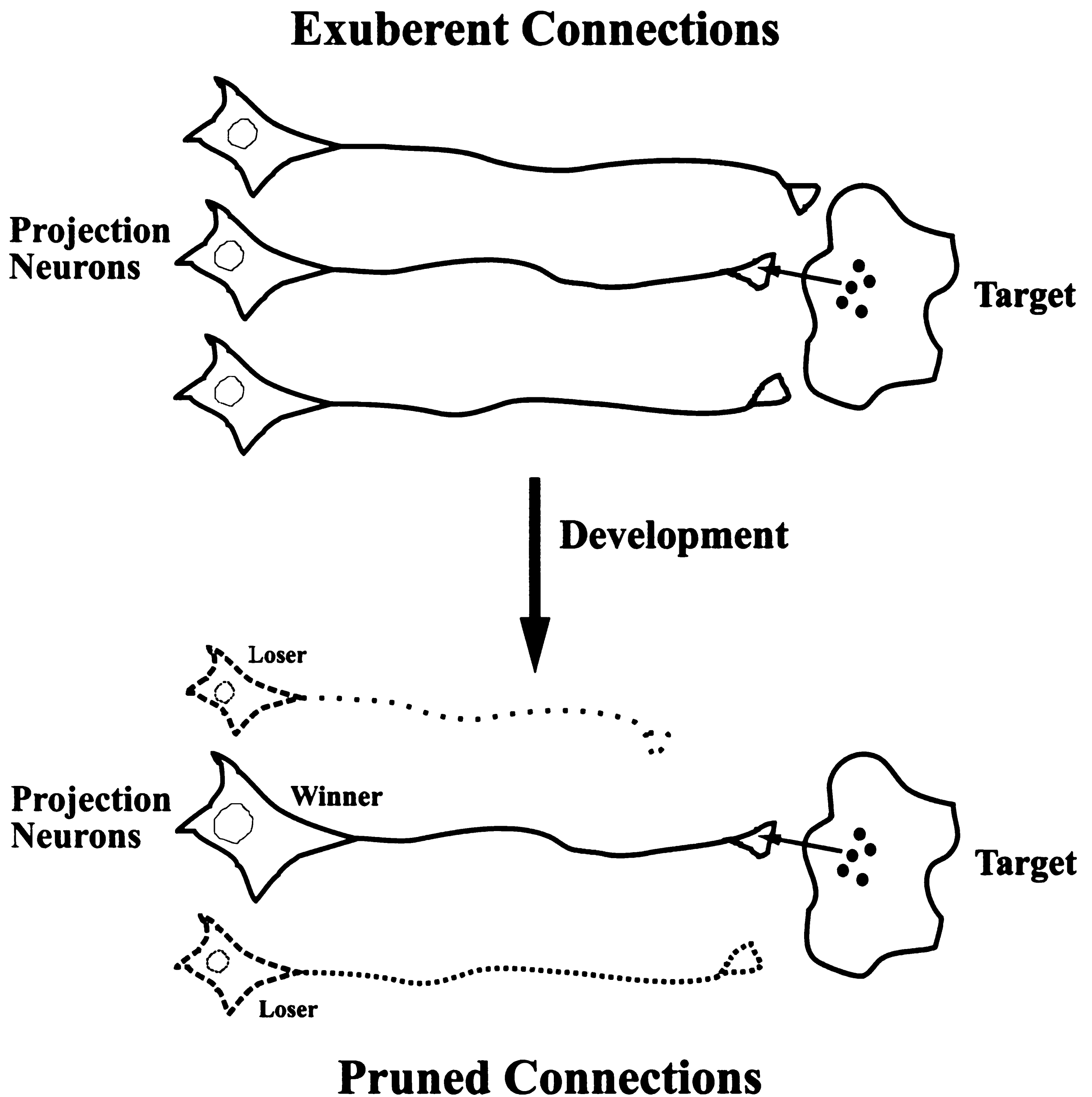

Figure 5.2.

Diagram illustrating the metamodulatory role of NGF. Acetylcholine modulates the efficacy and plasticity of the connection formed between presynaptic glutamatergic terminals and postsynaptic hippocampal and cortical neurons. Acetylcholine is in turn regulated and modulated by NGF. This places NGF in the role of indirectly but potently modulating hippocampal and cortical connectivity.

Figure 5.2

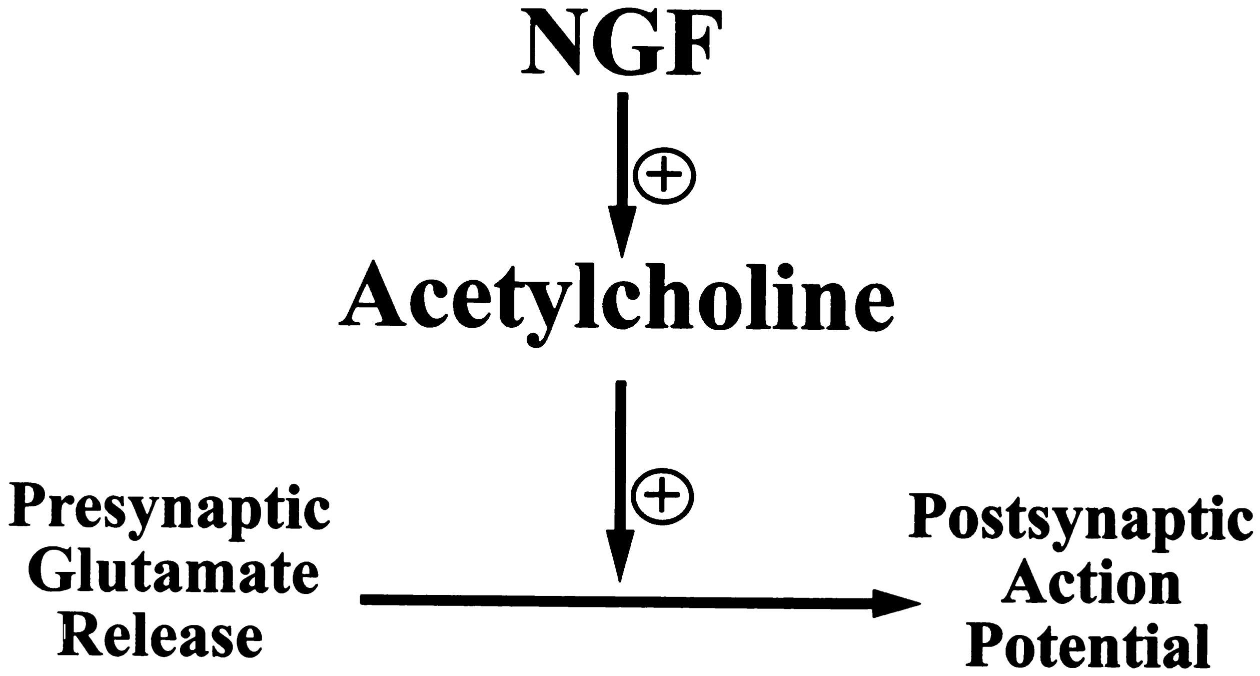

Figure 5.3.

Creation of a plasticity space at the convergence of acetylcholine, NGF, and cortical and hippocampal connectivity.

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Creation of a plasticity space at the convergence of acetylcholine, NGF, and cortical and hippocampal connectivity.

Figure 5.4.

Coronal (A) and sagittal (B) sections illustrating the general anatomical relationship between nuclei of the basal forebrain, and the pattern of their projections to cortical and hippocampal targets (B). MSN, medial septal nucleus. VDB, vertical limb of the diagonal band of Broca. HDB, horizontal limb of the diagonal band of Broca. NBM, nucleus basalis of Meynert.

Figure 5.4.

Coronal (A) and sagittal (B) sections illustrating the general anatomical relationship between nuclei of the basal forebrain, and the pattern of their projections to cortical and hippocampal targets (B). MSN, medial septal nucleus. VDB, vertical limb of the diagonal band of Broca. HDB, horizontal limb of the diagonal band of Broca. NBM, nucleus basalis of Meynert.

Figure 5.5.

Synaptic relationships between cholinergic and glutamatergic inputs and postsynaptic neurons in the hippocampus and neocortex. (A) Schematic representation of the anatomical relationship between presynaptic and postsynaptic elements. (B) One possible means by which cholinergic neurotransmission potentiates glutamatergic neurotransmission. Acetylcholine is released by the cholinergic terminal and binds receptors on the postsynaptic dendritic spine (B.1), stimulating an elevation in intraspine calcium levels, either via calcium influx or release from intracellular stores (B.2). Through an effect on membrane potential, the calcium acts to relieve the magnesium block on the NMDA receptor (B.3), thereby potentiating a coincident response to glutamate released by the presynaptic glutamatergic terminal (B.4).

(C) Another possible modulatory interaction between a cholinergic terminal and a primary synapse in the cortex or hippocampus. Acetylcholine released by the cholinergic terminal (C.1) activates acetylcholine receptors linked to kinase cascades (C.2) which lead either to phosphorylation of the NMDA receptor or to signaling pathways in common with downstream signaling from the NMDAR (C.3). Phosphorylation of NMDAR then potentiates the receptor's response to coincident release of glutamate by the presynaptic glutamatergic terminal (C.4).

Figure 5.5

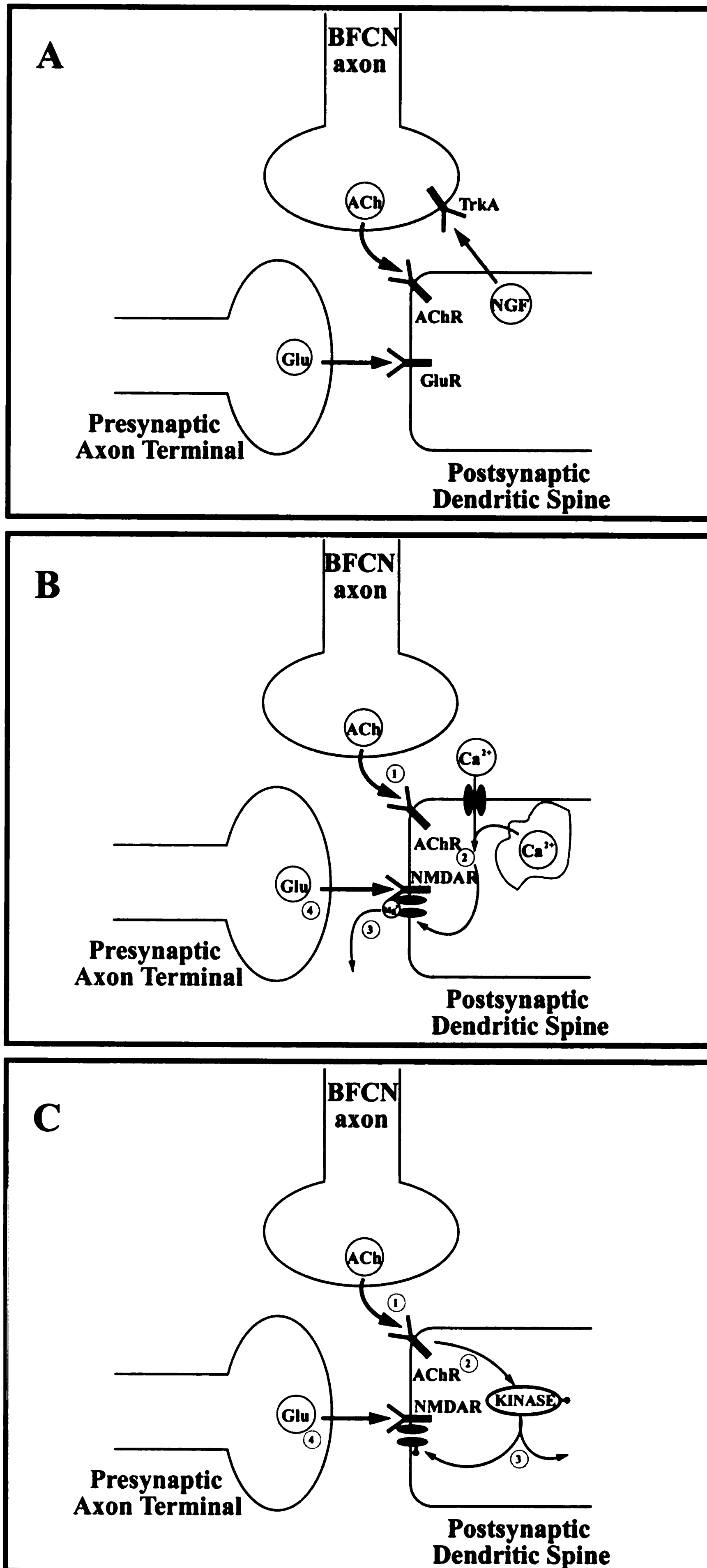

Figure 5.6.

Illustration of ocular dominance column development and plasticity. Thalamocortical inputs are initially widespread and overlapping, but eventually organize into highly patterned alternating regions of innervation within which cortical neurons respond preferentially to one eye or the other. During a critical period within this developmental sequence, depriving one eye of patterned input permits the non-deprived eye to acquire cortical territory normally occupied by the deprived eye.

Figure 5.6.

Illustration of ocular dominance column development and plasticity. Thalamocortical inputs are initially widespread and overlapping, but eventually organize into highly patterned alternating regions of innervation within which cortical neurons respond preferentially to one eye or the other. During a critical period within this developmental sequence, depriving one eye of patterned input permits the non-deprived eye to acquire cortical territory normally occupied by the deprived eye.

Figure 5.7.

Illustration of visual cortical neuron responsiveness after normal development (A), and following monocular deprivation (MD) (B). MD causes a predominant shift in responsivity from the contralateral deprived eye (DE) to the ipsilateral non-deprived eye (NDE). This shift, or ocular dominance plasticity, is prevented by treatment with NGF.

Figure 5.7.

Illustration of visual cortical neuron responsiveness after normal development (A), and following monocular deprivation (MD) (B). MD causes a predominant shift in responsivity from the contralateral deprived eye (DE) to the ipsilateral non-deprived eye (NDE). This shift, or ocular dominance plasticity, is prevented by treatment with NGF.

Figure 5.8.

Schematic representation of the metamodulation model. (A) The degree of plasticity within the hippocampus or cortex is dynamically dependent upon the concentration or availability of NGF. At some optimal level of NGF, plasticity is maximal, while lower or higher levels of NGF exhibit reduced plasticity (B).

(C) Possible relationships between NGF and plasticity in juvenile hippocampus and cortex versus adult hippocampus and cortex (See text for discussion).

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Schematic representation of the metamodulation model. (A) The degree of plasticity within the hippocampus or cortex is dynamically dependent upon the concentration or availability of NGF. At some optimal level of NGF, plasticity is maximal, while lower or higher levels of NGF exhibit reduced plasticity (B).

(C) Possible relationships between NGF and plasticity in juvenile hippocampus and cortex versus adult hippocampus and cortex (See text for discussion).

Chapter 6

Conclusions and Future Directions

Summary

The data presented in this thesis are the first evidence of purified signaling endosomes. As such, this work significantly extends our understanding of the biology of neurotrophin and growth factor signaling. In particular, these findings indicate that clathrin-coated vesicles are nascent signaling endosomes that can propagate the NGF signal, suggesting that the entire Ras signaling pathway is present and competent to signal from these membranes. The implications of these findings are that discrete, membrane-bounded signaling packets exist within cells and serve to transduce neurotrophic signaling from the plasma membrane to the nucleus.

Conclusions and Future Directions

Trophic state communication from the target of innervation to the innervating neuron in the developing and mature nervous system requires that a neurotrophin-induced signal be conveyed from the presynaptic terminal to the cell body, where it can induce pleiotropic effects on survival and differentiation. The means by which such a retrograde signal is propagated is currently unresolved, but three possible mechanisms have been proposed, as discussed in Chapter 1. The first potential mechanism is that target-derived neurotrophin is internalized at the presynaptic terminal and transported to the cell body, where it binds to receptors within the cytoplasm of the cell body to initiate local signaling cascades. The second mechanism is that target-derived neurotrophins activate presynaptic

neurotrophin receptors, and these receptors initiate signaling cascades that reach the cell body in a wavelike fashion. The third potential mechanism for communicating the trophic signal from axon terminals to neuron cell bodies involves activation of presynaptic neurotrophin receptors by target-derived neurotrophin, followed by internalization of these receptors in a ligand-bound manner into endocytic vesicles that are retrogradely trafficked to the cell body. Upon arrival in the cell body these "signaling endosomes" initiate local signal transduction cascades that mediate transcriptional events. As discussed at length in Chapter 1 and Chapter 3, evidence from several sources points to the validity of the third model, the signaling endosome hypothesis. The work presented in this thesis contributes to the body of evidence in support of the signaling endosome hypothesis by proving that NGF induces the formation of endocytic vesicles that embody the neurotrophin signal.

Based on previous work showing that activated TrkA within a gross endosomal fraction is associated with several signaling cascade components (Grimes and others 1996, 1997), I sought to isolate purified neurotrophic signaling endosomes and to address the endocytic events proximal to the generation of these organelles. Endocytosis of cell surface receptors for growth factors such as EGF and insulin via clathrin-mediated pathways has long been associated with a role in receptor recycling and termination of receptor signaling (Sorkin and Waters 1993). In the studies reported in this thesis, I

addressed the novel possibility that clathrin-mediated pathways play an important additional role in growth factor signaling – one that facilitates moving the signal from the cell surface to internal membranes, thereby creating signaling endosomes that are capable of undergoing retrograde transport from the axon tip to the cell body. In Chapter two, I showed that NGF signaling through TrkA regulates the extent to which clathrin assembles on surface membranes. In Chapter 3 I showed that NGF induces the formation of complexes containing TrkA together with AP2 and CHC, and induces the formation of clathrin-coated vesicles. Furthermore, by isolating a highly purified clathrin-coated vesicle fraction, I demonstrated that NGF signaling results in the formation of clathrin-coated vesicles containing NGF bound to activated TrkA receptors. Significantly, the activated TrkA receptors present in the clathrin-coated vesicle fraction were found together with activated Erk1/2, and isolated NGF-induced clathrin-coated vesicles were able to signal in a cell-free assay to phosphorylate Elk, a downstream target of the Erks. On the basis of these findings, I conclude that NGF signaling upregulates the endocytic machine to produce clathrin-coated vesicles that serve as signaling endosomes.

Whether the formation of signaling endosomes has a physiological role for signaling from TrkA and other receptor tyrosine kinases will require additional studies. However, the speculation that signaling from internalized activated receptors does play a role is supported by a number of recent observations. Studying the endocytosis of EGFR,

Schmid and colleagues found that receptor internalization was required for the full spectrum of normal signaling events ascribed to EGF treatment (Vieira and others 1996). In view of my finding that activated Erk was present in clathrin-coated vesicles isolated from NGF treated cells, it is interesting that I have observed decreased activation of Erk1/2 in cells that could not internalize TrkA through CCVs. It is conceivable that a significant fraction of Erk1/2 signaling is mediated through internalized receptors. Importantly, my findings would suggest that the receptors are present in highly organized signaling complexes that facilitate Erk activation. Decreased signaling through this pathway would be expected to have significant effects on cell growth and differentiation. In fact, TrkA internalization appears to be necessary for NGF induction of neurite outgrowth in PC12 cells (Zhang and others 2000). Moreover, the findings presented in Chapter 4 of this manuscript support the idea that TrkA endocytosis is a vital component of differentiative signaling, and they highlight the need for better understanding of the complex signaling pathways that converge upon control of receptor internalization. Overall, then, the work discussed in this manuscript offers the first proof of the existence of signaling endosomes, shows that these endosomes carry many active elements of the Ras signaling pathway, and describes one mechanism by which signaling via these endosomes may be controlled within the context of synaptic plasticity.

The work presented herein is really just the beginning of a long road that needs to be taken to understand not only the signaling events that initiate and control the generation of signaling endosomes, but also the nature and composition of such signaling endosomes. I propose that one of the most exciting avenues of exploration will be the characterization of signaling endosomes derived from clathrin-coated vesicles and signaling endosomes derived from other sources, within the context of signal propagation. Isolating signaling endosomes and injecting them into naive cells is potentially a very powerful means of characterizing the signaling potential contained within these vesicles. The prospect of causing differentiative changes in cells that never experienced external neurotrophic support simply by injecting signaling endosomes into the cell body is very exciting. It is also exciting to think about characterizing the signaling endosome proteome. In other words, describing all of the proteins associated with signaling endosomes generated in response to different trophic signals. Such an avenue of exploration may uncover a vast array of previously unrecognized signaling elements that are concentrated within the signaling endosome. Changes in such elements may go unnoticed or be averaged away within the large ocean of signaling mediators contained within the cell, but within the more restricted confines of the signaling endosome these elements may play critical roles. Finally, the creation of artificial signaling endosomes may hold therapeutic potential – could a nanoscale signaling machine be designed to propagate an artificial trophic signal within real neurons? While

highly speculative, such an idea is exciting, and holds within it the promise of ultimately better understanding cellular signaling by understanding where the cell signals.

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Appendix

A.1 Society for Neuroscience Annual Meeting Abstract 1998.

ANALYSIS OF NGF RECEPTOR BINDING, TRAFFICKING, AND SIGNALING IN MOUSE SYNAPTOSOMES.

Charles L Howe and William C Mobley

Synaptosomes are self-contained synaptic terminals and boutons formed by the pinching off of axonal membrane during homogenization in iso-osmotic sucrose. These membranes spontaneously reseal to form spheres that contain mitochondria, synaptic vesicles, and cytosolic components. Synaptosomes take up and metabolize glucose, and they respire and synthesize ATP. Synaptosomes also have functional transporters for neurotransmitter precursors such as choline and tyrosine, and, because they maintain a resting membrane potential equal to that of neurons, synaptosomes release various neurotransmitters in response to K^+- and transmitter-induced depolarization . Hence, the synaptosome has seen wide application as a model synapse. We have used mouse cortical and hippocampal synaptosomes to analyze nerve growth factor receptor binding, signaling, and trafficking.

Nerve growth factor (NGF) actions are mediated by two receptors, p75NTR and TrkA. TrkA is a receptor tyrosine kinase whose activation by NGF elicits a signal transduction cascade mediated by PLC-γ, SHC, and PI-3 kinase. We have shown that synaptosomes have surface TrkA and p75NTR, and that ^{125}I-NGF can be crosslinked to these receptors. Synaptosomal p75NTR and TrkA bind NGF, and NGF-receptor complexes are endocytosed in response to high-affinity binding. Synaptosomes have high-affinity binding sites for NGF that are comparable to PC12 cells. Finally, synaptosomal TrkA is activated by NGF with a time-course and dose response curve similar to PC12 cells. The downstream signaling consequences of TrkA activation in synaptosomes are under investigation, as is the use of synaptosomes to elucidate NGF signaling defects in mouse models of neurologic disease. This research was supported by an HHMI Predoctoral Fellowship (CLH), NIH grant NS24054, the Adler Foundation, and the McGowan Charitable Trust.

A.2 Society for Neuroscience Annual Meeting 1999

NGF-INDUCED TRKA-MEDIATED CLATHRIN COATED VESICLE FORMATION MAY CONTRIBUTE TO THE GENERATION OF A "SIGNALING ENDOSOME."

Charles L Howe, EC Beattie, and William C Mobley.

Nerve growth factor (NGF) binding to the receptor tyrosine kinase TrkA signals to promote the survival, differentiation, and phenotypic maintenance of specific neuronal populations. The signaling endosome hypothesis postulates that the signaling cascades responsible for these events may be initiated or mediated in part by endocytosis and retrograde transport of vesicular structures containing activated TrkA bound by NGF. Using PC12 cells as a model, we tested the hypothesis that TrkA, bound NGF, and associated signaling complexes are internalized from the plasma membrane via clathrin coated vesicles (CCVs). Biochemical analyses of a highly purified CCV population indicate that TrkA activation induces the formation of CCVs that contain TrkA, radiolabeled NGF, and several associated signaling molecules, including PLCγ and PI3 kinase. These data are consistent with our previous observation that TrkA signaling induces membrane redistribution and phosphorylation of the clathrin heavy chain (CHC). Extending these observations, we found that NGF induced an increase in the formation of

complexes containing TrkA, phosphorylated CHC, AP2, and pp60src. The role of pp60src in such complex formation was further elucidated by our finding that internalization of radiolabeled NGF was significantly inhibited in cells over-expressing a dominant-negative form of chicken pp60src (srcDN2 PC12). Finally, disparity between the absolute amount of internalized radiolabeled NGF and the extent of CCV formation suggests that TrkA may regulate the internalization of other surface receptors via a global increase in CCV trafficking. We propose a model in which TrkA signals to recruit several proteins involved in CCV-mediated endocytosis to the plasma membrane to create a signaling endosome in which internalized NGF and activated TrkA can be retrogradely transported. Supported by a HHMI Predoctoral Fellowship (CLH), the Adler Found-ation, the McGowan Charitable Trust, Alzheimer's Assoc., NS24054, NS38869, and AG16999.

A.3 Society for Neuroscience Annual Meeting 2000

Neurotrophic Signaling Endosomes and the Role of Internalization in Transmission of the Neurotrophin Signal.

Charles Leon Howe and William C Mobley

Nerve growth factor (NGF) binding to TrkA signals to promote survival, differentiation, and phenotypic maintenance of specific neuronal populations. The "signaling endosome" hypothesis postulates that signaling cascades responsible for these events are initiated or mediated in part by endocytosis and retrograde transport of vesicular structures containing activated TrkA bound by NGF. Based on our observation that TrkA signaling induced membrane redistribution of clathrin, we tested the hypothesis that TrkA, bound NGF, and associated signaling complexes are internalized from the plasma membrane via clathrin coated vesicles (CCVs). Biochemical analyses of a highly purified CCV population indicated that NGF induced the formation of CCVs containing phosphorylated TrkA bound by NGF. NGF also induced the formation of complexes containing phosphorylated TrkA, clathrin heavy chain, and the clathrin adaptor protein, AP2. We also found that NGF-induced CCVs contained Shc, Ras, and activated ERK. Importantly, these CCVs were able to signal in vitro to phosphorylate ELK, a downstream target of activated ERKs that is proximal to nuclear signaling events. Furthermore, inhibitors of

CCV formation decreased NGF endocytosis and concomitantly blocked NGF induction of ERK activation, suggesting that TrkA internalization is necessary for appropriate downstream signaling. We propose a model in which TrkA signals to recruit proteins involved in CCV-mediated endocytosis to the plasma membrane to create a signaling endosome in which internalized NGF, activated TrkA, and critical components of the NGF signaling cascade can be retrogradely transported. Support: HHMI Predoc Fellowship (CLH), Adler Foundation, McGowan Charitable Trust, Alzheimer's Assoc., NS24054, NS38869, and AG16999.

A.4 American Society for Cell Biology Annual Meeting 1998

TRKA SIGNALING REGULATES CLATHRIN COATED PIT FORMATION.

EC Beattie, CL Howe, and WC Mobley

Nerve growth factor (NGF) binding to the receptor tyrosine kinase TrkA signals to promote the survival and differentiation of a subset of neurons in the central and peripheral nervous system. This signaling is thought to be mediated in part by endocytosis and retrograde transport of activated TrkA bound by NGF. Using PC12 cells as a model, we tested the hypothesis that TrkA and bound NGF are internalized from the plasma membrane in clathrin coated pits (CCP). Confocal and biochemical analyses of various cell lines point to a role for clathrin in TrkA internalization. Our data indicate that TrkA signaling is responsible for rapid NGF-induced redistribution of clathrin and phosphorylation of the clathrin heavy chain (CHC). These findings are consistent with the earlier observation that NGF treatment caused an increase in CCP formation, and suggest that TrkA activation may regulate the internalization of other surface receptors via a general increase in CCP traffic. Moreover, we found that NGF caused an increase in association of clathrin, including phosphorylated CHC, with TrkA, and formation of complexes containing TrkA, AP2, clathrin, and pp60src. This extends previous work by Brodsky and Wilde, in collaboration with our lab, showing that pp60src overexpression

increases phosphorylation of CHC. These data are the first to describe ligand-induced tyrosine phosphorylation of CHC and the formation of a complex containing a growth factor receptor, pp60src, and clathrin. We propose a model in which TrkA interacts with several proteins involved in CCP-mediated endocytosis to create a microdomain through which the receptor can be rapidly internalized in response to ligand-binding.

A.5 American Society for Cell Biology Annual Meeting 1999

The Role of p75NTR in the Internalization of NGF, BDNF, and NT-3.

Charles L Howe, Alex Krüttgen, Eric Shooter, and William C Mobley

The p75 neurotrophin receptor (p75NTR) is a low-affinity receptor for nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF), and neurotrophin-3 (NT-3). Trk tyrosine kinase receptors also bind the neurotrophins, but with varying degrees of specificity (TrkA ≈ NGF, TrkB ≈ BDNF, TrkC ≈ NT-3). In contrast to Trk, little is known about the biological function of p75NTR. We have found that the neurotrophins exhibit differential surface binding on PC12 cells, which express p75NTR and TrkA, but not TrkB or TrkC, and that NGF, BDNF, and NT-3 are specifically internalized by PC12 cells. Furthermore, internalization kinetics are slower for BDNF and NT-3 than for NGF, and surface binding and internalization of BDNF and NT-3 display unique anti-p75NTR sensitivities in antibody blocking experiments. Moreover, crosslinking of radioiodinated neurotrophin to PC12 cell surface receptors shows that while NGF and NT-3 are crosslinked to both TrkA and p75NTR, BDNF was only crosslinked to p75NTR, even at high concentrations of BDNF. Hence, internalization of BDNF must be mediated by p75NTR. Finally, internalized neurotrophins are found in highly purified clathrin coated vesicle (CCV) preparations, suggesting that p75NTR is internalized via this pathway.

CCV-mediated p75NTR internalization suggests formation of intracellular endosomes which may participate in downstream signaling cascades. This work was supported by HHMI (CLH), NS04270 (AK, EMS), NS24054 (WCM).

A.6 Society for Neuroscience Annual Meeting Historical Section 2000

Marian Lydia Shorey and the Early Foundations of the Neurotrophic Factor Hypothesis.

Charles Leon Howe.

The Neurotrophic Factor Hypothesis postulates that neuronal survival and the development and maintenance of neuronal connectivity is dependent upon competition between axon terminals for a limited supply of target-derived trophic support. Neurons and neuronal connections which compete successfully for this limited supply of trophic factor survive and thrive, while those which fail, die. Early evidence in support of this hypothesis can be found in the work of Braus, Harrison, and Detwiler, among others, culminating in the experiments of Hamburger and Levi-Montalcini. However, one of the seminal observations supporting the Neurotrophic Factor Hypothesis was provided by Marian Lydia Shorey in 1909 (J Exp Zool 7:25-63). By showing that removal of a chick limb bud early in embryonic development resulted in a marked decrease in neuron number within the spinal cord at later developmental stages, she provided the first evidence that neuronal targets could influence the size of corresponding neural ganglia. She went on in subsequent work (J Exp Zool 9:85-93, 1911) to suggest that a metabolic product of the target is required for the formation and maintenance of neuronal

connections, presaging later discoveries of target-derived neurotrophic factors like nerve growth factor. While the prevailing theories of the time focused on control of neuronal differentiation, Shorey's experiments had far-reaching influence on subsequent work by Detwiler, Hamburger, and others, testing the role of the target in neuronal survival and connectivity. Supported by an HHMI Predoctoral Fellowship and the McGowan Charitable Trust.

All Along the Watchtower - Bob Dylan

There must be some kind of way out of here,
said the Joker to the Thief.
There's too much confusion,
I can't get no relief.

Business men they drink my wine,
plowman dig my earth.
None of them along the line
know what any of it is worth.

No reason to get excited,
the Thief, he kindly spoke.
There are many here among us
who feel that life is but a joke.

But you and I, we've been through that,
and that is not our fate.
So let us stop talking falsely now,
the hour is getting late.

All along the watchtower,
princes kept the view.
While all the women came and went,
barefoot servants, too.

Outside in the cold distance
a wildcat did growl.
Two riders were approaching,
and the wind began to howl.