UNIVERSITY OF
CALGARY

THE UNIVERSITY OF CALGARY

Trk Receptors in *Lymnaea*

by

Neal R. Melvin

A THESIS

SUBMITTED TO THE FACULTY OF GRADUATE STUDIES

IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE

DEGREE OF MASTER OF SCIENCE

DEPARTMENT OF NEUROSCIENCE

CALGARY, ALBERTA

AUGUST, 2001

Abstract

The neurotrophins and their corresponding Trk receptors play key roles in both the developing and mature nervous system of mammals. Recently, it has become apparent that some components of the neurotrophin system extend beyond the mammalian lineage. Despite physiological evidence that mammalian neurotrophins exert neurotrophin-characteristic effects on invertebrate neurons, no endogenous ligand has been identified outside the vertebrate lineage. However, one Trk-like receptor has been identified in the pond snail *Lymnaea*. This receptor appears unable to account for the effects of mammalian neurotrophins in *Lymnaea*. We therefore hypothesized that additional receptors must exist. To this end, we employed a panel of polyclonal, Trk-specific antisera to determine whether we could detect *Lymnaea* Trk-like proteins. Each antibody cross-reacted with antigens that had various properties associated with Trks, including one which is capable of becoming tyrosine phosphorylated after axotomy.

Acknowledgments

I was fortunate enough to be surrounded with several people, both within the laboratory and out, which made my studies a great experience. To begin with, I would like to thank Dr. Andrew Bulloch for affording me the opportunity to study in his laboratory. In addition, various members, both past and present have been great with offering advice or support throughout my project. To Garry and Matt, thank you for listening to my complaints and my bizarre jokes. I would like to give special thanks to both Drs. Wic Wildering and Petra Hermann for being a continuous source of invaluable expertise and advice. This project would have been impossible without their presence.

I would also like to acknowledge the Department of Neuroscience as a whole, for offering timely advice on my project.

I would also like to thank several friends who made my tenure here exciting. To Vinay Bharadia and Shane Ferguson, I will never forget the many lively discussions on various topics that we shared over the years. Particularly to Shane, without whose support I would not be where I am today.

Finally, to friends outside the lab and my family. To my father John, who offered advice when I needed to hear it. To Chris McLean and Phil Klassen, thank you for letting me vent when I needed to, and for a laugh when I needed it. To Melanie Popek and Karen Pelechaty, thank you for supporting me in the roles you both played in my life at different times. Finally and most importantly, to Christina Szekely, who will always be the most important person in my life. I hope you realize how much I appreciate every minute of our relationship.

Dedication

I would like to dedicate this thesis to my mother, June Melvin, who passed away shortly before I started graduate school. I think about you everyday, and I know you were with me every step of the way. I love you mom.

Table of contents

List of tables

List of figures

List of Abbreviations

5-HT	serotonin
Ap-Trk	*Aplysia* Trk
BSA	bovine serum albumin
BDNF	brain-derived neurotrophic factor
cDNA	complementary DNA
CM	brain-conditioned media
CNS	central nervous system
cRNA	complementary RNA
CRNF	cysteine-rich neurotrophic factor
DB	dorsal body
DM	defined medium
ECL	enhanced chemiluminescence
EGF	epidermal growth factor
Erk	extracellular-regulated kinase
EPSP	excitatory postsynaptic potential
FITC	fluorescein isothiocyanate
HRG	heregulin
HRP	horseradish peroxidase
HVA	high voltage activated
kD	kilodaltons
LBu	left buccal ganglion

LCe	left cerebral ganglion
Lf-NT	*Lampetra* neurotrophin
LPa	left parietal ganglion
LPe	left pedal ganglion
LPl	left pleural ganglion
*L*trk	*Lymnaea* Trk-like receptor
LTF	long-term facilitation
Mg-NT	*Myxine* neurotrophin
MYBP	million years before present
NGF	nerve growth factor
NGS	normal goat serum
NRS	non-immune rabbit serum
NT-3	neurotrophin-3
NT-4/5	neurotrophin-4/5
PBS	phosphate-buffered saline
PCR	polymerase chain reaction
PDGF	platelet-derived growth factor
PeA	pedal A neurons
PFA	paraformaldehyde
PLC-γ	phospholipase C-γ
pY	phosphotyrosine
RBu	right buccal ganglion
RCe	right cerebral ganglion

RPa	right parietal ganglion
RPA	right parietal A neurons
RPB	right parietal B neurons
RPD2	right parietal dorsal 2 neuron
RPe	right pedal ganglion
RPeD1	right pedal dorsal 1
RPl	right pleural ganglion
RTK	receptor tyrosine kinase
RT-PCR	reverse transcriptase-polymerase chain reaction
SCBT	Santa Cruz Biotechnology
TBS	tris-buffered saline
Trk	tropomyosin-related kinase
VD1	visceral dorsal 1 neuron
VD2/3	visceral dorsal 2/3 neuron
Vi	visceral ganglion
*z*TrkB1	zebrafish TrkB1 receptor
*z*TrkB2	zebrafish TrkB2 receptor
*z*TrkC1	zebrafish TrkC1 receptor
*z*TrkC2	zebrafish TrkC2 receptor
*z*NT-7	zebrafish neurotrophin-7

Chapter 1: Introduction

Background

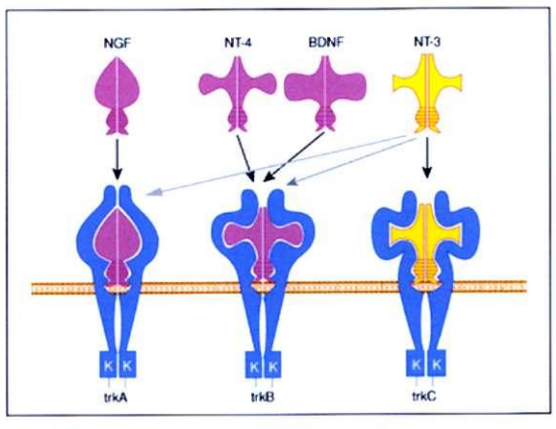
Neurotrophic factors are a group of polypeptide growth factors that play key roles in the developing nervous system. The neurotrophins, a subgroup of neurotrophic factors, are defined as being related in sequence to the first neurotrophin discovered, nerve growth factor (NGF) (Hallbook et al., 1991). Traditionally, the neurotrophins have been viewed as molecules having strictly developmental roles, including the regulation of cell survival (Levi-Montalcini, 1987; Buchman & Davies, 1993), cell death (Barker, 1998; Casaccia-Bonnefil et al., 1998), stimulation of neurite outgrowth (Levi-Montalcini, 1966; Reichardt and Farinas, 1997; Bosco & Linden, 1999), differentiation (de la Rosa et al., 1994; Peunove & Enikolopov, 1995; Brodski et al., 2000) and synapse formation (Wells et al., 1999). However, several recent papers have demonstrated that the neurotrophins also play key roles in the regulation of several phenomena in the adult central nervous system (CNS), including cell survival after injury (Giehl & Tetzlaff, 1996; Hammond et al., 1999; Novikova et al. 2000) axonal sprouting and regeneration (Bradbury et al., 1999; Oudega and Hagg, 1999; Mamounas et al., 2000) and long-term potentiation (Kang & Schuman, 1995; Kang et al., 1997; Korte et al., 1998; Arvanov et al., 2000). Thus, the neurotrophin system is a key component underlying many aspects of neural plasticity in a wide variety of contexts.

Neurotrophins, receptors, and their phylogenetic distributions

The neurotrophin system in mammals includes four neurotrophin ligands (nerve growth factor, NGF; brain-derived neurotrophic factor, BDNF; neurotrophin-3, NT-3; neurotrophin-4/5, NT-4/5), and two classes of receptors: three receptor tyrosine kinases (RTKs) known as the Trks, and a transmembrane protein with no intrinsic catalytic activity known as the p75 receptor. NGF binds preferentially to TrkA, BDNF and NT-4/5 bind to TrkB, and NT-3 preferentially binds to TrkC receptor, but is also capable of less efficiently binding to the TrkA and TrkB receptors (Yano & Chao, 2000; figure 1). Upon binding of a neurotrophin to its Trk receptor, receptor dimerization and subsequent autophosphorylation of intracellular tyrosine residues occurs. These phosphorylated residues serve as docking sites for several adaptor or effector proteins that subsequently mediate the various phenotypic effects of neurotrophins (Kaplan & Miller, 1997; Kaplan & Miller, 2000).

It is important to mention that there appear to be several different isoforms of Trk receptors. In addition to the full-length versions, both TrkB (Middlemas et al., 1991) and TrkC (Valenzuela et al., 1993), but not TrkA, have truncated isoforms that have extracellular and transmembrane regions identical to their full-length counterparts, but lack the intracellular tyrosine kinase domains. In addition, several other isoforms exist with more subtle changes, such as small inserts or deletions, which modulate the effects of the neurotrophins (Barbacid, 1994).

Figure 1: The neurotrophin system in mammals. This figures demonstrates the ligand-receptor pairings. NGF acts through the TrkA receptor, BDNF and NT-4 through the TrkB receptor, and NT-3, although capable of interacting with all Trks, primarily acts via the TrkC receptor. (Adapted from Zigmund et al. 1999).


NGF
NT-4
BDNF
NT-3
K K
K K
K K
trkA
trkB
trkC

The potential roles that the p75 neurotrophin receptor plays has only recently come to light (Barrett, 2000). These studies have been hampered by the fact that this transmembrane receptor contains a cytoplasmic segment with no obvious signaling domains. However, several recent studies demonstrate that p75 may play a prominent role in mediating neurotrophin-dependent cell death (Casaccia-Bonnefil et al., 1998; Casaccia-Bonnefil et al., 1999; Friedman, 2000), as well as modulating the neurotrophin-binding affinity and responsiveness of Trk receptors when these two receptors are co-expressed (Barker and Shooter, 1994; Verdi et al., 1994). However, since all the neurotrophins bind to the p75 receptor with similar affinities (Rodriguez-Tebar et al., 1992), it is generally concluded that the Trk receptor system mediates the specific effects of the neurotrophins. Indeed, most of the phenotypic affects that are associated with the neurotrophins (i.e., cell survival, differentiation, and neurite outgrowth) are attributed to signaling via the Trk receptors (Kaplan & Miller, 2000).

The neurotrophin system in non-mammalian vertebrates

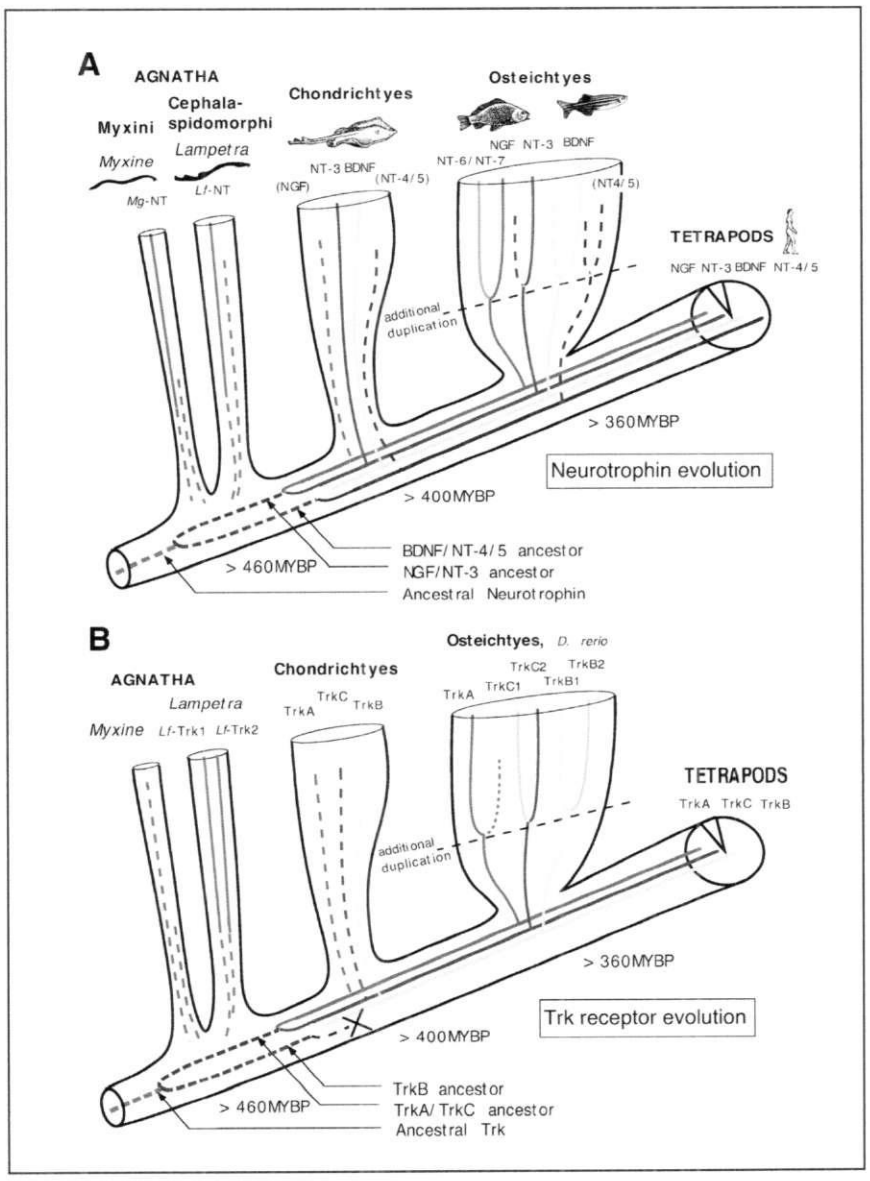
Until recently, it had been assumed that the neurotrophin system was a relatively modern evolutionary development (Barde, 1994). Although their presence is well documented in mammals, several recent studies have provided evidence that neurotrophins and Trk receptors are also present in several non-mammalian vertebrates.

The first direct evidence for a neurotrophin receptor system in a non-mammalian vertebrate species was provided by Martin et al. (1995), who cloned a total of five Trk receptors from the zebrafish *Danio rario* using a polymerase chain reaction (PCR)

homology-based approach. The clones proved to be homologues of each of the three subtypes of mammalian Trk receptors: a TrkA homolog, two TrkB homologs (*z*TrkB1 and *z*TrkB2), as well as two TrkC homologs (*z*TrkC1 and *z*TrkC2). Within the intracellular kinase domain, the deduced amino acid sequences revealed >90% similarity to mammalian Trks. Although it is not yet known whether there is conservation of function in zebrafish Trks, there appears to be a conservation of key amino acid residues and motifs known to mediate neurite outgrowth and cell survival (Heinrich & Lum, 2000). In support of this, a subsequent study has shown that the two TrkC homologues have expression patterns that correlate with axon formation, suggesting a role in neurite outgrowth (Martin et al., 1998). Furthermore, Williams et al. (2000) have shown that signaling via the *z*TrkC1 isoform can modulate cell death in the spinal cord. In the context of the current proposal, it is important to note that the impetus for these cloning experiments was based largely on their earlier findings of cross reactivity between antibodies designed against mammalian Trks and zebrafish antigens (Sandell et al., 1994); this antibody (known as anti-pan Trk) recognizes all Trk isoforms in mammals, and was designed against the same epitope as that used in the current study. These data indicate that the Trk receptor family had existed prior to the split of the Osteichtyes (bony fish) class from the vertebrate lineage (approximately 360 million years before present (MYBP)). It also suggests that, since zebrafish have five Trks, and mammals have only three, there must have been an additional gene duplication within the Osteichtyes lineage, giving rise to two additional members (Hallbook, 1999; figure 2).

Recently, a neurotrophin ligand has also been isolated from zebrafish. Nilsson et al. (1998) reported the discovery of *z*NT-7. This protein appears to be related to NGF and

Figure 2: Phylogenetic distribution of neurotrophins and Trks in vertebrates. **A)** A phylogenetic tree showing the evolutionary relationship between the various neurotrophins discovered in several groups of vertebrates. **B)** A phylogenetic tree showing the evolutionary relationship between the Trk receptors in various vertebrates. This diagram illustrates that components of the neurotrophin system are present in all major vertebrate groups, including Osteichtyes (bony fish), Chondrichtyes (cartilaginous fish), and Agnatha (jawless fish). (Taken from Hallbook, 1999).


A
AGNATHA
Myxini
Cephala-
spidomorphi
Myxine
Lampetra
Mg-NT
Lf-NT
Chondrichtyes
NT-3 BDNF
(NGF)
(NT-4/5)
Osteichtyes
NGF NT-3 BDNF
NT-6/NT-7
(NT4/5)
TETRAPODS
NGF NT-3 BDNF NT-4/5
additional
duplication
> 360MYBP
Neurotrophin evolution
> 400MYBP
> 460MYBP
BDNF/NT-4/5 ancestor
NGF/NT-3 ancestor
Ancestral Neurotrophin
B
AGNATHA
Lampetra
Myxine Lf-Trk1 Lf-Trk2
Chondrichtyes
TrkA TrkC TrkB
Osteichtyes, D. rerio
TrkA TrkC1 TrkC2 TrkB1 TrkB2
TETRAPODS
TrkA TrkC TrkB
additional
duplication
> 360MYBP
Trk receptor evolution
> 400MYBP
> 460MYBP
TrkB ancestor
TrkA/TrkC ancestor
Ancestral Trk

NT-6, a novel neurotrophin isolated from the platyfish *Xiphophorus*, another bony fish species (Gotz et al., 1994). Furthermore, Nilsson and colleagues demonstrated that recombinant *z*NT-7 is capable of binding to the human p75 receptor, and is also capable of inducing tyrosine phosphorylation of rat TrkA.

In addition to the aforementioned 'novel' isoforms of neurotrophin ligands, various homologues of the 'traditional' neurotrophins have also been isolated from several species of fish (Hallbook et al., 2000). NGF, BDNF, and NT-3 homologues have been described in the bony fish lineage (Gotz et al., 1992; Nilsson et al., 1998; Lai et al., 1998) and BDNF and NT-3 homologues have been found in the cartilaginous fish lineage (Hallbook et al., 1991). However, although neurotrophins are present in the jawless fish lineage (Hallbook et al., 1998), their primary sequences begin to exhibit less similarity to mammalian neurotrophins. For instance, although the *Lf*-NT sequence contains the six conserved cysteine residues typical of the neurotrophins, it also contains two insertions in the loop regions. One insertion is found in a region where other insertions can be found in NT-6, NT-7, and mammalian NT-4/5; the location of the other insertion is unique to *Lf*-NT. These findings demonstrate the subtle divergence of structure that begins to occur in phylogenetically older groups of animals. Taken together, the data has indicated that the neurotrophin system dates back at least as far as the earliest known vertebrates, 460 MYBP (figure 2).

In addition to the discovery of neurotrophin system components in bony fish species, neurotrophin ligands and receptors have also been identified in species that diverged even earlier. Using a PCR homology-based strategy, Hallbook and colleagues (1998) obtained two partial sequences of Trks from the river lamprey *Lampetra*.

Lampetra is a representative of the jawless fishes, which diverged from the common lineage approximately 460 MYBP (Kumar & Hedges, 1998), and are thought to be some of the earliest known vertebrates (Halstead, 1993; figure 2). Using a similar strategy, the authors obtained full-length clones of two neurotrophins, one from *Lampetra* (*Lf*-NT), and one from the hagfish *Myxine* (*Mg*-NT), another jawless fish. Hallbook points out that although the neurotrophin binding characteristics of *Lf*-Trk1 and *Lf*-Trk2 are not yet known, it is likely that *Lf*-NT binds at least one of them (Hallbook, 1999).

Evidence for the existence of the neurotrophin system in invertebrates

The idea that the neurotrophin system may extend even further back than the earliest vertebrates and into the invertebrate lineage is a relatively recent idea. This claim is supported by several studies that provide both indirect and direct evidence of a parallel neurotrophin-like system in a variety of invertebrate species. It is relevant to mention that the majority of this data comes from studies on various species within the Phylum mollusca.

Early evidence that a neurotrophin-like ligand may exist in invertebrates was provided by the studies of Ridgway et al., (1991) using the pond snail *Lymnaea stagnalis*, a gastropod mollusc. A central tenet of cell culture studies in this and closely related species holds that in order for neurons to sprout neurites *in vitro*, brain-derived (*Lymnaea* and *Helisoma*; Wong et al., 1981; Wong et al., 1984; Ridgway et al., 1993) or blood-derived proteins (*Aplysia*; Schacher & Proshansky, 1983) are required. Thus, it is standard practice to culture neurons from *Lymnaea* in brain-conditioned media (CM),

which is made by incubating 2 brains in 1 ml of a modified Leibowitz-15 (L-15; defined media, DM) solution for 72 hours. During this time, a variety of factors are released into the media that contain 'neurotrophic activity'; in other words, factors that are capable of stimulating neurite outgrowth.

In order to gain insights into the factors that mediate outgrowth, Ridgway et al. (1991) tested the possibility that the mammalian neurotrophin NGF, which mediates neurite outgrowth from various subpopulations of mammalian neurons, might provide neurotrophic activity for *Lymnaea* neurons. To this end, the authors tested various phenotypic classes of neurons cultured in a control media (DM), or DM plus NGF. It was found that both motor neurons and interneurons, but not secretory neurons, sprouted neurites in the presence of NGF. Importantly, this effect could be blocked with an anti-NGF antibody. This suggested the possibility that there may be an endogenous NGF-like molecule present in *Lymnaea* that may mediate CM-induced outgrowth. In order to test for the presence of endogenous NGF-like molecules, the authors pre-absorbed CM with an anti-NGF antibody, and tested the resulting CM for neurotrophic activity. When cultured in this media, the neurotrophic activity was abolished for both motor neurons and interneurons, but secretory neurons were still able to sprout. Thus, immunoprecipitating CM with an anti-NGF antibody selectively removes the endogenous neurotrophic factor(s) for the classes of neurons that are responsive to NGF. These data provide support for the contention that the *Lymnaea* nervous system contains an endogenous molecule that cross reacts with an anti-NGF antiserum, and which can function in a manner typical of the neurotrophins.

Two subsequent studies have shown that mammalian NGF has additional effects on *Lymnaea* neurons. Wildering et al. (1995) demonstrated that mammalian NGF has acute electrophysiological effects on *Lymnaea* neurons. Treating right parietal A (RPA) motor neurons with NGF resulted in a dose-dependent enhancement of high voltage activated (HVA) currents within 2 minutes of application; this effect was reversible upon washout. It should be noted that acute electrophysiological effects of the neurotrophins have also been described in the literature. For example, Lohof et al. (1993) showed that BDNF and NT-3 rapidly potentiate synaptic activity at *Xenopus* neuromuscular junctions; this effect is mediated by Trk receptors. In addition, BDNF has been shown to very rapidly modulate a subtype of glutamate current in rodent hippocampal neurons (Jarvis et al., 1997). A further study characterizing the effects of NGF in *Lymnaea* comes from the work of Syed et el. (1996). This study showed that, in addition to being a neurotrophic factor, it is also capable of supporting the formation of synapses between interneurons and their postsynaptic neurons *in vitro*.

Despite the suggestion by the aforementioned studies that a neurotrophin-like homologue may exist in *Lymnaea*, no such molecule has yet been identified. In an attempt to identify a neurotrophin-like ligand in *Lymnaea*, Fainzilber et al. (1996) decided to adopt a functional approach. Since all neurotrophins bind to the p75 receptor, the authors postulated that a *Lymnaea* neurotrophin homolog might also bind the p75 receptor. Using a cell line that expresses high levels of p75, but not Trk receptors, CM and hemolymph (i.e., blood) from *Lymnaea* were tested for their ability to displace radiolabelled NGF in a binding assay. Both extracts were shown to displace NGF binding in a dose-dependent manner. Upon sequencing, however, the 13.1kD protein that was

responsible for this effect (called cysteine-rich neurotrophic factor or CRNF) had no sequence similarity either to mammalian neurotrophins nor any other known molecule. *In situ* hybridization studies revealed that CRNF was normally expressed in the epithelial cells of the foot. Expression in the CNS, however, was only seen after injury. CRNF was also capable of stimulating neurite outgrowth in RPA motor neurons, as was the case for mammalian NGF. In addition, CRNF was also capable of modulating HVA currents but, unlike NGF, this effect was not reversible upon washing.

Since NGF stimulates neurite outgrowth through the TrkA receptor in mammals (Stephens et al., 1994), and NGF elicits this same effect in *Lymnaea,* it seemed plausible that *Lymnaea* may contain endogenous Trk-like proteins. In order to address this issue, van Kesteren et al., (1998) employed a homology-based PCR approach using primers directed against conserved sequences within the tyrosine kinase domain of most RTKs. Although several fragments were isolated, one had a significantly higher sequence similarity to the Trks, and was subsequently used to screen a *Lymnaea* cDNA library to identify a full-length clone. The full-length clone, known as *Lymnaea* Trk (*Ltrk*), was a 2829 base pair cDNA that encodes a 794 amino acid protein. The deduced protein sequence revealed several features characteristic of mammalian Trk receptors, as well as some novel domains.

*L*trk exhibits several characteristics that are consistent with mammalian Trk receptors. To begin with, *L*trk is most similar to TrkC, with an overall sequence similarity of 34% at the protein level, whereas the similarity to both TrkA and TrkB is 31%. Intracellularly, the sequence similarity is much higher, being 61% similar to TrkC in its tyrosine kinase domain. Many of the amino acid sequences that are specific to Trks are

also seen in *L*trk, including the Shc-binding site, and the phospholipase C-γ (PLC- γ) binding site, both of which may be cooperatively involved in mediating neurite outgrowth (Stephens et al., 1994). Furthermore, the autophosphorylation site is also well conserved. In its extracellular region, *L*trk has two cysteine clusters that surround a leucine-rich motif that is characteristic of Trk receptors (Barbacid, 1994). There are, however, two key differences in the extracellular portion of *L*trk that distinguish it from mammalian Trks: *L*trk lacks immunoglobulin domains, and has a novel N-terminal extension of approximately 100 amino acids.

Experiments designed to address the binding characteristics and expression pattern of *L*trk were also completed by van Kesteren et al. (1998). By exogenously expressing *L*trk in cell lines, it was demonstrated that it is capable of binding TrkC's native ligand, NT-3, but not NGF, BDNF, or CRNF. However, despite possessing the ability to bind a mammalian neurotrophin, NT-3 was unable to induce a response characteristic of Trk activation, namely Erk (extracellular-regulated kinase) phosphorylation or an increase in cell survival. This finding would seem to suggest at least two possibilities: either the mammalian ligand is different enough from *L*trk's endogenous ligand so as to not sufficiently activate its signaling capacity, or the kinase domain of *L*trk is a non-functional evolutionary relic. Doubt has been cast on the latter explanation recently by experiments conducted in the laboratory of our colleague, Michael Fainzilber, using a protein chimera approach. By fusing the extracellular domain of the platelet-derived growth factor (PDGF) to the intracellular domain of *L*trk, Fainzilber and colleagues have shown that *L*trk is indeed capable of autophosphorylation and Erk phosphorylation (Fainzilber et al., 2000). *In situ* hybridization experiments

revealed that *L*trk has a very limited distribution within the nervous system, being predominantly expressed in a pair of neuroendocrine organs called the dorsal bodies, as well as some neurons in the right parietal and visceral ganglia. Interestingly, reverse-transcriptase PCR (RT-PCR) experiments showed that expression was seen at various points in embryonic animals, in sub-adults, and in the adult CNS only when the dorsal bodies were present. However, recent experiments performed by Garry Hauser using RT-PCR analysis on individual ganglia from adult animals indicates that *L*trk is expressed in virtually all ganglia, albeit at different levels (unpublished observations). The function of endogenously expressed *L*trk is not currently known.

NGF also has physiological actions in the squid *Loligo*, a cephalopod mollusc. In a very comprehensive series of experiments, Moreno et al. (1998) provided convincing evidence that a TrkA-like receptor may exist in this species. They showed that mammalian NGF acutely reduced the amplitude of synaptic potentials at the squid giant synapse. Related neurotrophins (NT-3 and BDNF) and other growth factors (epidermal growth factor (EGF), and heregulin, (HRG)) did not induce a significant reduction of synaptic amplitude. In addition, the effects of NGF could be blocked with the tyrosine kinase inhibitor k252a, which has been shown to inhibit Trk receptor activation (Angeles et al., 1998). Because these electrophysiological and pharmacological experiments suggested the presence of a TrkA-like receptor, further biochemical experiments were performed to identify such a protein. Using an antibody directed against mammalian Trk receptors, it was demonstrated that two immunoreactive bands were present in the squid: a ~ 140 kDa protein, as well as a more prominent ~ 95 kDa protein. In addition, using immunoprecipitation with the anti-Trk antisera, it was shown that the 95 kDa protein

undergoes tyrosine phosphorylation in response to NGF treatment. Importantly, this NGF-induced phosphorylation could be prevented with k252a. This elegant series of experiments provided convincing evidence that a TrkA-like receptor might exist in the squid. It is important to note that the antibody used in this study is the same antibody we used in the current study.

NGF is not the only mammalian neurotrophin that has also been shown to have physiological effects on molluscan neurons. McKay et al. (1999) have reported that BDNF can cause an increase in the amplitude of excitatory postsynaptic potentials (EPSPs) after 18-24 hours in the marine mollusc *Aplysia*. This effect, known as long-term facilitation (LTF), is known to be elicited under normal physiological conditions by the neurotransmitter serotonin (5-HT) (Mauelshagen et al., 1996). When 5-HT-induced LTF is elicited *in vitro*, the use of TrkB receptor bodies, which would chelate potential BDNF-like molecules, abolishes this effect (Giustetto et al., 1999). These results imply that *Aplysia* may have an endogenous BDNF-like molecule.

In addition to altering the electrophysiological properties of *Aplysia* synapses, BDNF can also modify elements of the cytoskeleton (McKay et al., 1999). Using an antibody against tyrosine-phosphorylated residues, this study showed that the application of BDNF to cultured neurons leads to a decrease in tyrosine phosphorylation within the filopodia of extending neurites. 5-HT was also able to down-regulate this phosphorylation over a similar time course.

Subsequent western blotting experiments using an antibody directed against the mammalian TrkB revealed the presence of a ~ 140 kDa protein, which the authors suggested may represent the Aplysia TrkB receptor (Giustetto et al., 1999).

Immunocytochemical experiments demonstrated both a pre- as well as postsynaptic localization of the TrkB-like antigen. Giustetto and colleagues (2000) have recently reported the partial complementary DNA (cDNA) cloning of a Trk-like receptor from *Aplysia* (referred to as *Ap*-Trk). From the sequence obtained, the authors report that *Ap*-Trk exhibits high sequence similarity with both vertebrate and invertebrate Trks.

Data supporting the existence of neurotrophins from non-molluscan invertebrates has also emerged. A paper from Hayashi et al. (1992) using *Drosophila* as a model system provided further evidence that invertebrates may use neurotrophin-like ligands. The authors used the chick sensory ganglion outgrowth assay (Levi-Montalcini & Hamburger, 1953) to determine whether conditioned media made from embryonic *Drosophila* cells contains factors with neurotrophic activity. When chick sensory ganglia were cultured in this media, neurite outgrowth was observed that could be blocked by concomitant incubation with an anti-NGF antibody. Also, the addition of mouse NGF to *Drosophila* embryonic neural precursors resulted in an increase in cell number. Lastly, Hayashi et al. (1992) used Northern blotting to show that a mouse complementary RNA (cRNA) probe hybridizes to a 1.4 Kb *Drosophila* transcript. Importantly, hybridization of this probe to the mouse NGF mRNA reveals a 1.3 Kb product (Masonpierre et al., 1990). Taken together, these results provided further circumstantial evidence that an NGF-like molecule may exist in an invertebrate species. However, the recent sequencing of the *Drosophila* genome has failed to yield any neurotrophins or Trk homologues (Adams et al., 2000; Myers et al., 2000). However, Pulido and colleagues (1992) describe a molecule that does have some characteristics in common with vertebrate Trks, but is

sufficiently different in its extracellular organization, and is thus assigned to a distinct family of kinases (Jaaro et al., 2001).

Finally, a study using the annelid worm *Eisenia* has provided putative immunochemical evidence for the existence of Trk-like proteins. Using immunohistochemistry and western blotting with antibodies directed against mammalian Trk proteins, Lucini et al., (1999) demonstrated that Trk-like antigens with molecular weights consistent with those seen in mammals are detected in both neuronal and non-neuronal cell types. It would be interesting to see if any of these antigens become tyrosine phosphorylated in response to treatment with mammalian neurotrophin ligands.

The full-length cloning of *Ltrk* in *Lymnaea* and a partial Trk clone in *Aplysia* provides definitive evidence for the emergence of Trk receptors prior to the divergence of the molluscan and vertebrate lineages, approximately 600 MYBP (Jaaro et al., 2000). Combined with the data discussed above in other molluscan species, it seems plausible that other members of the Trk family of proteins may exist in *Lymnaea*. In particular, since mammalian NGF has a variety of physiological actions in *Lymnaea*, and *L*trk does not bind NGF, and because the effects of NGF are consistent with those know to result from Trk receptor activation, we hypothesized that at least one other Trk-like receptor exists in this species that may account for the effects of NGF.

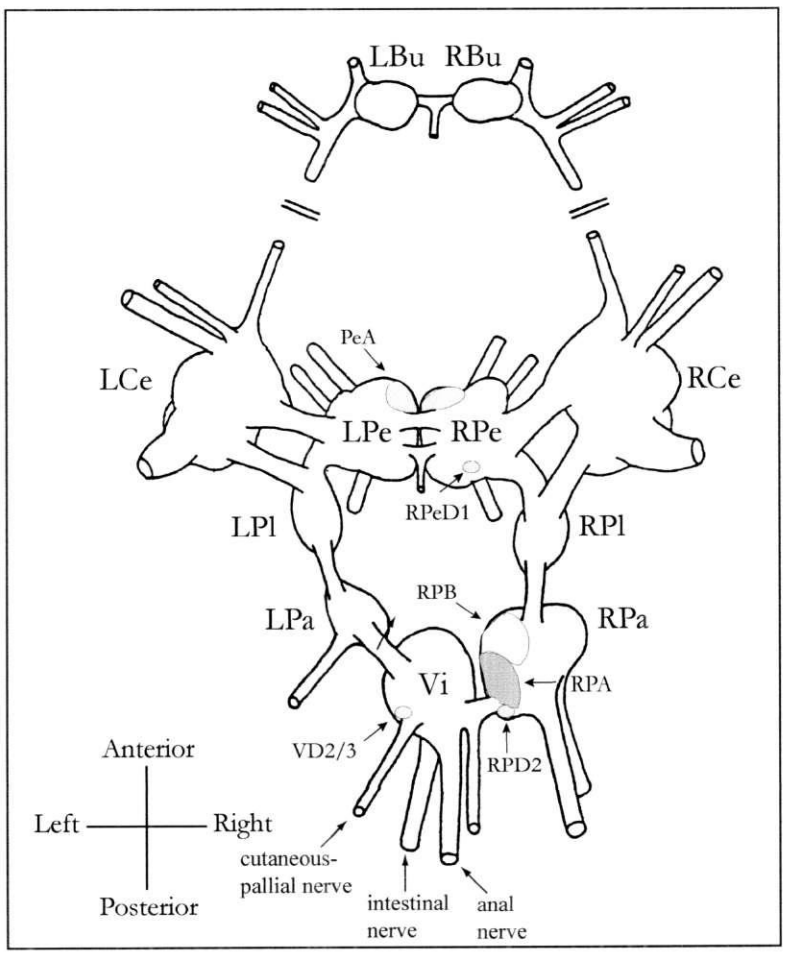
The model system

Due to its complexity, the mammalian CNS is often not a suitable model system to study the cellular and molecular basis of neural plasticity. Accordingly, many

researchers have adopted a reductionist approach, opting to study organisms with 'simpler' nervous systems. A heavily exploited organism, particularly for electrophysiologists, has been the nervous systems of a variety of molluscan species. Indeed, much of our current knowledge of the electrical properties of neurons (Hodgkin & Huxley, 1939; Hodgkin & Huxley, 1949; Hodgkin & Huxley, 1952) and the cellular and molecular basis of learning and memory (Castellucci et al., 1986; Montarolo et al., 1986; Dale et al., 1988; Wu & Schacher, 1994; Benjamin et al., 2000; Crow & Xue-Bian, 2000) has come from research conducted using molluscan nervous systems.

Relatively 'simpler' model systems, such as the nervous system of the pond snail *Lymnaea stagnalis* (figure 3), have provided a wealth of information to neurobiologists, particularly with respect to various aspects of neural plasticity (Bulloch & Ridgway, 1989; Bulloch & Syed, 1992; Syed et al., 1992; Magoski & Bulloch, 1998). Specifically, *Lymnaea* offers several significant advantages for studies in neurobiology: (1) the nervous system contains several easily identified and well characterized neurons, with well defined connections (Bulloch & Ridgway, 1989), and (2) adult neurons are capable of axonal regeneration and the re-establishment of appropriate synaptic connections both *in vitro* and *in vivo* (e.g., Murphy and Kater, 1980; Syed et al., 1992; Moffet, 2000). In addition to these more 'practical' issues, the study of the neurotrophin system in *Lymnaea* may also have important evolutionary implications.

Figure 3: Schematic illustration of the CNS of *Lymnaea*. Abbreviations: LBu, RBu (left and right buccal ganglia, respectively); LCe, RCe (left and right cerebral ganglia, respectively); LPe, RPe (left and right pedal ganglia, respectively); LPl, RPl (left and right pleural ganglia, respectively); LPa, RPa (left and right parietal ganglia, respectively); Vi (visceral ganglion); PeA (pedal A neurons); RPeD1 (right pedal dorsal 1 neuron); RPB (right parietal B cells); RPA (right parietal A cells); RPD2 (right parietal dorsal 2 neuron); VD 2/3 (visceral dorsal 2/3 neuron).

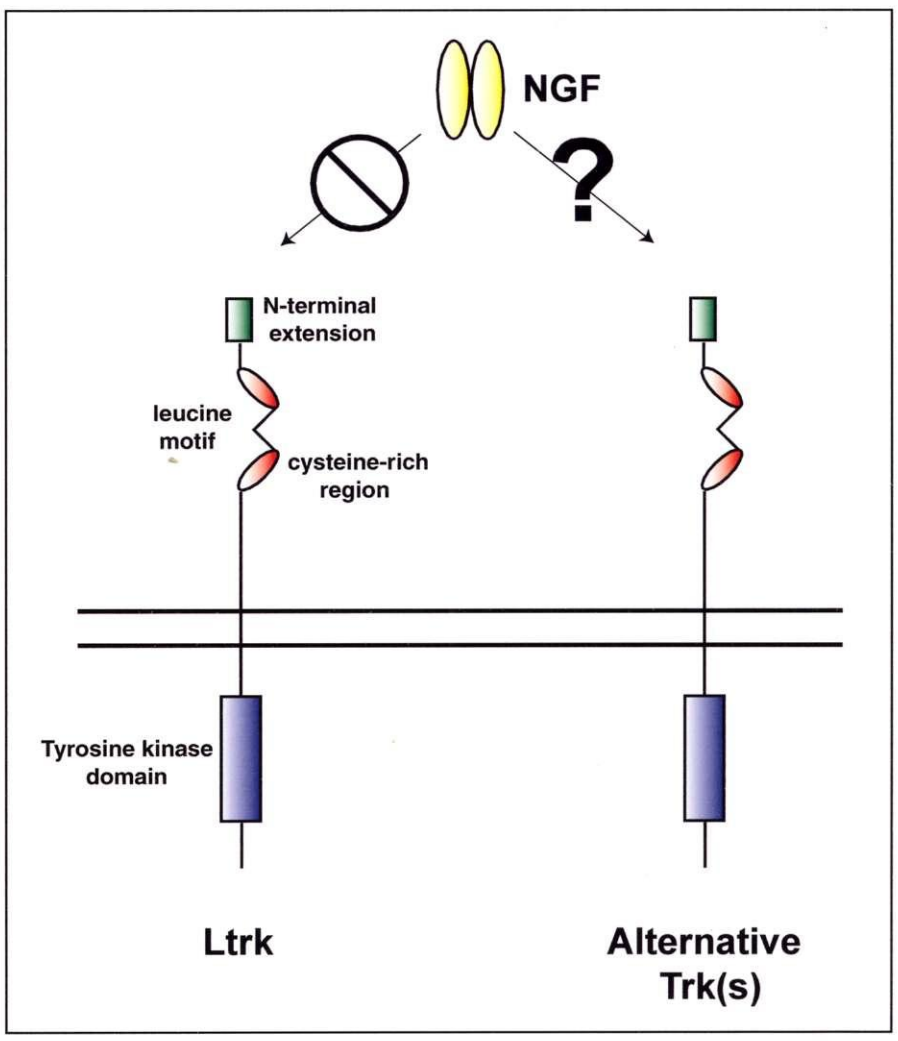

LBu
RBu
PeA
LCe
RCe
LPe
RPe
RPeD1
LPl
RPl
RPB
LPa
RPa
Vi
RPA
VD2/3
RPD2
Anterior
Left
Right
Posterior
cutaneous-
pallial nerve
intestinal
nerve
anal
nerve

The approach

The hypothesis that other Trk receptors may be present in *Lymnaea* is supported by several studies conducted in a variety of molluscan species, including *Lymnaea*. Although much of this evidence is circumstantial, it nevertheless provides sufficient reason to believe that Trks exist as a family in molluscs as they do in mammals. To begin with, three studies from the laboratory of Dr. Bulloch (Ridgway et al., 1991; Wildering et al., 1995; Syed et al., 1996) show that mammalian NGF has a variety of activities on *Lymnaea* neurons, which suggests the existence of a TrkA-like receptor. Moreno et al. (1998) provided additional evidence for the existence of a TrkA-like receptor in the squid *Loligo*. Finally, Giustetto et al. (1999) report experiments suggesting the existence of a TrkB-like receptor, and have obtained a partial cDNA sequence that is Trk-like. If TrkA-like and TrkB-like receptors are in fact present in these molluscs, it is reasonable to assume that the *Lymnaea* nervous system may also contain similar proteins. Since *L*trk appears to be a TrkC homolog, does not bind NGF, and is not expressed in areas where NGF-responsive cells are found, it cannot account for the effects of NGF on *Lymnaea* neurons. Thus, at least one other Trk-like receptor, possibly similar to TrkA, is likely to exist in *Lymnaea* (figure 4).

Curiously, several experiments designed to exploit nucleic acid sequence similarities to isolate additional Trk-like receptors have failed (PCR-based homology; Hauser & Bulloch, unpublished observations). This series of experiments suggests one of two conclusions: (1) *L*trk is the only Trk-like receptor present in *Lymnaea* or (2) these methods are unable to detect other Trk-like proteins. Several variables may explain the

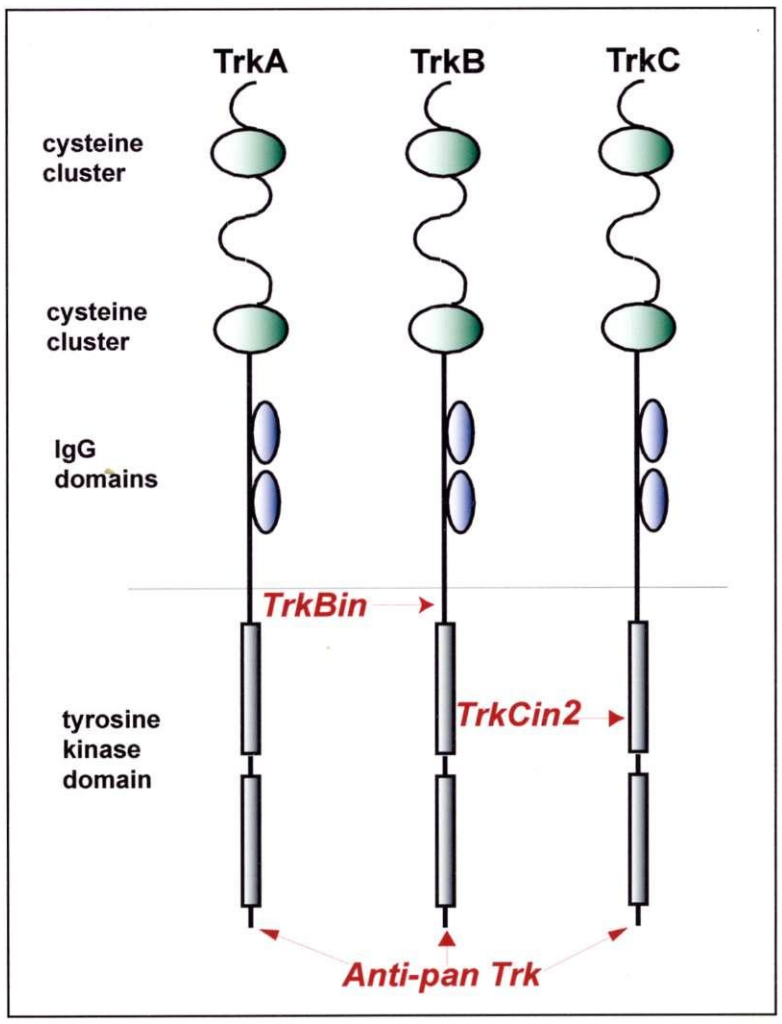
Figure 4: Schematic illustration of the general hypothesis. Mammalian NGF exerts several physiological effects on the *Lymnaea* nervous system. However, it does not bind *L*trk, the one known Trk-like receptor in *Lymnaea*. Therefore, we hypothesize that alternative Trk receptors exist in *Lymnaea* that may account for these effects.


NGF
?
N-terminal extension
leucine motif
cysteine-rich region
Tyrosine kinase domain
Ltrk
Alternative Trk(s)

latter result, such as the design of PCR primers, reaction conditions, or the choice of an optimal tissue source. In the current proposal, I suggest that an alternative strategy might be useful. I propose that exploiting the potential homology between mammalian Trk receptors and as yet unidentified molluscan Trks at the protein level might be effective in isolating additional members of this protein family from *Lymnaea*. To this end, a variety of polyclonal antibodies directed against mammalian Trks have been used as probes to identify potential Trk receptor members (figure 5). There are certainly many risks involved in using this approach, but our goal is simply to determine whether this alternative approach might be useful where the nucleic acid based methods have failed. Indeed, this approach has been used previously with some success. As discussed above, the impetus to clone the zebrafish Trks came from initial evidence using Trk-specific antibodies that cross-reacted with antigens in zebrafish (Sandell et al., 1994). Furthermore, the motivation for pursuing the cloning of an *Aplysia* Trk was based on cross-reactivity between a mammalian Trk antibody and an *Aplysia* protein (Giustetto et al., 1999; Giustetto et al., 2000).

Utilizing an approach based on amino acid similarities may in fact be a more fruitful approach when one considers the concept of synonymous and non-synonymous nucleotide substitutions in DNA. Due to the degeneracy of the genetic code, it is possible for a change in DNA sequence to occur without causing a change in the subsequent amino acid sequence it encodes. This is referred to as a synonymous substitution; an alteration in the DNA sequence that results in a change in the encoded protein is called a non-synonymous substitution (Futuyma, 1997). There is evidence to indicate that synonymous substitutions are much more likely to occur than non-synonymous

Figure 5: Schematic illustration of antibody epitopes. Epitopes recognized by each antibody are shown in red. Anti-pan Trk detects all mammalian Trks by virtue of its highly conserved epitope at the carboxyl terminal. TrkBin and TrkCin2 detect only TrkB and TrkC isoform, respectively by recognizing epitopes unique to these receptors.


TrkA
TrkB
TrkC
cysteine cluster
cysteine cluster
IgG domains
TrkBin
TrkCin2
tyrosine kinase domain
Anti-pan Trk

substitutions. In an analysis of mitochondrial DNA from several primate species, Brown et al. (1982) found that synonymous substitutions have occurred at six times the rate of non-synonymous substitutions. Supporting data can also be found in bacteria (Dubose & Hartl, 1991) and *Drosophila* (Kreitman, 1983). It follows that Trks might also be more conserved on the protein level, and suggests that nucleic acid based strategies may not have detected additional Trks because of changes in the nucleotide sequence.

One of the antibodies used in the current studies --- anti-pan Trk --- is directed against a highly conserved epitope that corresponds to the PLC-γ binding site of Trks. The original antigen used to raise this antibody was a peptide corresponding to the last 14 amino acids at the carboxyl terminus of the human TrkA receptor. Anti-pan Trk recognizes Trks A, B and C due to the presence of this highly conserved epitope (Belliveau et al., 1997; figure 5). This site may be important for playing a role in neurite outgrowth (Stephens et al., 1994) and axon elongation (Ming et al., 1999; but for a counter argument, see Atwal et al., 2000). This site appears to be reasonably well conserved in *L*trk as well (van Kesteren et al., 1998), although it is not yet clear whether the anti-pan Trk antiserum recognizes *L*trk.

Originally, we planned on using only this antibody, as we reasoned that because it recognizes an epitope found in all Trk isoforms and corresponds to a highly conserved functional site, it would give us the best chance to identify other Trk-like proteins in *Lymnaea*. However, we also obtained antibodies specific to the TrkB (anti-TrkBin; Fryer et al., 1996) and TrkC (anti-TrkC2; Belliveau et al., 1997) isoforms, and decided to include them in the current study. These antibodies recognize epitopes that are specific to TrkB and TrkC, respectively, and thus are not highly conserved between Trk isoforms

(figure 5). It is important to note that the epitopes for all the antibodies used in this study are located on the intracellular portion of the receptors. These were chosen based on the fact that the degree of conservation between *L*trk and mammalian Trks is much higher intracellularly than extracellularly.

The objective of this project was to complete an immunohistochemical mapping of the distribution of *Lymnaea* antigens that are recognized by each antibody, and to employ protein chemistry techniques to further investigate the characteristics of the corresponding antigens. These techniques included conducting western blots with each antibody on adult *Lymnaea* CNS samples, and immunoprecipitation followed by anti-phosphotyrosine western blots for the anti-pan Trk antiserum. The TrkB and TrkC antibodies do not work under immunoprecipitating conditions, and as a result, these studies were limited to western blotting (Belliveau et al., 1997; Donna Senger, personal communication).

Chapter 2: Materials and Methods

Animals

All animals utilized in these studies had a shell length of 25mm (approximately 3 months of age), and were taken from laboratory stocks maintained at the University of Calgary. These animals were reared in aerated artificial pond water (0.25g/L Instant Ocean, Aquarium Systems) maintained at room temperature, and were fed lettuce and Trout Chow (Purina). For all procedures except where noted, animals were de-shelled and anaesthetized in a 25% Listerine solution in HEPES-buffered saline (referred to as 'normal saline'; 51.3 mM NaCl, 1.7 mM KCl, 4.1 mM $CaCl_2$, 1.5 mM $MgCl_2$, 5.0 mM N-3-hydroxyethylypiperazine-N'-2-ethanesulfonic acid), pH 7.9, for 3-5 minutes.

All biochemical experiments were conducted a minimum of two times, with the exception of the CM-challenge experiment and the TrkBin and TrkCin2 western blots of SCBT anti-pan Trk immunoprecipitated samples. All immunohistochemistry experiments were done a minimum of 5 times.

Antibodies

Anti-pan Trk 203A was a generous gift from Dr. David Kaplan of the Montreal Neurological Institute (Hempstead et al., 1992). A second anti-pan Trk antibody was also utilized (Santa Cruz Biotechnology (Trk (C-14)); referred to as SCBT anti-pan Trk). Both of these antibodies were rabbit polyclonals, and were both raised against an identical epitope. Specifically, the epitope was the last 14 carboxyl terminal amino acid residues of the human TrkA sequence (ALAQAPPVYLDVLG), a region that is highly conserved

among all vertebrate Trk receptors. Both of these antisera recognize full-length TrkA, TrkB, and TrkC receptors. The TrkBin and TrkCin2, both provided by Dr. David Kaplan, were also rabbit polyclonal antibodies raised against the following epitopes: anti-TrkBin: HISNGSNTPSSSEGGPDAVI; anti-TrkCin2: MILVDGQPRQAKGELGL. A mouse monoclonal anti-phosphotyrosine antibody (clone # 4G10; Upstate Biotechnology) was a kind gift from Dr. Stephen Robbins.

Secondary antibodies were as follows: goat anti-rabbit linked to horseradish peroxidase (HRP; Jackson), goat anti-rabbit linked to fluorescein (FITC; Jackson), and sheep anti-mouse conjugated to HRP (Amersham).

Anti-pan Trk 203A frozen section immunohistochemistry

Animals were dissected, brains were pinned out, and immediately placed in 4% paraformaldehyde (PFA; BDH) dissolved in phosphate-buffered saline (PBS; 50 mM Na_2HPO_4, 140 mM NaCl, pH 7.2) overnight at 4 °C. Brains were then transferred to 30% sucrose in PBS overnight, and subsequently mounted in OCT Compound (Sakura Finetechnical Co.) and cut on a cryostat at a thickness of 12 µm. After drying, sections were encircled with a hydrophobic pen and a 1:500 dilution of the 203A anti-pan Trk in PBS with 5% normal goat serum (NGS; Jackson) and 0.3% Triton X-100 (Sigma) was applied overnight at 4 °C. After several washes in PBS, a 1:1,000 dilution of FITC-conjugated anti-rabbit secondary in PBS was applied for 1 hour at room temperature. After washing, slides were coverslipped with an anti-fade reagent (all chemicals from

Sigma; 2.5% 1,4-diazabicyclo[2.2.2]octane, 9.6% polyvinyl alcohol, 24% glycerol, in 100 mM Tris base, pH 8.0) and photographed using 400 ASA film.

SCBT anti-pan Trk wholemount immunohistochemistry

Immediately after dissection and pinning out, brains were placed in 0.5% type XIV protease ('pronase'; Sigma) in normal saline for 5.5 minutes to aid in subsequent antibody penetration (Croll et al., 1999; Voronezhskaya et al., 1999). Brains were subsequently fixed overnight at 4 °C in 4% PFA. After 3 rinses in PBS, brains were incubated in PBS plus 4% Triton X-100 for 12 hours at 4 °C to further aid in antibody penetration. Primary antibodies were diluted in this same buffer with 5% NGS at a dilution of 1:2,000 (anti-TrkBin and anti-TrkCin) or 1:200 (SCBT anti-pan Trk) for 3 days at 4 °C. Samples were subsequently rinsed 6 times over a 3-4 hour period in PBS before incubating in a 1:1000 dilution of FITC-conjugated goat anti-rabbit secondary antibody in PBS for 12 hours at 4 °C. Brains were then rinsed multiple times in PBS overnight before mounting on slides and coverslipping with an antifade-containing medium. Images were then captured on a Spot digital camera as a black and white image, imported into Adobe Photoshop, and pseudocoloured green to enhance visualization.

Organ culture

All organ culture experiments were conducted at room temperature. For the CM-challenge experiments, stock CM was diluted 1:1 in DM (serum-free Leibowitz L-15

medium (GIBCO), with added inorganic salts and 20 μg/ml gentamicin (Sigma)) with 1 mM sodium orthovanadate (Sigma). Brains were incubated in CM for the chosen time periods (acute, 5, 10, 20, 40, 80 minutes), where acute refers to brains immediately frozen on dry ice after dissection.

NGF-challenge experiments were carried out on brains from animals that were not anaesthetized in Listerine. This was done in order to minimize the time from dissection until brains were placed into the media. In this case, brains were removed from the animals by cutting the head off, and making a rostral incision in the skin to expose the brain. Brains were removed and placed in either 200 ng/ml NGF (Chemicon; NGF time-course experiment, 5, 10, and 20 minutes in NGF) or 400 ng/ml NGF (acute, 20 minute +/- NGF). In all cases, NGF was added to normal saline containing 150 μg/ml of the antibiotic gentamycin supplemented with 0.1% bovine serum albumin (BSA; Sigma). In the NGF time course experiment, sodium orthovanadate was not included in the incubation solution.

In all experiments, 20 brains were used per condition. After incubations, samples were frozen on dry ice and kept at -80 °C until processed for immunoprecipitation and phosphotyrosine western blotting as described below.

Immunoprecipitation

All Immunoprecipitation experiments were conducted using 20 brains per condition. After dissection, brains were either snap frozen on dry ice immediately, or after the required treatments, after which they were stored at –80 °C until processed. Upon thawing, 1 ml of Kaplan buffer (20 mM Tris base, 137 mM NaCl, pH 8.0, 10%

glycerol, 1% NP-40 (USB),1 mM sodium orthovanadate) supplemented with protease inhibitors (all from Sigma; 10 μg/ml aprotinin, 1 μg/ml leupeptin, 1 mM phenylmethanesulfonyl fluoride) was added and samples were homogenized using a Tissue Tearor (Biospec Products Inc.). Samples were centrifuged at 10,000 x g for 10 minutes at 4 °C to pellet debris, and the resulting supernatant (referred to as the lysate) was used for immunoprecipitations. Primary antibodies (1 μg of SCBT anti-pan Trk or non-immune rabbit serum (NRS; Cedar Lane), as a negative control) were added to lysates and mixed for 1.5-2 hours at 4 °C. 50 μl of protein A-agarose beads (Boehringer Mannheim) were added and mixed end-over-end for 1 hour. Beads were recovered by centrifuging for 5 minutes at 2,500 rpm. Immune complexes were washed 3 times in Kaplan buffer for 15 minutes at 4 °C. After a final wash in sterile-filtered, double distilled H_2O, 50 μl of sample buffer (62.5 mM Tris base, pH 6.8, 10% glycerol, 2% SDS, 0.1% dithiothreitol, 0.001% bromophenol blue; all from Bio-Rad) was added and samples were boiled for 5 minutes at 95°. Beads were pelleted and the supernatant was loaded on either 7.5% or 10% gels and electrophoresed at 50 mA. For SCBT anti-pan Trk blots, 10μl was loaded per lane; for anti-phosphotyrosine, 30μl was loaded per well. Pre-stained molecular weight markers (broad range; Bio-Rad) were run along with samples to delineate molecular weights.

Electrotransfer of proteins to nitrocellulose membranes (Bio-Rad) was done using 30 V constant voltage for 18 hours with the electrotransfer unit immersed in ice. After transfer, membranes were rinsed briefly in tris-buffered saline (TBS; pH 7.6) before being placed in blocking solution (TBS, pH 7.6, 0.1% Tween-20 (Bio-Rad), 0.5% NP-40, and 3% BSA for 1-2 hours. Membranes were then incubated in primary antibody in

blocking solution overnight at 4 °C (SCBT anti-pan Trk, 1:2,000) or 1 hour at room temperature (mouse anti-phosphotyrosine, 1:10,000). After rinsing membranes in TBS with 0.2% Tween-20 3 times for 10 minutes each, appropriate secondary antibodies were diluted in this solution (goat anti-rabbit linked to HRP at 1:10,000; sheep anti-mouse linked to HRP at 1:1,000) for 40 minutes to 1 hour. After rinsing 3 times for 10 minutes in blocking buffer, blots were rinsed briefly in TBS alone, and developed with enhanced chemiluminescence (ECL; Amersham). It should be noted that all samples that were processed for anti-phosphotyrosine immunoreactivity were also processed for SCBT anti-pan Trk immunoreactivity as a positive control to ensure that the antigen(s) had been successfully isolated.

SCBT anti-pan Trk preabsorption

In an attempt to block the products obtained with immunoprecipitations, SCBT anti-pan Trk antibody was incubated with a 5-fold excess (w/w) of the immunizing peptide prior to western blotting, according to the manufacturer's instructions. The preabsorption was done in a 200 µl volume of TBS, pH 7.6 with 1 µl of antibody and 5 µl of peptide for 2 hours at room temperature. Then, the mix was diluted into 1,800 µl of buffer to obtain a final concentrations of 0.1% Tween-20, 0.5% NP-40, and 3% BSA, and a 1:2,000 final dilution of antibody. This mixture was then applied to western blots of immunoprecipitated samples as noted above.

Anti-TrkBin and anti-TrkCin2 western blots

Tissue preparation for anti-TrkBin and anti-TrkCin2 Western blots involved homogenizing brains in 50 mM Tris base, pH 8.0, 150 mM NaCl, 1% Triton X-100, 0.25% sodium deoxycholate (Sigma), including a protease inhibitor cocktail (Sigma) (Suneja & Potashner, 1998). Tissues were homogenized at a ratio of 100 mg:200 µl of lysis buffer, diluted 10-fold into sample buffer and boiled for 5 minutes at 95 °C. After electrophoresis through 6% a gel, proteins were blotting at 30V overnight to nitrocellulose. Blocking was done in PBS plus 0.5% gelatin (Fisher) at 37 °C for 6-8 hours. Blots were then incubated in blocking solution containing a 1:2,000 dilution of primary antibody overnight at 4 °C. After several PBS rinses, blots were incubated in a 1:10,000 dilution of anti-rabbit conjugated to HRP for 1 hour at room temperature. After rinsing, blots were developed using enhanced chemiluminescence.

SCBT anti-pan Trk western blots

Tissue was prepared and blotted as noted above, except that the tissue was lysed in Kaplan buffer. Proteins were blotted to PVDF membranes (Bio-Rad), rinsed briefly in TBS, and blocked in 5% non-fat milk powder (Carnation) and 0.5% BSA in TBS for 2 hours at room temperature. SCBT anti-pan Trk was diluted into blocking buffer at 1:2,000. After an overnight incubation, blots were rinsed in TBS, and placed in anti-rabbit conjugated HRP at a 1:10,000 dilution for 1 hour. Blots were developed with ECL.

Anti-pan Trk 203A western blots

These experiments were conducted by Andrew Chojnacki. Cultured neural stem cells were spun down at 800 rpm for 5 minutes, the media removed, replaced with 1.5 mls of ice-cold PBS, and transferred to 1.5 ml microcentrifuge tubes. The cells were then pelleted and the supernatant aspirated, frozen in liquid nitrogen, proteins extracted using SDS-lysis buffer (60mM Tris-HCl pH 6.8, 100 mM 2-mercapto-ethanol, and 2% SDS), and subsequently boiled for 10 min. 25 μg of protein were run on 7.5% SDS-polyacrylamide gels and electroblotted to nitrocellulose membranes (Bio-Rad). The blots were blocked in blocking buffer for 1hr (25 mM Tris-HCl pH 7.5, 0.5 M NaCl, 0.3% Tween 20, and 3% non fat skim milk), and incubated in the 203A antiserum overnight at 4 °C in the blocking buffer, washed with blocking buffer, and then incubated with blocking buffer plus goat anti-rabbit conjugated to horseradish peroxidase (Chemicon). Blots were then washed 3 times in blocking buffer followed by two washes in TBS. Blots were subsequently developed using ECL.

Chapter 3: Anti-pan Trk Results

Introduction

The objective of this thesis was to use antibodies specific to vertebrate Trk receptors as probes to identify other potential members of this protein family from *Lymnaea*. Initially, we reasoned that to identify additional Trk family members in *Lymnaea* at the protein level, an antibody that recognizes most, if not all, vertebrate Trks would be the most useful. Such an antibody does indeed exist for the Trk receptors, and is referred to generically as 'anti-pan Trk'.

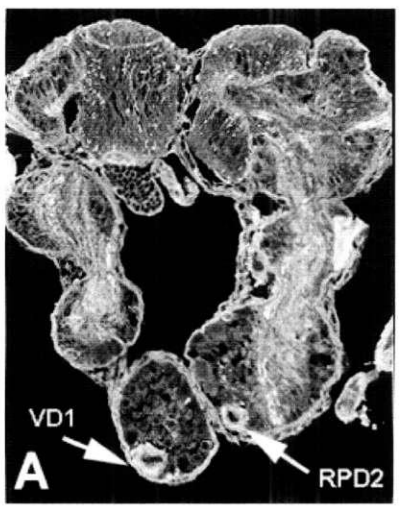
In the current study, two different anti-pan Trk antibodies from two different sources were employed. Both antibodies were raised against identical epitopes, the PLC-□binding site of the human TrkA receptor, which is highly conserved in all Trks. The first anti-pan Trk antiserum (anti-pan Trk 203A) was provided courtesy of Dr. David Kaplan (figure 5). The second was a commercially available antiserum from Santa Cruz Biotechnology (SCBT anti-pan Trk). Anti-pan Trk 203A has been shown to recognize Trk receptors from several vertebrates, including the chick, rodents, humans, monkeys, and frogs (David Kaplan, personal communication). The SCBT anti-pan Trk is known to cross react with rat, mouse and human Trks (Santa Cruz Biotechnology product insert), and would be expected to cross-react with other vertebrate Trks as well.

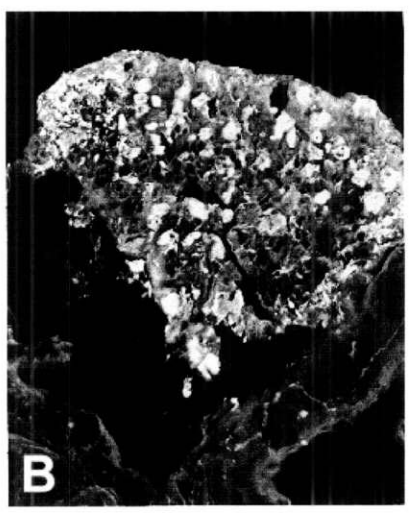
Anti-pan Trk 203A immunohistochemistry

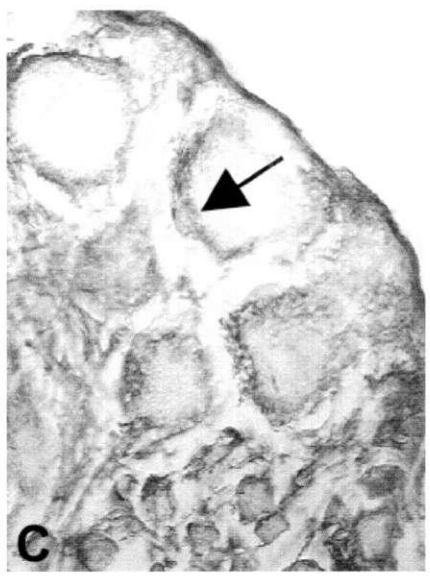
Our first step was to examine the pattern of expression of the anti-pan Trk 203A antigen in frozen sections of adult nervous systems. Using immunofluorescence, immunoreactivity was detected in several areas of the brain. Notably, many axon tracts coursing between various ganglia appeared immunoreactive (figure 6a). In addition, two very prominently stained neuronal cell bodies could also been seen. In the visceral ganglion, a large cell whose size and location are consistent with that of VD1 was stained (figure 6b), as well as the RPD2 neuron within the right parietal ganglion. These two large peptidergic neurons are known to be electrically coupled to one another, and form part of a network that controls respiratory behaviour (Benjamin & Pilkington, 1986; Kerkhoven et al., 1990). Interestingly, immunoreactivity was also prominent within the dorsal bodies of adult nervous systems (figure 6b), where *Ltrk* transcripts are known to be expressed in abundance (van Kesteren et al., 1998; Melvin & Logan, unpublished observations).

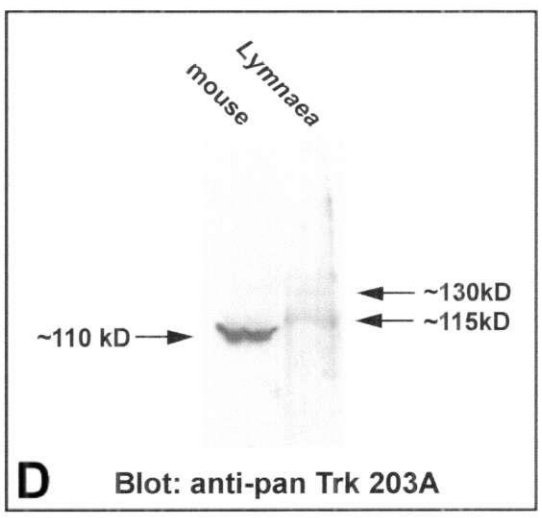
Performing immunohistochemistry with enzyme-linked secondary antibodies is generally more sensitive than immunofluorescence due to the fact that the enzymatic reaction accumulates. In order to observe staining patterns under these conditions (Davis, 1993), some sections were processed with an enzyme-linked secondary antibody, and photographed using Nomarski optics. In a high-power magnification of the visceral ganglion, immunoreactivity could be seen surrounding several cell bodies (figure 6c). This staining revealed a 'wicker basket-like' pattern, which is clearly localized to the periphery of the cell bodies. This may represent staining of axon terminals that synapse

Figure 6: Characteristics of *Lymnaea* antigens cross-reacting with the anti-pan Trk 203A antiserum. **A)** Frozen section immunohistochemistry of the adult *Lymnaea* CNS. This section reveals an abundance of staining through the CNS, particular in inter-ganglionic axon tracts, connective tissues surrounding each ganglia, and in two identified neurons, VD1 and RPD2. **B)** Section of the dorsal body (DB) showing ubiquitous staining. Interestingly, this structure is known to express *Ltrk* transcripts. **C)** A section processed with an enzyme-linked secondary antibody. The arrow points to the staining of 'wicker basket-like' structures surrounding a cell body, which may represent presynaptic terminals. **D)** A western blot with the 203A antiserum. The underglycosylated 110 kD Trk receptor is present in the mouse positive control lane. The *Lymnaea* lane reveals a prominent band at approximately 115 kD, as well as a weak band present at about 130 kD, and other higher molecular weight bands.


VD1
RPD2
A


B


C


Lymnaea
mouse
~130kD
~115kD
~110 kD
D
Blot: anti-pan Trk 203A

directly onto these cell bodies. This pattern was not observed using immunofluoresence, and may be due to the higher sensitivity of enzyme-linked immunohistochemistry (Davis, 1993). These results suggest that in some areas of the nervous system, the anti-pan Trk 203A antigen may be localized presynaptically. Finally, robust immunoreactivity was also present in the connective tissue surrounding the ganglia (figure 6a).

Anti-pan Trk 203A western blots

Our next step was to use the anti-pan Trk 203A in western blotting experiments to determine whether it is capable of detecting a *Lymnaea* antigen of a similar molecular weight to that found in mammalian tissue. As a positive control, mouse embryonic stem cells grown in culture were run alongside the *Lymnaea* sample. As can be seen, a single band was present at ~ 110kD, previously shown to represent the underglycosylated version of the Trk receptors (figure 6d; Martin-Zanca et al., 1989; David Kaplan, personal communication). Generally, a band at ~ 140 kD is also seen, which represents the fully glycosylated Trk receptor (Watson et al., 1999; David Kaplan, personal communication). When homogenates from the adult *Lymnaea* nervous system were run under similar conditions, a prominent band at approximately 115kD was detected, in addition to a weakly immunoreactive band at approximately 130 kD (figure 6d; blot performed courtesy of Andrew Chojnacki).

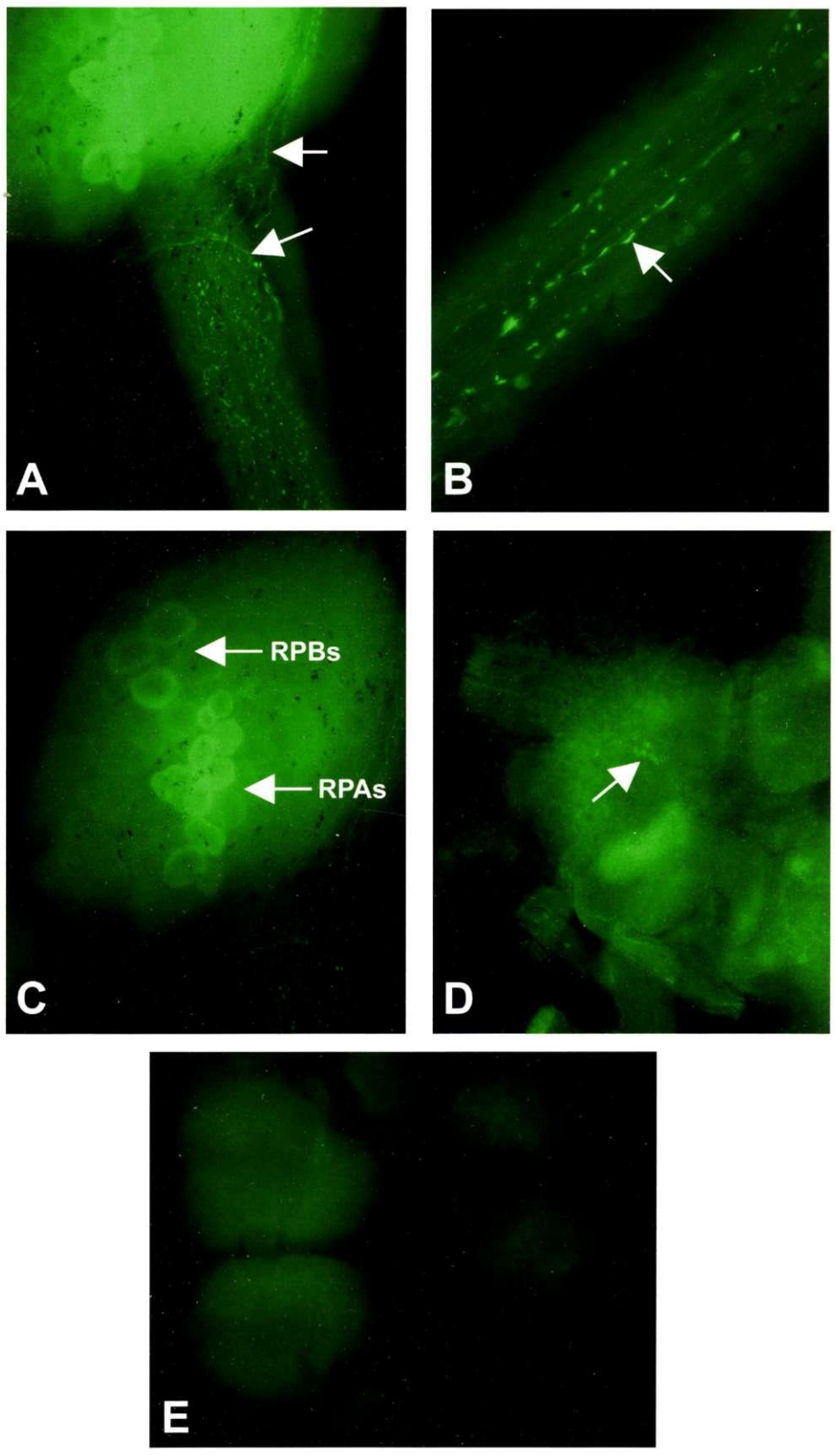
SCBT anti-pan Trk immunohistochemistry

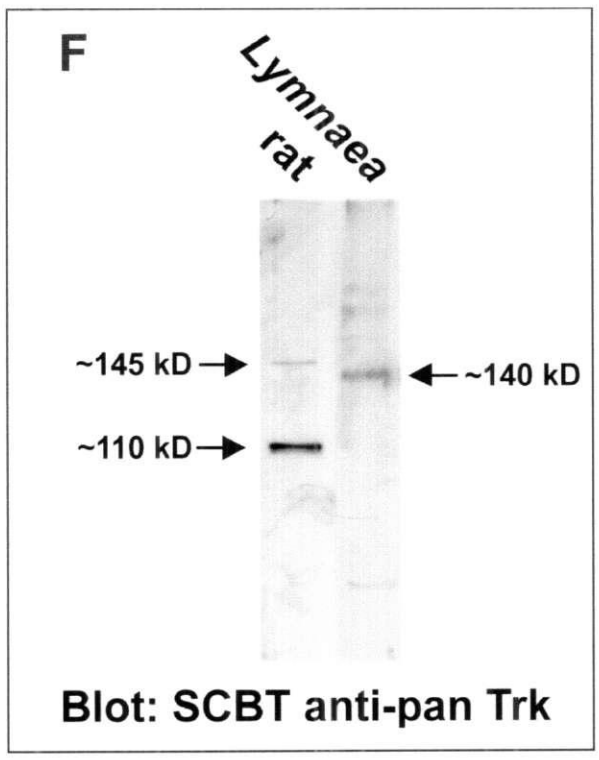
To allow specific cells to be more easily identified, whole-mount immunofluorescence was employed to visualize the expression of the antigen(s) that cross-reacted with SCBT anti-pan Trk. This technique was also attempted with anti-pan Trk 203A, but the staining in the connective tissues surrounding the ganglia prevented the visualization of immunoreactivity within the ganglia (see figure 6a).

The SCBT antiserum primarily stained neuronal cell bodies, but also stained some axons as well as an unidentified cell type present within peripheral nerves. Staining of axons was most prominent at the point where they exited a particular ganglion and extended into peripheral nerves (figure 7a). It is not yet known whether these axons are continuous with those seen exiting a ganglion. The staining in peripheral nerve axons was weak compared to another element that stained positively in this same area. These structures appeared to be thin, elongated cells whose axis ran parallel to that of axons within the nerve (figure 7b). Although we do not know the identity of this cell type, it is presumed that they may correspond to glial cells or some component of connective tissue present inside the nerve.

The most prominent immunoreactive components present with this antiserum however, were three distinct groups of neuronal cell bodies. These cell bodies were present in clusters and found within two ganglia. Most prominent were two clusters present within the right parietal ganglion. Specifically, a small number of medium size cells in the rostromedial portion of the ganglion were immunoreactive, the size and

Figure 7: Characteristics of *Lymnaea* antigens cross reacting with the SCBT anti-pan Trk antiserum. **A)** The arrows depict axons emanating from the right parietal ganglion. **B)** Immunoreactivity is present in small, elongated cells (arrow) within peripheral nerves. **C)** Prominent immunoreactivity is found in two cell groups within the right parietal ganglion, specifically in RPA and RPB neurons. **D)** A third group of cell bodies (arrow) stained positively in the cerebral ganglia. **E)** Specimens processed in the absence of primary antibody revealed no staining. This control applies to TrkBin and TrkCin2 immunohistochemistry as well, as all used the same secondary antibody. **F)** Western blot demonstrated a prominent ~ 140 kD band in the *Lymnaea* lane. The positive control rat lane shows the presence of the fully glycosylated 145 kD antigen, and the 110 kD underglycosylated form.


A
B
RPBs
RPAs
C
D
E


F
Lymnaea
rat
~145 kD
~110 kD
~140 kD
Blot: SCBT anti-pan Trk

position of which are consistent with them being members of the right parietal B cell cluster (figure 7c). In addition, a spatially segregated group of cells with varying sizes were also positively stained within this ganglion. Their size and position indicated that they may be members of the right parietal A group of motor neurons (figure 7c). Importantly, these cells correspond to those previously shown to be responsive to mammalian NGF (Ridgway et al., 1991; Wildering et al., 1995). Finally, a discrete group of small-sized neurons were also present within the center of each cerebral ganglion, the identity of which is unknown (figure 7d). Very light staining was observed in the dorsal bodies.

Samples processed in the absence of primary antibody resulted in no staining, indicating that the immunoreactivity observed was not due to non-specific cross-reactions with the secondary antibody (figure 7e). Incidentally, this control is also applicable to the whole-mount immunofluoresence preparations developed for the anti-TrkBin and anti-TrkCin2 antibodies as well, since they used the same secondary antibody.

SCBT anti-pan Trk western blots

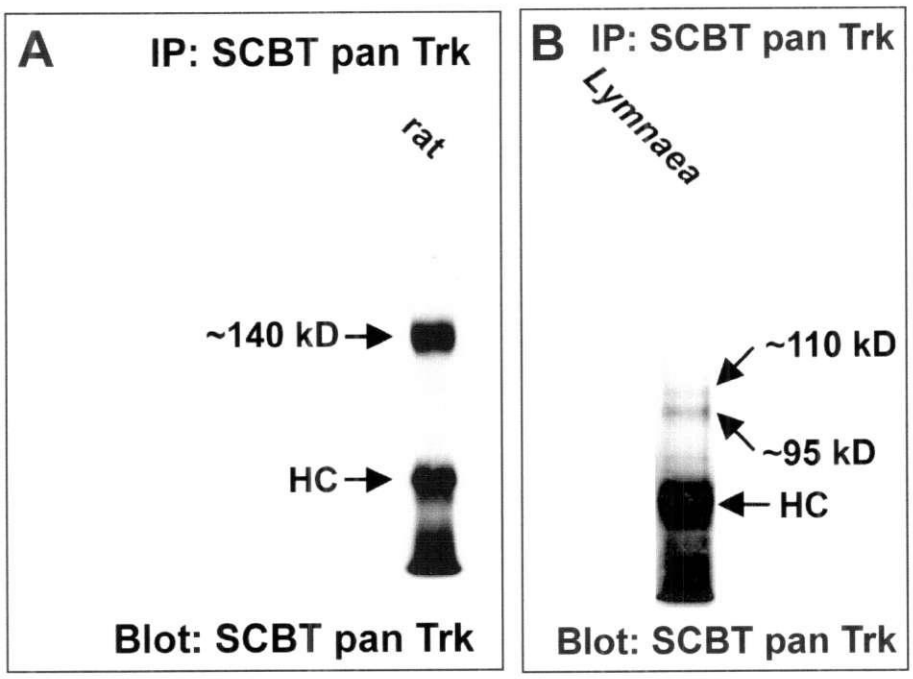
In an effort to further characterize cross-reacting proteins recognized by the SCBT anti-pan Trk antiserum, we conducted western blots on adult *Lymnaea* CNS samples. Although several weak bands are detected that have an apparent molecular weight of about 200 kD, the most prominent band detected was at approximately 140 kD (figure 7f). In this experiment, the positive control sample from adult rat cortex revealed two bands. The more intensely labelled band was at 110 kD, and a less prominent band

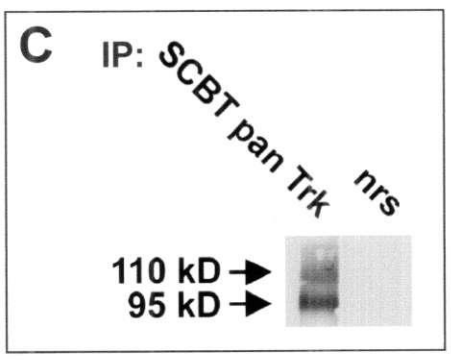
was also detected at approximately 145 kD (figure 7f). As mentioned above, the 110 kD protein represent the underglycosylated isoform of Trk, and the 145 kD protein is the fully glycosylated Trk (Martin-Zanca et al., 1989; Watson et al., 1998; David Kaplan, personal communication).

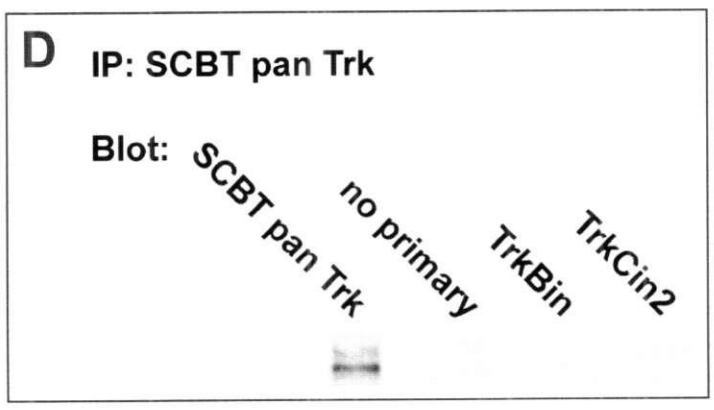
SCBT anti-pan Trk immunoprecipitation

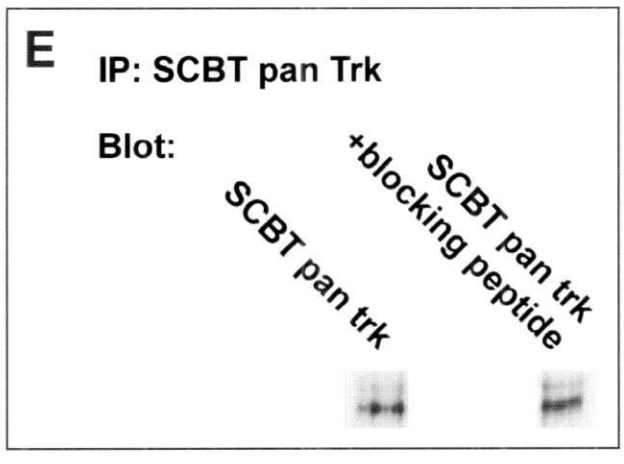
Since the ultimate goal with the SCBT antiserum was to determine whether cross-reacting antigens in *Lymnaea* are tyrosine phoshphorylated, we next employed the technique of immunoprecipitation. Adult rat cortex was employed as a positive control. As can be seen, the SCBT antibody was able to immunoprecipitate the full-length ~140 kD Trk protein (figure 8a). Under these conditions, however, two different cross-reacting antigens were isolated. When lysates from adult *Lymnaea* CNS samples were immunoprecipitated with SCBT anti-pan Trk, and subsequently probed on western blots with SCBT anti-pan Trk, a ~ 110 kD band, as well a more intense ~ 95 kD band were detected (figure 8b). As a negative control, an equivalent amount of non-immune rabbit serum was used, and this did not result in the isolation of either the 110 kD or the 95 kD antigen (figure 8c). In addition, these products were not due to cross-reactivity to the secondary antibody, as probing the western blot with the secondary alone did not reveal either band (figure 8d). Importantly, these two antigens are generally comparable in their molecular weights to those isolated under similar conditions from the squid (Moreno et al., 1998). The potential reasons for the differences between the results obtained by

Figure 8: SCBT anti-pan Trk immunoprecipitations with associated control experiments. **A)** Positive control demonstrating that the SCBT antiserum successfully precipitates full-length Trks. **B)** Same experiment as in A), but with *Lymnaea* tissue. As indicated, two antigens were purified: a 110 kD and a 95 kD antigen. **C)** Negative control experiment showing that immunoprecipitation with NRS does not result in the isolation of either product. **D)** This experiment demonstrates that the 110 kD and 95 kD antigens immunoprecipitated from *Lymnaea* are distinct from those recognized by either anti-TrkBin or anti-TrkCin2. Also, the no primary blot shows that the signals present in *Lymnaea* immunoprecipitates are not due to non-specific cross-reactions with the secondary antibody. **E)** This experiment demonstrates that pre-incubating the SCBT antiserum with its blocking peptide prior to western blotting does not block the signal from either band. Abbreviations: HC (heavy chain of the immunoprecipitating antibody); NRS (non-immune rabbit serum).


A
IP: SCBT pan Trk
rat
~140 kD
HC
Blot: SCBT pan Trk
B
IP: SCBT pan Trk
Lymnaea
~110 kD
~95 kD
HC
Blot: SCBT pan Trk


C
IP: SCBT pan Trk
nrs
110 kD
95 kD


D
IP: SCBT pan Trk
Blot:
SCBT pan Trk
no primary
TrkBin
TrkCin2


E
IP: SCBT pan Trk
Blot:
SCBT pan trk
+blocking peptide
SCBT pan trk

western blotting and immunoprecipitation experiments will be addressed in the discussion.

TrkBin and TrkCin2 probes of immunoprecipitates

Since SCBT anti-pan Trk recognizes TrkA, TrkB, and TrkC in mammals, I conducted experiments aimed at determining whether the pan Trk antigens isolated under immunoprecipitation conditions were the same as those detected on western blots with either the TrkBin or TrkCin2 antibodies. If the pan Trk serum recognizes all Trks in *Lymnaea*, and the TrkBin and TrkCin2 antigens are truly Trk receptors, then the pan Trk antigens should be detectable by the TrkBin and TrkCin2 antisera on western blots. On western blots, full-length TrkB and TrkC receptors run at about 145kD. As shown in figure 8d, neither TrkBin nor TrkCin2 antibodies recognized the pan Trk antigens isolated by immunoprecipitation, indicating that they are distinct from that recognized by both the TrkB or TrkC-specific antisera.

SCBT anti-pan Trk preabsorbtion

If the two pan Trk antigens were truly Trk-like antigens, one would expect that preincubating the antisera with its original antigen would block the signal detected after western blotting. However, when this experiment was performed according to the manufacturer's instructions, the signal was not blocked. As can be seen in figure 8e, pre-absorbing the western blotting antibody does not block the detection of either pan Trk

antigen. This would seem to indicate that the antigens isolated are in fact not Trk-like, an issue will be addressed further in the discussion.

Assessments of tyrosine phosphorylation

A basic feature of the activation of receptor tyrosine kinases is the autophosphorylation that occurs on intracellular tyrosine residues in response to ligand acitivation (Hubbard & Till, 2000). Our eventual goal with this antiserum was to show that the antigens isolated through immunoprecipitation are capable of being tyrosine phosphorylated. To this end, we combined immunoprecipitations with western blots using an antibody specific to phosphorylated tyrosine residues.

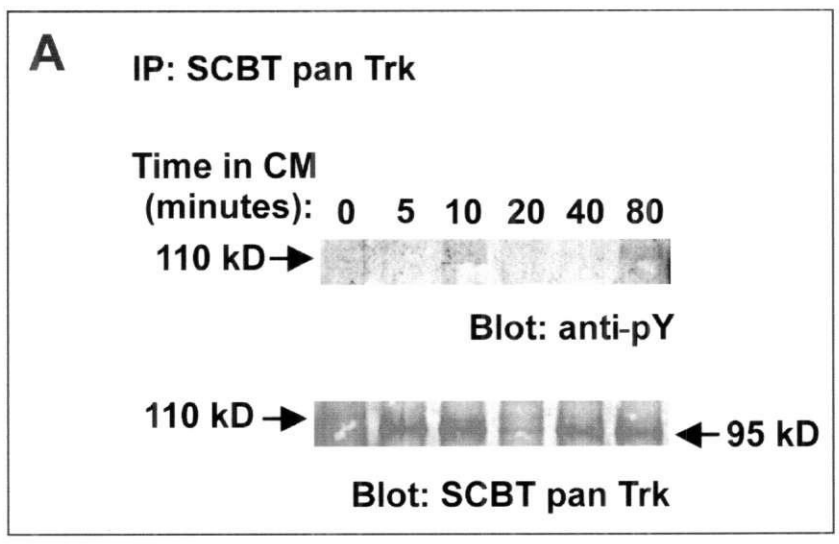
We assumed that if the antigens isolated via immunoprecipitation were Trk-like, they should become tyrosine phosphorylated after neurotrophin treatment. However, the initial studies of neurotrophins in *Lymnaea* conducted by Ridgway et al. (1991) described the presence of protein(s) in CM, capable of cross-reacting with anti-NGF antibodies. When these cross-reacting proteins were removed from CM via immunoprecipitation, selected classes of neurons were no longer able to sprout neurites. Importantly, it was these same classes of neurons that did exhibit neurite outgrowth in response to mammalian NGF. Coupled with our findings that the SCBT anti-pan Trk antiserum detected cross-reacting antigens in some of these same neurons, we reasoned that it might be possible that treating brains with CM might serve to 'activate' this cross-reacting antigen. If it were in fact Trk-like, and CM contained an endogenous ligand capable of

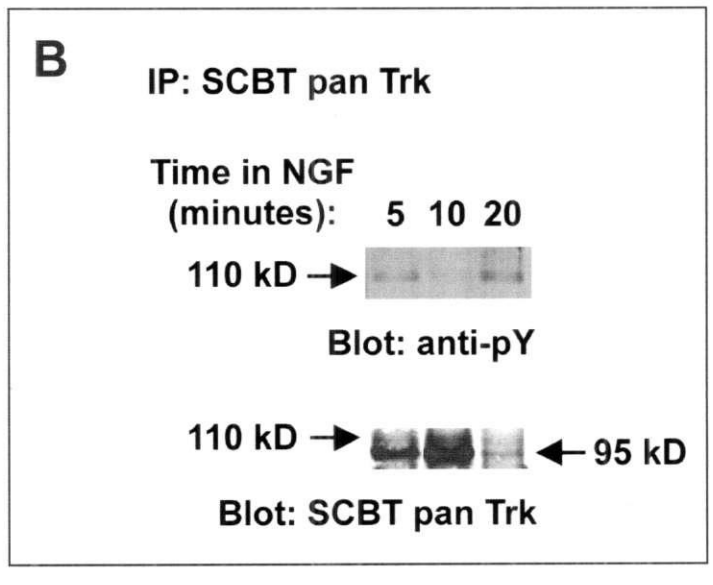
activating it, then treating brains with CM should result in its tyrosine phosphorylation. Therefore, our first experiment was to treat organ cultured brains with CM.

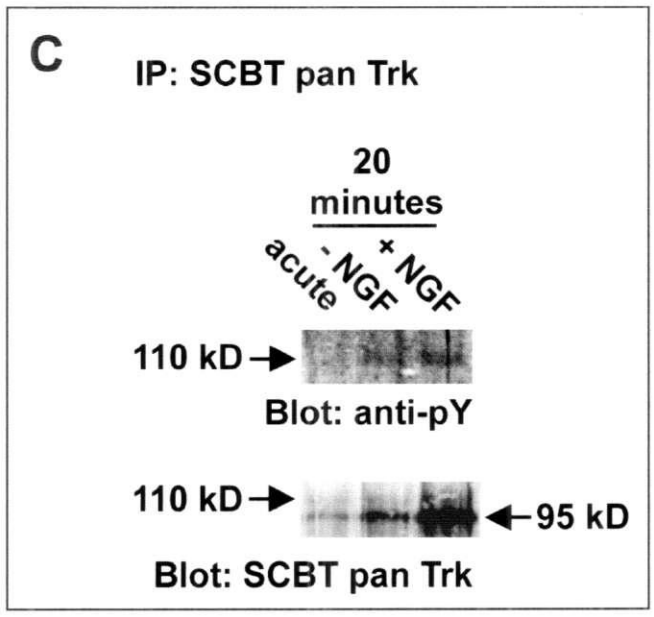
CM-challenge experiment

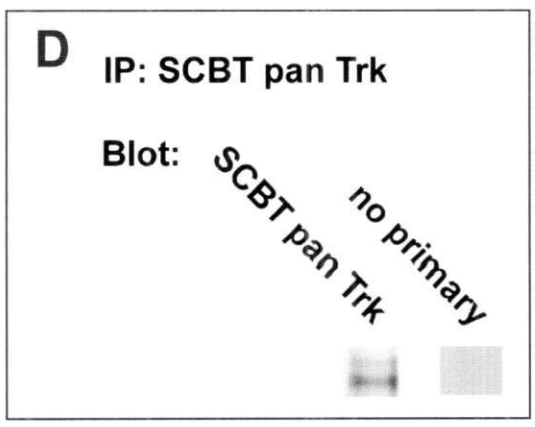
We reasoned that a ligand capable of activating the pan Trk antigens would most likely be found in CM, which contains a variety of endogenous proteins with neurotrophic functions (Ridgway et al., 1993). To accomplish this, we employed an organ culture strategy that involves incubating dissected nervous systems in multi-well dishes. The initial experiment was done as a time-course including 0 (immediately frozen after dissection), 5, 10, 20, 40, and 80 minutes in CM. Samples were subsequently immunoprecipitated with the SCBT anti-pan Trk antisera and immunoblotted with anti-phosphotyrosine. A weak tyrosine phosphorylation signal of the 110 kD band can be detected in the 10 minute and 80 minute condition, but not at the other time points (figure 9a, top panel). The absence of a signal in the other time points is likely due to inadequate loading in these lanes (figure 9a, bottom panel). It should be noted that shortly after this initial experiment, it was found that the batch of CM used was found to be inactive, as tested in a neurite outgrowth assay on cultured *Lymnaea* neurons (Wilhelm Wildering, personal observation).

Figure 9: Tyrosine phosphorylation experiments. **A)** This experiment showed weak tyrosine phosphorylation of the 110 kD band in *Lymnaea* immunoprecipitates after 10 and 80 minutes in CM (top panel). The absence of signals at other time points may be due to inadequate loading (bottom panel). **B)** A 'bi-phasic' pattern of tyrosine phosphorylation was seen when *Lymnaea* brains were cultured in NGF (top panel). Note the down-regulation of tyrosine phosphorylation in the 10 minute sample, despite containing more antigen (bottom panel). **C)** This experiment shows no detectable tyrosine phosphorylation in acutely isolated brains. In addition, 20 minutes of organ culture alone is sufficient to induce tyrosine phosphorylation, regardless of the presence of NGF (top panel). The slight difference between the - NGF and + NGF conditions is likely due to unequal loading (bottom panel). **D)** The tyrosine phosphorylation signal is not due to non-specific cross-reactions with the secondary antibody.


A
IP: SCBT pan Trk
Time in CM
(minutes): 0 5 10 20 40 80
110 kD
Blot: anti-pY
110 kD
95 kD
Blot: SCBT pan Trk


B
IP: SCBT pan Trk
Time in NGF
(minutes): 5 10 20
110 kD
Blot: anti-pY
110 kD
95 kD
Blot: SCBT pan Trk


C
IP: SCBT pan Trk
20
minutes
- NGF
+ NGF
acute
110 kD
Blot: anti-pY
110 kD
95 kD
Blot: SCBT pan Trk


D
IP: SCBT pan Trk
Blot:
SCBT pan Trk
no primary

NGF-challenge experiments

When onc considers that some of the neurons that are responsive to mammalian NGF express pan Trk antigens as detected with the SCBT antiserum, it seemed reasonable to hypothesize that one or both of these antigens may in fact bc a TrkA-like receptor. If this were the case, one would expect this protein to become tyrosine phosphorylated upon exposure to NGF. To address this question, we once again employed our organ culture system.

In our first attempt, we decided to conduct a time series of 5, 10, and 20 minutes in NGF. After incubating for the times indicated, the brains were lysed subjected to immunoprecipitation with thc SCBT anti-pan Trk antiserum, followed by a western blot with the anti-phosphotyrosine antibody to assess the phosphorylation status of the antigens. The data shown in Figure 9b demonstrates an apparently 'bi-phasic' pattern of tyrosine phosphorylation. After 5 minutes in NGF, the 110 kD product becomes tyrosine phosphorylated. After 10 minutes, the phosphorylation signal becomes down regulated, and by 20 minutes, the signal returns. Importantly, this particular experiment did not include an acute control (frozen immediately after dissection), or time-matched groups incubated without NGF.

Accordingly, our next step was to conduct the same experiment including both an acute condition as well as a time-matched control in thc absence of NGF. For simplicity, we chose to work with only one time point. Since the 20 minute time point gave thc most prominent phosphorylation signal (figure 9b), we chose this time point for further analysis. As shown in Figure 9c, in the acute sample there is no detectable

phosphorylation signal present. This suggests that under basal conditions, the 110 kD antigen is not tyrosine phosphorylated. When brains incubated in the presence or absence of NGF for 20 minutes were analyzed for tyrosine phosphorylation, the intensity of the bands appeared similar (figure 9c). Thus, the 110 kD band becomes tyrosine phosphorylated after cutting all peripheral nerves (a consequence of the organ culture procedure), regardless of the presence of NGF. A potential explanation is put forth in the discussion.

Finally, the tyrosine phosphorylation signal was not due to non-specific binding of the secondary antibody, as sample processed in the absence of the primary antibody revealed no positively stained bands (figure 9c).

Chapter 4: TrkBin and TrkCin2 Results

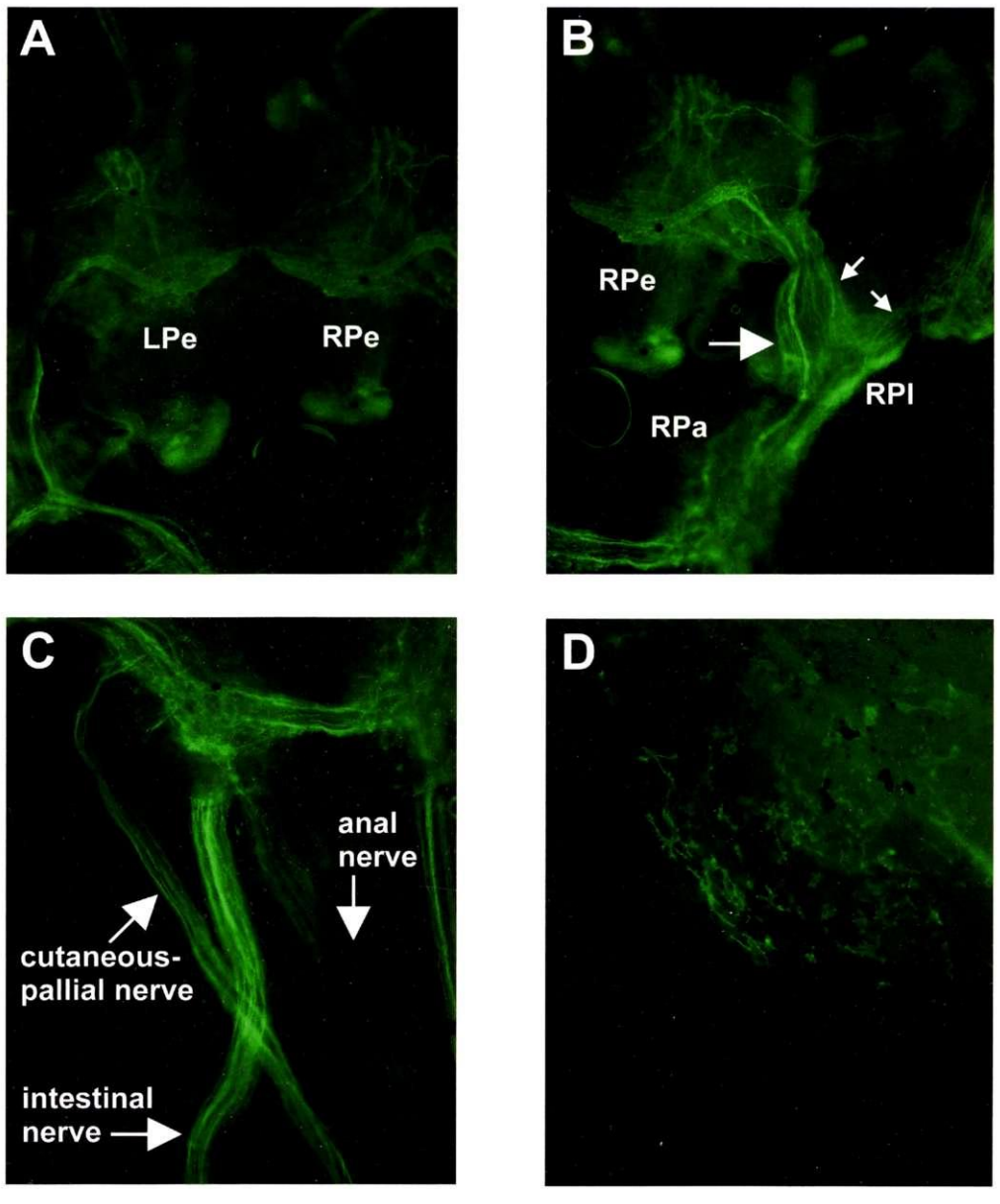
Introduction

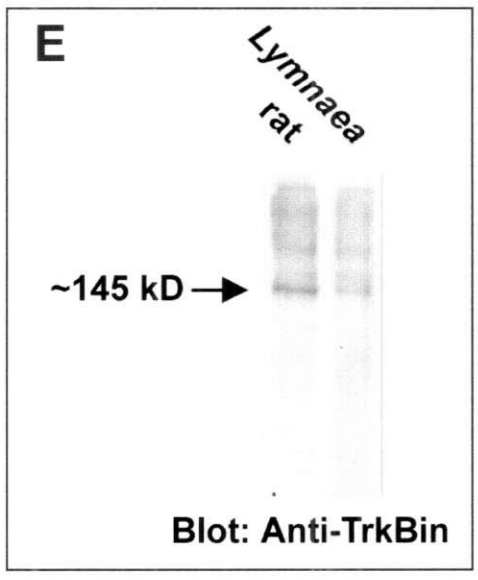
In our initial search for Trk-specific antibodies, we were also able to obtain two isoform-specific antisera. One, referred to as anti-TrkBin, recognizes an epitope that is present only in full-length TrkB receptors. The other antiserum (TrkCin2) recognizes an epitope only found in full-length TrkC receptors. Thus, as opposed to the two anti-pan Trk antibodies, anti-TrkBin and anti-TrkCin2 cross-react with epitopes that are not evolutionary conserved between isoforms. However, we reasoned that it might be worthwhile to employ these antibodies in the current studies, especially since an anti-TrkB antibody provided the impetus for Giustetto et al., (1999) to clone *Ap-Trk.* Since neither antibody works well under immunoprecipitating conditions (Donna Senger, personal communication), we were limited to using only immunohistochemistry and western blotting to characterize the corresponding antigens.

TrkBin whole-mount immunohistochemistry

As a first step, we used whole-mount immunohistochemistry to analyze the distribution of the cross-reacting *Lymnaea* antigen in adult CNS preparations. Interestingly, the expression of this antigen appeared to be limited to axons within neural tissue (figure 10a). The most prominent components immunoreactive with this antibody were groups of axons that coursed throughout the central ring. There appeared to be two distinct bundles of axons stained: one that formed an apparently continuous circle

Figure 10: Characterization of TrkBin-like immunoreactivity. **A)** TrkBin stains primarily axons with the adult *Lymnaea* CNS course throughout all central ganglia. **B)** The axonal bundle bifurcates within the pleural ganglia, projecting one bundle towards the cerebral ganglia, and the other into the pedal ganglia (small arrows). **C)** Staining within the cutaneous-pallial and intestinal nerves was relatively abundant; virtually no immunoreactivity is detected within the anal nerve. **D)** Cells with a stellate-like morphology were stained in the dorsal body. **E)** Western blot analysis showed a band of similar molecular weights in both the positive control rat sample, as well as the *Lymnaea* sample.


A
LPe
RPe
B
RPe
RPa
RPl
C
anal
nerve
cutaneous-
pallial nerve
intestinal
nerve
D


E
Lymnaea
rat
~145 kD
Blot: Anti-TrkBin

running along the inside of all ganglia of the central ring except the cerebrals, and a second group ran parallel to the inside bundle. This outer group of axons appeared to bifurcate in the pleural ganglia and project two separate bundles into the cerebral ganglia, and another into the pedal ganglia (figure 10b, small arrows). This pedal projection appeared as though it may continue on into the buccal ganglia rostrally.

In addition, immunoreactivity was also seen in some, but not all, peripheral nerves. For example, of the three nerves that emerge from the visceral ganglion, only the cutaneous-pallial nerve and the intestinal nerve expressed the antigen, whereas the anal nerve was largely devoid of staining (figure 10c). This peripheral immunoreactivity appeared to be emanating from the outer axonal bundle in the CNS described above.

Finally, elements with a stellate-like morphology present within the dorsal bodies were also stained with the anti-TrkBin antiserum (figure 10d). The identity of this cell type cannot be confirmed as little is know about the inherent morphology of cell types in the intact dorsal body, but it is possible that these may be the same components that express *Ltrk* mRNA (van Kesteren et al., 1998; Cairine Logan and Neal Melvin, personal observations).

TrkBin western blotting

Our next step was to determine whether cross-reacting antigens in *Lymnaea* had a similar molecular weight to mammalian Trks. To this end, we used the anti-TrkBin antiserum to conduct western blots on adult *Lymnaea* nervous systems. A positive control sample, rat cerebral cortex, was run in parallel with samples from adult *Lymnaea*. As

expected, a prominent band was seen at 145 kD in the positive control, which corresponds to the full length TrkB receptor (figure 10e). Lysates from adult *Lymnaea* samples revealed the presence of a protein with a similar molecular weight. This finding is generally consistent with that reported by Giustetto et al. (1999) in *Aplysia*, in which a ~ 140 kD band is obtained on western blots with a TrkB-specific antiserum.

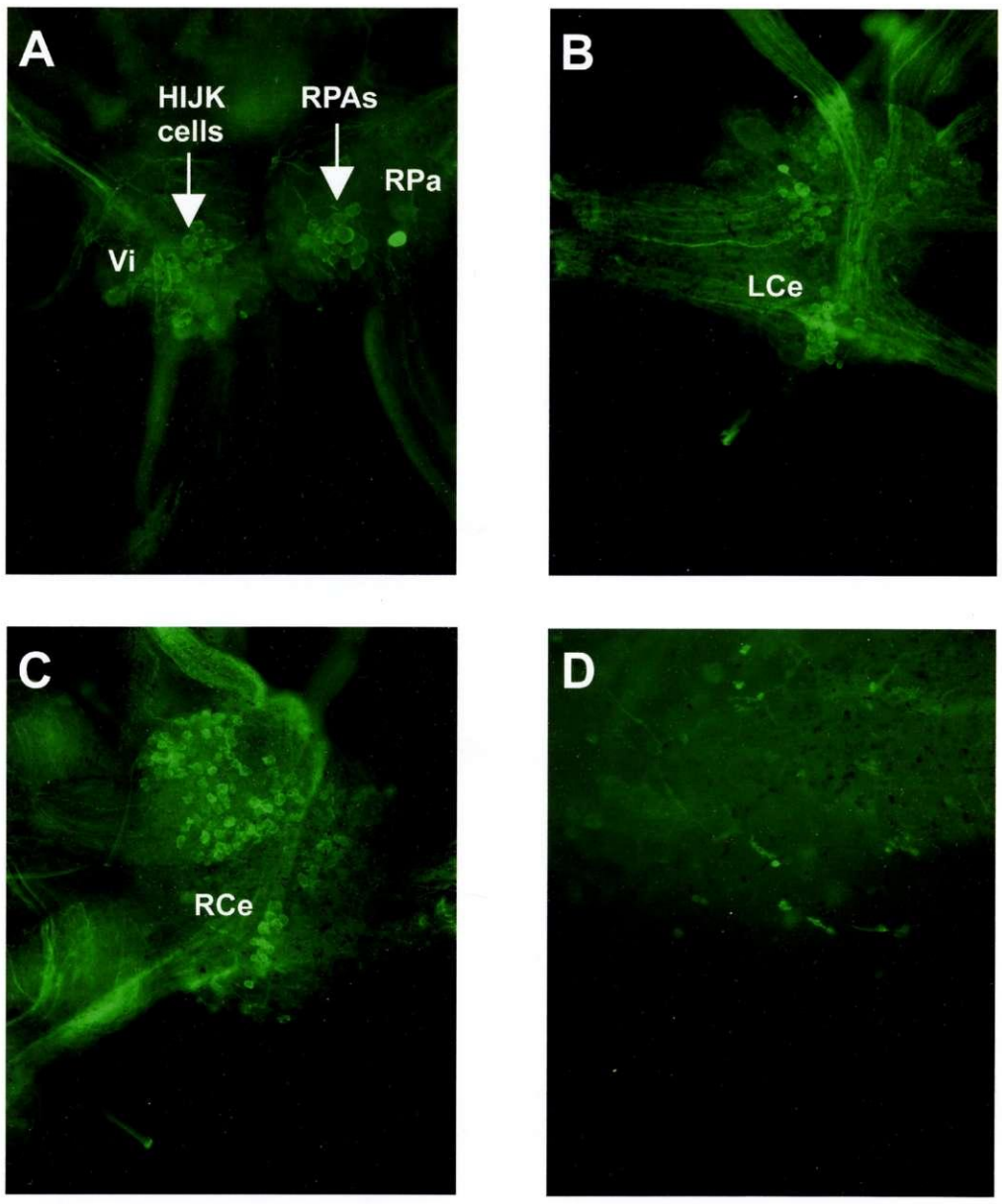
TrkCin2 whole-mount immunohistochemistry

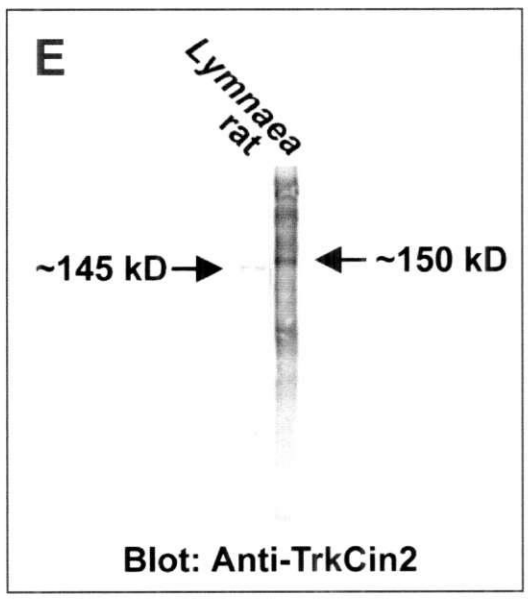
To map the spatial distribution of cross-reacting antigens in *Lymnaea*, we conducted whole-mount immunohistochemistry with the anti-TrkCin2 antiserum. In stark contrast to the TrkB antiserum, anti-TrkCin2 labelled primarily cell bodies that could be distinguished in three separate ganglia. A large group of cell bodies in the visceral ganglion was found to be immunopositive (figure 11a). The position of these cells is consistent with the so-called "HIJK" cells (Wilhelm Wildering, personal communication). These cells mediate a number of functions, including the control of cardiac and respiratory functions.

Immunoreactivity was also present in a distinct cluster of neuronal cell bodies within the right parietal ganglion (figure 11a). These cells correspond to the right parietal A group of motor neurons previously shown to be physiologically responsive to mammalian NGF (Ridgway et al., 1991; Wildering et al., 1995), and to the *Lymnaea* neurotrophic factor CRNF (Fainzilber et al., 1996).

Prominent immunoreactivity was also observed in several cell bodies bilaterally in the cerebral ganglia (figure 11b, c). Some of these cells may correspond to the caudodorsal cells, which are a group of approximately 100 peptidergic, electrotonically-

Figure 11: Characterization of TrkCin2 cross-reacting antigens. **A)** This antiserum labelled both axons as well as cell bodies. Prominent among positively stained cell bodies were the HIJK cells in the visceral ganglion, and the RPA neurons in the right parietal ganglion. **B), C)** Other immunoreactive cell bodies were present in the cerebral ganglia bilaterally. **D)** Cells of a stellate-like morphology were positively stained in the dorsal bodies. **E)** Western blot analysis with *Lymnaea* samples revealed their presence of many bands, including one slightly above the full-length 145 kD TrkC receptor.


A
HIJK cells
RPAs
RPa
Vi
B
LCe
C
RCe
D


E
Lymnaea
rat
~145 kD
~150 kD
Blot: Anti-TrkCin2

coupled cells that play key roles in egg laying behaviour (Hermann et al., 1997). In addition, elements within the dorsal bodies were also stained with this antiserum.

Lastly, this antiserum also stained some components within the dorsal bodies (figure 11d). The morphology of these cells appeared similar to that described for the TrkBin immunohistochemistry.

TrkCin2 western blotting

As with the anti-TrkB antiserum, we conducted both western blotting with the TrkCin2 antiserum to see if it might also cross-react with a protein of a similar molecular weight to that found in mammals. Western blotting was done using adult *Lymnaea* brain homogenates, with rat cerebellum homogenates as a positive control. As expected, the positive control sample revealed a single band at approximately 145 kD, corresponding to the full-length TrkC receptor (figure 11e). The *Lymnaea* lane, although containing several bands, contained a prominent band running slightly above the rat sample, at approximately 150 kD.

Chapter 5: Discussion

Introduction

Our original hypothesis set out to test the notion that the *Lymnaea* nervous system contains additional Trk-like proteins. Previously, methods based on nucleic acid sequence similarities have been employed in an attempt to isolate additional family members. However, since these methods have not been successful, we decided to employ an alternative approach based on the exploitation of potential sequence similarities at the protein level. To this end, we used a panel of Trk-specific polyclonal antibodies to detect and characterize cross-reacting proteins from *Lymnaea*.

Anti-pan Trk results

We used two different anti-pan Trk antibodies directed against the same epitope to determine the expression patterns and molecular weights of cross-reacting antigens. We decided to employ a second source of anti-pan Trk (SCBT anti-pan Trk) for several reasons: (1) we had little success using the 203A antibody for western blotting and whole-mount immunohistochemistry experiments with *Lymnaea* tissue, (2) it would potentially provide comparative data between the two antibodies, and (3) the SCBT anti-pan Trk had been used by Moreno et al. (1998) for similar experiments with some success in another molluscan species.

Curiously, the patterns of immunoreactivity revealed by each antibody were very different (see table 1). In addition, each antisera appeared to detect proteins of different apparent molecular weights in our western blotting experiments as well (see table 1). The difference in results may be attributable to several causes.

To start with, it is likely that the antibodies themselves are different. Although each antibody was directed against the same epitope, the antisera were produced separately and thus will certainly vary in their average affinities, avidities, and the degree to which they were purified. Polyclonal sera, by their nature, contain the entire repertoire of antibodies that were circulating within the immunized animal at the time the serum was collected (Harlow & Lane, 1999). Depending on the efficiency of affinity purification, some contaminating antibodies will remain in the final antiserum and can cause non-specific staining. Thus, the amounts of different antibodies within a polyclonal mixture will undoubtedly vary from batch to batch, and from animal to animal (Glick & Pasternak, 1998).

However, the observed differences may not be solely attributable to differences between antibodies. A potentially critical factor may be differences between individual animals as well as seasonal variations. Dramatic variations in physiology, undoubtedly the reflection of underlying molecular differences, have been documented in *Lymnaea*. For example, Magoski and Bulloch (1999) have shown that the synaptic connection between identified neurons RPeD1 and VD4 is extremely variable between animals, being either inhibitory, biphasic, or completely undetectable. These types of changes were even observed within a particular brain, often occurring within minutes (Magoski & Bulloch, 1999). Interestingly, changes in electrical activity have been shown to elicit

Table 1: Summary of major findings. This table summarizes the data gathered with each antibody in various experiments.

	Anti -pan Trk 203A	SCBT anti-pan Trk	Anti-TrkBin	Anti-TrkCin2
Immunohistochemistry	- inter-ganglionic axons - VD1/RPD2 - Connective tissue - DB	- axons entering peripheral nerves - RPA cells - RPB cells - Ce cells - Small cells in peripheral nerve	- inter-ganglionic axon bundle (bifurcates) - some peripheral nerves - DB	- some axons - RPA cells - HIJK cells - Ce ganglion cells - DB
Western blotting	~ 115 kD ~ 130 kD	~ 140 kD	~ 145 kD	~ 150 kD
Immunoprecipitation	not applicable	~ 110kD and ~95 kD	not applicable	not applicable

changes in neurotrophin and Trk receptor expression in several systems (Castren et al., 1992; Mannion et al., 1999). A recent study has also provided evidence of significant seasonal variations in the physiology of *Lymnaea* neurons. Copping et al. (2000) demonstrated that RPeD1 was disconnected from its 'normal' synaptic partners at different times of the year. These cells were also differentially responsive to RPeD1's neurotransmitter, dopamine, suggesting a seasonal variation of receptor expression. Thus the physiology and underlying molecular components can vary significantly both between and within animals.

If these antisera were indeed capable of detecting *Lymnaea* versions of Trks, we expected the cross-reacting antigens to have molecular weights similar to those found in mammals; *in vitro* translated, non-glycosylated *L*trk weighs 89kD and 78kD. Accordingly, we conducted western blotting experiments to determine the molecular weights of these *Lymnaea* antigens.

As with our immunohistochemistry data, we also observed differences in the molecular weights of antigens detected by each anti-pan Trk antibody on western blots of *Lymnaea* tissue (see table 1). In addition to those discussed above in the context of immunohistochemistry, differences obtained in western blotting experiments may also be attributable to other factors.

To begin with, each blot was performed by different investigators using different sources of some reagents. The 203A western blot included in this thesis was performed on our behalf by Andrew Chojnacki. We were never able to obtain this same result in our laboratory, despite trying the same protocol. Only subsequent to these initial experiments did we learn that the 203A antiserum does not generally work well under simple western

blotting conditions (Donna Senger, personal communication). In fact, this antiserum is primarily used after first partially purifying a sample via immunoprecipitation with the 203A antibody, followed by a western blot with the same antibody (Belliveau et al., 1997).

An alternative explanation is that the 130 kD and the 115 kD antigens detected with the 203A antiserum, and the 140 kD antigen detected with the SCBT antiserum, may in fact be alternative versions of the same core protein. Differences in apparent molecular weights may result from differential posttranscriptional or posttranslational modifications of the same gene product. Indeed, both of these types of modifications are common to many proteins, including the Trk receptors. A common type of posttranscriptional regulation in Trk receptors is alternative mRNA splicing, which results in the production of Trk isoforms with various inserts (Barbacid, 1994; Karchewski et al., 1999; Forooghian et al., 2000) or deletions (Backstrom et al., 1997). Thus, it is plausible that the different apparent molecular weights detected on western blots by each antibody reflect alternately spliced versions of a Trk-like protein that retains the epitope detected by these antibodies. In addition, these differences in molecular weights may also be due to slightly different protein isoforms being expressed between animals.

An example of a posttranslational modification that is known to have significant effects on the molecular weight of Trk receptors is glycosylation. When deglycosylated, mammalian Trk receptors have a molecular weight of about 80 kD (Martin-Zanca et al., 1989; Watson et al., 1999). As indicated above, there are two primary glycosylation states detected in cells expressing Trks: the fully glycosylated ~ 140 kD form, and the underglycosylated 110 kD form. Accordingly, the ~ 140 kD antigen detected by the

SCBT antiserum, and the ~ 130 kD and the ~ 115 kD antigens detected by the 203A antiserum may simply represent differential glycosylation of the same core protein. This hypothesis could be tested by deglycosylating *Lymnaea* lysates, followed by analysis by western blotting. If these different molecular weight products do in fact represent the same protein in different glycosylation states, then only one low molecular weight band should be present.

To facilitate a valid comparison between these two antisera, they should be tested side-by-side by the same investigator in both immunohistochemical and western blotting experiments.

Immunoprecipitation and functional characterization

If these Trk-specific antibodies were in fact capable of cross-reacting with additional Trk-like proteins in *Lymnaea*, we would expect these proteins to exhibit functional properties consistent with those of Trk receptors in other species. A hallmark feature of RTKs, including the Trks, is the ability to undergo autophosphorylation on intracellular tyrosine residues upon ligand binding (Hubbard & Till, 2000). This serves to 'activate' the receptor, and is the first step in triggering the subsequent intracellular signalling cascades that underlie the various phenotypic outcomes of RTK stimulation.

A common technique used to determine whether or not a protein becomes tyrosine phosphorylated in response to a particular stimulus is to first immunoprecipitate the protein in question, then subject it to western blot analysis with an antibody that specifically recognizes phosphorylated tyrosine residues. Unfortunately, neither the

TrkBin nor the TrkCin2 antisera work well for immunoprecipitations, so we were not able to assess the potential tyrosinc phosphorylation characteristics of cross-reacting *Lymnaea* antigens. Given that Moreno et al. (1998) successfully used the SCBT antiserum to detect an NGF-responsive protein in another molluscan species, and that an initial attempt to use the 203A antiserum to immunoprecipitate antigens from *Lymnaea* lysates failed, we decided to use the SCBT antiserum for our immunoprecipitation experiments.

To this end, our first step was to determine what antigens become purified after immunoprecipitating *Lymnaea* samples with the SCBT antiserum. This was done by immunoprecipitating from lysates with the SCBT antiserum, and probing western blots with this same antiserum. As can be seen from our positive control experiments, the ~ 140 kD Trks were successfully immunoprecipitated with this antiserum, indicating that the antiserum was capable of selectively isolating Trks from mammalian lysates. In our experiments with *Lymnaea* lysates, however, we isolated two antigens of different molecular weights than those seen in the western blotting experiments. Under immunoprecipitating conditions, a ~ 110 kD as well as a ~ 95 kD antigen were purified (see table 1).

Our immunoprecipitation results were initially surprising, as we expected to isolate the ~ 140 kD band that was most prominent on western blots with this antiserum. However, after our initial concerns, we realized that the molecular weights of these antigens were generally consistent with those detected by Moreno et al. (1998), where they immunoprecipitated a ~ 140 kD antigen as well as a ~ 95 kD antigen from the squid

brain using this same antiserum. The differences observed between our western blotting and immunoprecipitation experiments may be due to several reasons.

To begin with, under immunoprecipitating conditions the affinity of an antibody for its antigen can often be higher than under western blotting conditions (Harlow & Lane, 1999). In practical terms, this suggests that strength of the interaction between antibody and antigen can be higher using immunoprecipitation. This may result in it being 'easier' for the antibody to bind more tightly to the 'correct' *Lymnaea* antigen under immunoprecipitating conditions, while at the same time effectively reducing the likelihood of non-specific interactions. Thus, it may be the case that the lower affinity seen with western blotting allows irrelevant background bands to be more prevalent, while at the same time masking the 110 and 95 kD antigens from being detected. As noted by van Regenmortel and Azimzadeth (2000), the affinity of an antibody is a crucial factor in how they perform in various immunological assays.

Secondly, it seems plausible that the SCBT antiserum requires an initial partial purification provided by immunoprecipitation before the 'correct' *Lymnaea* antigens can be effectively detected. This idea seems particularly interesting in light of the fact that some antibodies simply do not perform well under simple western blotting conditions, and often require this initial 'enrichment' step provided by immunoprecipitation. Indeed, as mentioned, this is generally the case for the 203A antiserum when used on mammalian samples (Donna Senger, personal communication).

Another alternative that may account for the differences obtained between western blotting and immunoprecipitation is the state of the antigen when it initially reacts with the antibody. When detected on a simple western blot, the antigen is

denatured and immobilized on the blotting membrane. Under the relatively mild conditions used for immunoprecipitation, the antibody initially reacts with cross-reacting antigens in solution, where the antigen is in a conformation closer to its native state. Interestingly, some antibodies will only react with their corresponding antigens when they are in their native conformation (Willingham, 1999). The binding of the correct antigen to the SBCT antiserum is clearly not 'conformationally dependent' when detecting Trks from mammalian tissue, as evidenced by the fact that it reacts with proteins of the appropriate molecular weights under both simple western blotting and under immunoprecipitation conditions. However, this does not necessarily imply that the antiserum is not conformationally dependent when detecting *Lymnaea* versions of Trk-like proteins, which will almost certainly be different to some extent from mammalian Trks in their corresponding epitopes. This explanation is less likely to account for our data, however, as the issue of conformational dependence is generally only encountered when using monoclonal antibodies (Willingham, 1999).

If the antigen(s) isolated by immunoprecipitations with the SCBT antiserum were truly Trk-like, one would predict that their signal could be blocked by pre-incubating the primary antiserum with its blocking peptide. This was done in the current experiments by incubating the western blotting primary with a 5-fold excess by weight of peptide, according to the manufacturer's instructions. This did not block the signal from either the 110 kD band or the 95 kD band seen under immunoprecipitating conditions, nor ~ 140 kD protein detected on western blots of *Lymnaea* lysates (data not shown). The peptide was, however, capable of blocking both the 140 kD and the 110 kD bands seen on western blots of mammalian tissues (data not shown). Although the peptide blocking

experiment was not tried on immunoprecipitates from mammalian tissues, it would be expected to block the signal.

Several possibilities exist to explain these findings. First, it may be the case that the peptide was not truly in excess, and that a larger amount of peptide is required to block the signal(s) from *Lymnaea* tissues. Indeed, some researchers claim that a 10-100 fold excess of peptide by weight is often required (Keith Sharkey, personal communication). Of course, an alternative explanation is that the proteins isolated via western blotting or immunoprecipitating conditions are not truly Trk-like proteins, and thus not capable of being blocked with the peptide at any concentration. However, until a strict series of experiments are conducted using a variety of peptide concentrations, these results are inconclusive (see appendix).

It should be mentioned that several authors discount the validity of a preabsorption control in immunochemical assays (Willingham, 1999; Burry, 2000). Preabsorption is a control that confirms the specificity of the antibody for the peptide against which it was raised. Success in this type of control does not necessarily determine whether the protein recognized in tissues or on blots is the same as the peptide. As mentioned by Burry (2000), it is often difficult to obtain complete inhibition of antibody staining even in the presence of a competing peptide. This is thought to occur because a small but significant dissociation can occur between the antibody and the peptide during the incubation, thus allowing unbound antibody to bind to its protein antigen. Therefore, drawing conclusions from such experiments are not always straightforward, as there have been situations reported in which this experiment should work in theory, but has failed (Burry, 2000). Thus, in the absence of protein sequence information, it is difficult to

determine which molecular weight product detected by the SCBT antiserum, if any, might be Trk-like (see appendix).

The pan-Trk antibodies recognize all full-length Trk receptors in mammals. In an attempt to determine whether this was also the case in *Lymnaea*, we probed SCBT immunoprecipitates with the TrkB and TrkC-specific antibodies. Because no bands were present using either TrkB or TrkC-specific probes, we conclude that the 110 kD and 95 kD immunoprecipitated proteins are distinct antigens from that recognized by either the TrkB or TrkC antisera. The results of this experiment imply two different conclusions.

To begin with, it might be the case that the TrkB and TrkC-specific antisera are cross-reacting with other proteins that are not Trk-like, while the SCBT pan Trk antibody may be detecting Trk-like proteins. Of course, the converse could also be true. The latter seems unlikely though, as we would expect the SCBT antiserum to have a higher probability of detecting Trk-like proteins because its epitope is highly conserved in all Trk isoforms and all known species, whereas the TrkB and TrkC epitopes are not conserved across isoforms.

Although it is not possible at this stage to determine which *Lymnaea* protein(s), if any, might be Trk-like, our immunoprecipitation results were generally consistent with those described for the squid, in which a ~140 kD as well as a ~ 95 kD antigen were isolated with this antiserum (Moreno et al., 1998). As our original goal with this antiserum was to use it for immunoprecipitation combined with western blotting to determine tyrosine phosphorylation characteristics, we continued working with the 110 kD and 95 kD proteins isolated in immunoprecipitation experiments.

If the antigens isolated via immunoprecipitation were Trk-like, they should become tyrosine phosphorylated after neurotrophin treatment. Our first experiment, however, was to treat organ cultured brains with CM. As described by Ridgway et al. (1991), CM contains protein(s) that cross-react with an anti-NGF antibody. When these protein(s) are removed from CM via immunoprecipitation, neurons that would previously exhibit robust neurite outgrowth in response to CM could no longer do so. Importantly, it is these same neurons that are responsive to mammalian NGF. Furthermore, our immunohistochemical mapping of antigens revealed that some of these NGF-responsive cells cross-react with the SCBT antiserum. We thus reasoned that if the SCBT anti-pan Trk antiserum was cross-reacting with a potentially Trk-like protein in *Lymnaea*, this protein may become 'activated' (as assessed by detecting tyrosine phosphorylation) in response to CM treatment.

Subsequent to this initial experiment, it was determined that the particular batch of CM used for these experiments was not active. We determine the quality of our CM via a neurite outgrowth assay. When *Lymnaea* neurons were cultured in this batch of CM, virtually no outgrowth was seen, indicating that it was no longer active (Wilhelm Wildering, personal observation). The reason for the presence of a tyrosine phosphorylation signal at some time points would become apparent in our later experiments.

Our next step was to determine whether either antigen might become phosphorylated in response to treatment with NGF. This was a particularly interesting option in light of the fact that we obtained positive immunolabelling with the SCBT anti-

pan Trk antiserum in some cell groups that had previously been shown to be responsive to NGF.

Our first course of action was to determine a time course of tyrosine phosphorylation. We observed a tyrosine phosphorylation signal of the 110 kD band, but not the 95 kD band, at the 5 and 20 minute time points, but a dramatic decrease in the 10 minute time point, despite there being more antigen present in this condition. It should be noted that in our experiments with NGF, we did not include the phosphatase inhibitor sodium orthovanadate. This was done to determine the phosphorylation patterns in the presence of endogenously active phosphatases. Since this experiment did not include an acute condition (i.e., time 0), or time-matched controls incubated in the absence of NGF, our next step was to repeat this experiment including these controls.

For simplicity, we decided to examine phosphorylation in the presence or absence of NGF using only one time point. Since the 20 minute condition gave the strongest tyrosine phosphorylation signal, we chose to work with this time point. In the acute condition, no tyrosine phosphorylation signal was detected. However, at the 20 minute time points, a phosphorylation signal was detected regardless of the presence of NGF. These experiments suggest that, under basal conditions, the 110 kD antigen is not phosphorylated. Further, it demonstrates that the 110 kD antigen is capable of being tyrosine phosphorylated independent of NGF under organ culture conditions.

This was initially surprising, as we expected to see no phosphorylation signal in the absence of NGF. However, since the organ culture procedure involves severing all peripheral nerves, it may be the case that this injury causes the release of an endogenous ligand which activates the 110 kD antigen, resulting in its subsequent tyrosine

phosphorylation. These experiments do not rule out the idea that the 110 kD antigen is responsive to NGF; this may still be the case, but any potential effects that NGF may have would be masked by this axotomy-induced phosphorylation. If a procedure can be developed to allow the application of NGF in a situation that does not involve peripheral axotomy, this could be determined. If such a procedure were implemented, it might also be useful to test the potential effects of CM, in addition to other mammalian neurotrophins (i.e., BDNF, NT-3, and NT-4/5). This axotomy-induced phosphorylation likely accounted for the presence of a phosphorylation signal in our original CM experiments, despite the fact that the CM used was subsequently determined to be inactive.

It is tempting to speculate that the phosphorylation of the 110 kD antigen after axotomy may play a role in the subsequent regeneration process. Indeed, several authors have shown that the neurotrophin system may play a key role in facilitating regeneration after axotomy in mammalian nervous systems (Bradbury et al., 1998; Bradbury et al., 1999; Oudega & Hagg, 1999; Mamounas et al., 2000). In light of this, it would be instructive to examine potential changes in the patterns of SCBT anti-pan Trk immunoreactivity after organ culture. As another characteristic of mammalian Trks is their retrograde transport to the nucleus after ligand activation (Reynolds et al., 2000), it would be interesting to determine whether, after axotomy, there would be an accumulation of immunoreactivity in cell bodies. This could be combined with the phosphotyrosine antibody in double labelling experiments to determine the loci of increased tyrosine phosphorylation.

Several possibilities exist to explain the presence of the 95 kD antigen, which did not become tyrosine phosphorylated under any conditions tested here. To begin with, this antigen may simply be another form of the 110 kD antigen. It is conceivable that this protein might be the product of either alternative mRNA splicing of a single gene product, or may reflect the differential utilization of start codons within its mRNA. In support of this, it has been shown using *in vitro* translation that *L*trk is capable of using two different start codons to produce two different sized proteins with molecular weights of 89kD and 78kD (van Kesteren et al., 1998). Of course, it is also possible that the 95 kD antigen is also not a Trk-like protein, but is detected non-specifically by the pan Trk antiserum.

TrkBin and TrkCin2 results

Unfortunately, our experiments with the TrkBin and TrkCin2 antibodies were limited by the fact that neither of them work well under immunoprecipitating conditions. Therefore, we conducted only immunohistochemical and western blotting studies.

Each antibody revealed different patterns of immunoreactivity in whole-mount preparations. Whereas the TrkBin antiserum detected primarily axons, the TrkCin2 antiserum detected some axons, but primarily cell bodies (see table 1). Our western blotting experiments with these antibodies demonstrated the presence of cross-reacting antigens whose apparent molecular weight were similar to those seen in mammalian samples. Blots with the TrkBin antiserum showed a prominent immunoreactive band that ran slightly heavier than its mammalian counterpart. Although the TrkCin2 antiserum

detected many bands on western blots, the most prominent was one which was also slightly heavier than its mammalian counterpart, having an apparent molecular weight of about 150 kD. This result is consistent with that described by Guistetto et al., (1999), where they obtained a ~ 140 kD band from the marine mollusc *Aplysia* on western blots using a TrkB-specific antibody.

Despite the fact that the anti-TrkBin and anti-TrkCin2 antibodies recognize proteins of similar molecular weights as compared to mammals, conclusions regarding their ability to cross-react with Trk-like proteins in *Lymnaea* should be viewed with particular skepticism. As mentioned, the epitopes used to raise these antibodies are specific to these Trk subtypes in vertebrates, and thus are not highly conserved through evolution. However, there are some limited similarities between the TrkBin and TrkCin2 epitopes and Ltrk, leaving open the possibility of cross-reactions between these antisera and Ltrk.

The goal of this thesis was to determine the potential utility of antibodies to identify additional Trk-like family members in *Lymnaea*. Naturally, taking antibodies designed to detect mammalian proteins across this scale of evolutionary distance carries significant risks, and the conclusions reached by using such an approach should be treated with caution. Although the *Lymnaea* antigens identified with these antibodies exhibit some properties that are characteristic of Trk receptors in vertebrates, it is also a distinct possibility that these antisera are cross-reacting with proteins which are not Trk-like.

The conclusive identity of these antigens, however, can only be provided by obtaining protein sequences directly, or by cloning their corresponding cDNAs. There are

potentially two approaches that we could take to acquire such information. First, the technique of cDNA expression cloning could be employed. This involves screening a *Lymnaea*-based expression library with each antibody to identify clones that produce cross-reacting proteins. If full-length clones are not detected, partial cDNAs sequences of the corresponding clones can then be obtained, and used to design PCR primers in an attempt to isolate a full-length cDNA sequence from *Lymnaea.* Certainly a major problem with this approach is the potential to identify several false positives, and would likely involve sequencing a number of cDNAs for potential Trk-like sequences. This situation could be improved by constructing specific cDNA libraries from areas that are known to express high levels of each particular antigen. For example, high levels of SCBT immunoreactivity are seen in the right parietal ganglion. Making an expression library consisting of cDNAs from this ganglion alone would likely decrease the number of false positives when compared to a whole brain cDNA library.

A second approach involves first obtaining partial peptide sequences directly from western blotted material. After blotting, the corresponding area of the membrane can be excised and the bound protein partially sequenced. From these peptide sequences, PCR primers could be designed and used to isolate full-length cDNAs from standard cDNA libraries. This may in fact be a more viable option for our situation, as we have collaborators whose expertise is in sequencing proteins. This approach would avoid the potential of sequencing numerous false positives as in the case of expression cloning.

Although previous attempts using PCR-based homology and library screeing have thus far failed to yield additional Trk-like sequences from *Lymnaea*, another nucleic acid-based technique may be useful in identifying other Trks from *Lymnaea*. Low stringency

Southern blots using Ltrk-specific probes, or more generally, tyrosine kinase-specific probes, would surely detect many more tyrosine kinases. Thus, if additional Trks are present in *Lymnaea*, sequencing all cross-reacting products would likely facilitate their identification.

Potential significance and perspectives

One largely neglected area of research into the neurotrophin system has been their phylogenetic history. This was primarily because it had been assumed that this system was limited to the vertebrate lineage (Barde, 1994). If sequence analysis demonstrates that we have identified additional Trk-like proteins, it would provide key information regarding the evolutionary history of the Trk receptors.

A phylogenetic analysis of Trk receptor evolution has recently been conducted by Finn Hallbook (Hallbook et al., 1998; Hallbook, 1999, Hallbook et al., 2001). He suggests a single ancestral Trk, from which all subsequent Trks emerged, existed prior to the emergence of vertebrates from the common lineage. Further, Hallbook suggests that *L*trk may be this ancestral Trk (Hallbook et al., 1998). Identifying another Trk-like sequence from *Lymnaea* would suggest one of several possibilities: (1) either the Trk family first appeared in this lineage and the gene was duplicated at least once to give rise to additional family members, (2) the original ancestral Trk may have emerged in a species that diverged from the common lineage even earlier than the molluscs.

In addition to providing valuable evolutionary insights, the identification of additional Trk-like proteins in *Lymnaea* would further facilitate our goals to elucidate the

cellular and molecular mechanisms of neural plasticity in the adult CNS. The identification of a 110 kD protein whose tyrosine phosphorylation is induced after axotomy is particular interesting in this respect. Cloning other Trk-like sequences from *Lymnaea* would enable us to employ various molecular manipulations, such as antisense knockdown, in an attempt to ascertain their roles in neural plasticity.

One unresolved issue in the study of the evolutionary history of the neurotrophin system is the apparent absence of neurotrophin-like ligands in invertebrates (Chao, 2000, Jaaro et al., 2001). However, Jaaro et al. (2001) note that it might be that the nervous systems of these animals contains other molecules that are structural homologues of the neurotrophins, despite lacking significant amino acid similarities. Indeed, this type of structural conservation in the absence of defined sequence similarity is not without precedent. Coagulogen, a clotting protein from the horseshoe crab *Limulus*, exhibits no amino acid similarity with NGF, but contains a domain that is structurally homologous to one found in the NGF monomer (Bergner et al., 1996). Another example of structural homology is seen in *Drosophila*. The ligand Spatzle has been demonstrated to form a disulfide-dimer that adopts a conformation similar to that seen in NGF and coagulogen (DeLotto & DeLotto, 1998). As noted by Jaaro et al. (2001) however, functional similarities do not necessarily follow from structural similarities.

According to the phylogenetic trees constructed by Hallbook (Hallbook et al., 1998; Hallbook, 1999; Hallbook et al., 2001; see figure 2), there is a striking correlation between the Trk receptors and the neurotrophins. This suggests that the ligand and receptors may have co-evolved. Although a Trk-like receptor has been cloned from *Lymnaea*, no ligand with sequence similarity to vertebrate neurotrophins has yet been

isolated from this species. According to the model proposed by Hallbook, however, it would not be surprising to find at least on neurotrophin-like ligand, which may represent the ligand for *L*trk.

This does not necessarily imply, however, that all invertebrates should contain sequences with homology mammalian Trk receptors and neurotrophin ligands. Indeed, the genome of two prominent invertebrate model systems – *Drosophila* and *C. elegans* – were a disappointment to some researchers interested in the evolutionary history of the neurotrophin system in that no neurotrophin-like or Trk-like sequences were identified (Jaaro et al., 2001; Myers et al., 2000; *C. elegans* Sequencing Consortium, 1998). However, classical morphology-based phylogenetic trees had initially placed arthropods (i.e., *Drosophila*) and nematodes (i.e., *C. elegans*) on different evolutionary branches. The fact that neither lineage has Trk-like or neurotrophin-like sequences would have thus required independent gene loss in each lineage. However, the explosion of molecular biology has stimulated the cloning of several genes, and facilitated the construction of a phylogenetic tree based on these sequences. When a tree is reconstructed using ribosomal RNA or netrin gene sequences, arthropods and nematodes are placed on the same branch of the phylogenetic tree (Adoutte et al., 2000; Jaaro et al., 2001). This classification scheme would require ligand and receptor gene loss only in the common ancestor of these two phyla, a much more likely scenario. Taken together, this data suggests that Trk and possibly neurotrophin-like sequences may have emerged prior to the split between the arthropod/nematode lineage and the molluscan lineage. Certainly, at least one Trk-like sequence (i.e., *L*trk) was retained in the molluscan lineage, while these sequences were likely lost in the arthropod/nematode lineage. Given the possible co-evolution of Trk

receptors and neurotrophin ligands (Hallbook et al., 1998; Hallbook, 1999; Hallbook et al., 2001), it is possible that a neurotrophin-like sequence may yet be indentified in *Lymnaea*.

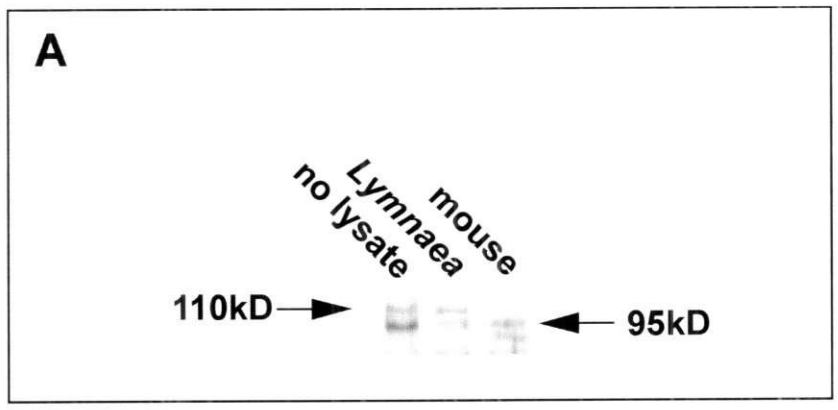
Appendix

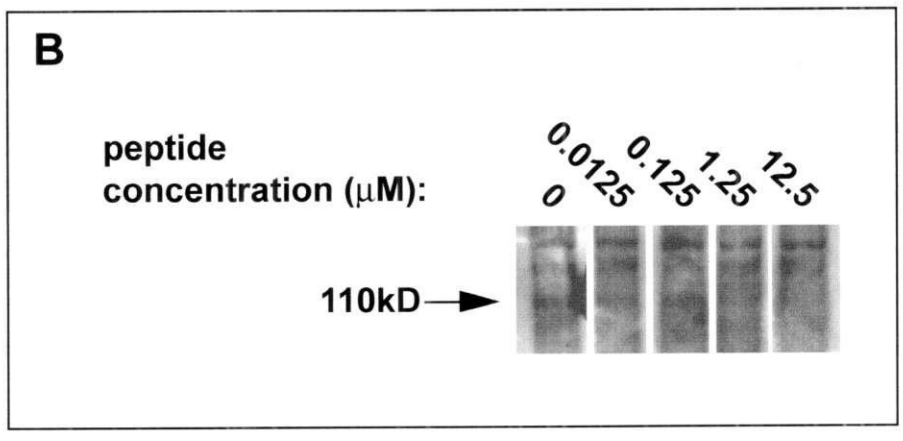
As a requirement for the completion of this thesis, two additional control experiments were conducted by Chi Diep in our laboratory. The experiments were as follows: 1) a no tissue immunoprecipitation control to determine whether the ~ 110kD and the ~ 95kD bands present actually represented undenatured IgGs used to immunoprecipitate, and 2) a repeat of the peptide preabsorption using a variety of peptide concentrations to determine which, if any, product was blocked.

As seen in figure 1a, completing the immunoprecipitation procedure in the absence of lysate reveals both the ~ 110kD and the ~ 95kD bands. Unfortunately, due to our inability to completely denature the immunoprecipitating antibody, we were unable to determine which proteins, if any, represent Trk-like proteins. Despite using fresh reagents and multiple denaturants (i.e., DTT and mercaptoethanol), complete denaturation of the immunoprecipitating antibody was not possible in our hands.

The second experiment was designed to determine whether the signal from the ~ 110kD band or the ~ 95kD band could be attenuated with increasing concentrations of immunizing peptide. This would be expected if an endogenous *Lymnaea* protein with a similar epitope was present (indistinguishable from the undenatured antibody signal based on molecular weight) at either molecular weight. When blots from immunoprecipitated *Lymnaea* samples were incubated with increasing peptide concentrations, the signal from the ~ 110kD product becomes attenuated (figure 1b). This suggests that embedded in the ~ 110kD band is an endogenous *Lymnaea* protein that has a similar epitope to that used originally to raise the anti-pan Trk antiserum. The signal from the ~ 95kD band was relatively unaffected.

Figure 1: Additional control experiments conducted by Chi Diep. **A)** Negative control experiment performing the immunoprecipitation in the absence of lysate. Note that both the 110 kD and the 95 kD band are present; thus, making it difficult to ascertain which, if any, proteins isolated using *Lymnaea* lysate, were in fact Trk-like. **B)** The 110 kD signal from *Lymnaea* lysates becomes attenuated when blots are incubated with increasing concentrations of blocking peptide. This suggests that an endogenous *Lymnaea* protein also contributes to the signal seen at 110 kD, and that its epitope is similar to that used to raise the antibody.


A
no lysate
Lymnaea
mouse
110kD
95kD


B
peptide
concentration (μM):
0
0.0125
0.125
1.25
12.5
110kD

Combining these results with my original data showing that only the ~ 110kD band becomes tyrosine phosphorylated after axotomy would suggest that this product may in fact be a true Trk-like *Lymnaea* protein.

References

Adoutte, A., Balavoine, G., Lartillot, N., Lespinet, O., Prud'homme, B., & de Rosa, R. (2000). The new animal phylogeny: reliability and implications. Proceedings of the National Academy of Sciences, USA, 97, 4453-4456.

Angeles, T.S., Yang, S.X., Steffler, C., & Dionne, C.A. (1998). Kinetics of trkA tyrosine kinase activity and inhibition by K-252a. Archives of Biochemistry, 349, 267-274.

Arvanov, V.L., Seebach, B.S., & Mendell, L.M. (2000). NT-3 evokes an LTP-like facilitation of AMPA/kainate receptor-mediated synaptic transmission in the neonatal rat spinal cord. Journal of Neurophysiology, 84, 752-758.

Atwal, J.K., Massie, B., Miller, F.D., & Kaplan, D.R. (2000). The TrkB-Shc site signals neuronal survival and local axon growth via MEK and P13-kinase. Neuron, 27, 265-277.

Backstrom, A., Soderstrom, S., & Ebendal, T. (1997). Cloning of a new chicken trkC extracellular isoform and its mRNA expression in E9 sensory and autonomic ganglia. International Journal of Developmental Neuroscience, 15, 275-284.

Barbacid, M. (1994). The Trk family of neurotrophin receptors. Journal of Neurobiology, 25, 1386-1403.

Barker, P.A. (1998). p75NTR: A study in contrasts. Cell Death and Differentiation, 5, 346-356.

Barker, P.A., & Shooter, E.M. (1994). Disruption of NGF binding to the low affinity neurotrophin receptor p75LNTR reduces NGF binding to TrkA on PC12 cells. Neuron, 13, 203-215.

Barrett, G.L. (2000). The p75 neurotrophin receptor and neuronal apoptosis. Progress in Neurobiology, 61, 205-229.

Belliveau, D.J., Krivko, I., Kohn, J., Lachance, C., Pozniak, C., Rusakov, D., Kaplan, D., & Miller, F.D. (1997). NGF and neurotrophin-3 both activate TrkA on sympathetic neurons but differentially regulate survival and neuritogenesis. Journal of Cell Biology, 136, 375-388.

Benjamin, P.R., & Pilkington, J.B. (1986). The electrotonic location of low-resistance intercellular junctions between a pair of giant neurones in the snail Lymnaea. Journal of Physiology, 370, 111-126.

Benjamin, P.R., Staras, K., & Kemenes, G. (2000). A systems approach to the cellular analysis of associative learning in the pond snail Lymnaea. Learning and Memory, 7, 124-131.

Bergner, A., Oganessyan, V., Muta, T., Iwanaga, S., Typke, D., Huber, R., & Bode, W. (1996). Crystal structure of a coagulogen, the clotting protein from horseshoe crab: a structural homologue of nerve growth factor. EMBO Journal, 15, 6789-6797.

Bosco, A., & Linden, R. (1999). BDNF and NT-4 differentially modulate neurite outgrowth in developing retinal ganglion cells. Journal of Neuroscience Research, 57, 759-769.

Bradbury, E.J., King, V.R., Simmons, L.J., Priestley, J.V., & McMahon, S.B. (1998). NT-3, but not BDNF, prevents atrophy and death of axotomized spinal cord projection neurons. European Journal of Neuroscience, 10, 3058-3068.

Bradbury, E.J., Khemani, S., Von, R., King, Priestley, J.V., & McMahon, S.B. (1999). NT-3 promotes growth of lesioned adult rat sensory axons ascending in the dorsal columns of the spinal cord. European Journal of Neuroscience, 11, 3873-3883.

Brodski, C., Schnurch, H., & Dechant, G. (2000). Neurotrophin-3 promotes the cholinergic differentiation of sympathetic neurons. Proceedings of the National Academy of Sciences, USA, 97, 9683-9688.

Brown, W.M., Prager, E.M., Wang, A., & Wilson, A.C. (1982). Mitochondrial DNA sequences of primates: tempo and mode of evolution. Journal of Molecular Evolution,18, 225-239.

Bulloch, A.G., & Ridgway, R.L. (1989). Neuronal plasticity in the adult invertebrate nervous system. Journal of Neurobiology, 20, 295-311.

Bulloch, A.G., & Syed, N.I. (1992). Reconstruction of neuronal networks in culture. Trends in Neuroscience, 15, 422-427.

Buchman, V.L., & Davies, A.M. (1993). Different neurotrophins are expressed and act in a developmental sequence to promote the survival of embryonic sensory neurons. Development, 118, 989-1001.

Burry, R.W. (2000). Specificity controls for immunocytochemical methods. Journal of Histochemistry and Cytochemistry,48, 163-166.

C. elegans Sequencing Consortium. (1998). Genome sequence of the nematode *C. elegans*: a platform for investigating biology. Science, 282, 2012-2018.

Casaccia-Bonnefil, P., Gu, C., & Chao, M.V. (1999). Neurotrophins in cell survival/death decisions. Advances in Experimental Medicine and Biology, 468, 275-282.

Casaccia-Bonnefil, P., Kong, H., & Chao, M.V. (1998). Neurotrophins: the biological paradox of survival factors eliciting apoptosis. Cell Death and Differentiation, 5, 357-364.

Castellucci, V.F., Frost, W.N., Goelet, P., Montarolo, P.G., Schacher, S., Morgan, J.A., Blumenfeld, H., & Kandel, E.R. (1986). Cell and molecular analysis of long-term sensitization in Aplysia. Journal of Physiology, 81, 349-357.

Castren, E., Zafra, F., Thoenen, H., & Lindholm, D. (1992). Light regulates expression of brain-derived neurotrophic factor mRNA in rat visual cortex. Proceedings of the National Academy of Sciences, USA, 89, 9444-9448.

Chao, M.V. (2000). Trophic factors: An evolutionary cul-de-sac or door into higher neuronal function? Journal of Neuroscience Research, 59, 353-355.

Copping, J., Syed, N.I., & Winlow, W. (2000). Seasonal plasticity of synaptic connections between identified neurones in Lymnaea. Acta Biologica Hungarica, 51, 205-210.

Croll, R.P., Voronezhskaya, E.E., Hiripi, L., & Elekes, K. (1999). Development of catecholaminergic neurons in the pond snail, Lymnaea stagnalis: II. Postembryonic development of central and peripheral cells. Journal of Comparative Neurology, 404, 297-309.

Crow, T., & Xue-Bian, J.J. (2000). Identification of a 24 kDa phosphoprotein associated with an intermediate stage of memory in Hermissenda. Journal of Neuroscience, 20, RC74.

Dale, N., Schacher, S., & Kandel, E.R. (1988). Long-term facilitation in Aplysia involves increase in transmitter release. Science, 239, 282-285.

Davis, C.A. (1993). Whole-mount immunohistochemistry. Methods in Enzymology, 31, 502-516.

DeLotto, Y., & DeLotto, R. (1998). Proteolytic processing of the Drosophila Spatzle protein by easter generates a dimeric NGF-like molecule with ventralising activity. Mechanisms of Development, 72, 141-148.

DuBose, R.F., & Hartl, D.L. (1990). The molecular evolution of bacterial alkaline phosphatase: correlating variation among enteric bacteria to experimental manipulations of the protein. Molecular Biology and Evolution,7, 547-577.

de la Rosa, E.J., Arribas, A., Frade, J.M., & Rodriguez-Tebar, A. (1994). Role of neurotrophins in the control of neural development: neurotrophin-3 promotes both neuron differentiation and survival of cultured chick retinal cells. Neuroscience, 58, 347-352.

Fainzilber, M., Smit, A.B., Syed, N.I., Wildering, W.C., Hermann, P.M., van der Schors, R.C., Jimenez, C., Li, K.W., van Minnen, J., Bulloch, A.G., Ibanez, C.F., & Geraerts, W.P. (1996). CRNF, a molluscan neurotrophic factor that interacts with the p75 neurotrophin receptor. Science,274, 1540-1543.

Fainzilber, M. (2000). NGF 2000 Meeting, Montreal, Canada.

Forooghian, F., Kojic, L., Gu, Q., Wong, C.A., & Prasad, S.S. (2000). Molecular analysis of trkC in the cat visual cortex. Journal of Molecular Neuroscience, 14, 39-51.

Friedman, W.J. (2000). Neurotrophins induce death of hippocampal neurons via the p75 receptor. Journal of Neuroscience, 20, 6340-6346.

Fryer, R.H., Kaplan, D.R., Feinstein, S.C., Radeke, M.J., Grayson, D.R., & Kromer, L.F. (1996). Developmental and mature expression of full-length and truncated TrkB receptors in the rat forebrain. Journal of Comparative Neurology, 374, 21-40.

Futuyma, D.J. (1997). Evolutionary Biology. Sunderland, MA: Sinauer Associates.

Giehl, K.M., & Tetzlaff, W. (1996). BDNF and NT-3, but not NGF, prevent axotomy-induced death of rat corticospinal neurons in vivo. European Journal of Neuroscience, 8, 1167-1175.

Giustetto, M., Patterson, S. Martin, K.C., & Kandel, E.R. (1999). The role of TrkB-ligands in the long-term facililation of *Aplysia* sensory-motor neuron connections in culture. Society for Neuroscience Abstract, 792.5.

Giustetto, M., Bartsch, D., & Kandel, E.R. (2000). Cloning of *Aplysia*-Trk receptor and its possible contribution to long-term synaptic plasticity. Society for Neuroscience Abstract, 405.3.

Glick, B.R., & Pasternak, J.J. (1998). Molecular biotechnology: Principles & applications of recombinant DNA. American Society for Microbiology.

Gotz, R., Raulf, F., & Schartl, M. (1992). Brain-derived neurotrophic factor is more highly conserved in structure and function than nerve growth factor during vertebrate evolution. Journal of Neurochemistry, 59, 432-442.

Gotz, R., Koster, R., Winkler, C., Raulf, F., Lottspeich, F., Schartl, M., & Thoenen, H. (1994). Neurotrophin-6 is a new member of the nerve growth factor family. Nature, 372, 266-269.

Hallbook, F., Ibanez, C.F., & Persson, H. (1991). Evolutionary studies of the nerve growth factor family reveal a novel member abundantly expressed in Xenopus ovary. Neuron, 6, 845-858.

Hallbook, F., Lundin, L.G., & Kullander, K. (1998). Lampetra fluviatilis neurotrophin homolog, descendant of a neurotrophin ancestor, discloses the early molecular evolution of neurotrophins in the vertebrate subphylum. Journal of Neuroscience, 18, 8700-8711.

Hallbook, F. (1999). Evolution of the vertebrate neurotrophin and Trk receptor gene families. Current Opinion in Neurobiology, 9, 616-621.

Hallbook, F., Kullander, K., Lundin, L.G. (2000). Evolution of the neurotrophins and their receptors. In I. Mocchetti (Ed.), Neurobiology of the neurotrophins (pp. 99-134). Johnson City, TN: FP Graham Publishing.

Halstead, L.B. (1993). Agnatha. In M.J. Benton (Ed.), The fossil record 2 (pp. 573-581). London: Chapman and Hall.

Hammond, E.N., Tetzlaff, W., Mestres, P., & Giehl, K.M. (1999). BDNF, but not NT-3, promotes long-term survival of axotomized adult rat corticospinal neurons in vivo. Neuroreport, 10, 2671-2675.

Harlow, E., & Lane, D. (1999). Using antibodies: A laboratory manual. New York: Cold Spring Harbour Laboratory Press.

Hayashi, I., Perez-Magallanes, M., & Rossi, J.M. (1992). Neurotrophic factor-like activity in Drosophila. Biochemical and Biophysical Research Communications, 184, 73-79.

Heinrich, G., & Lum, T. (2000). Fish neurotrophins and Trk receptors. International Journal of Developmental Neuroscience, 18, 1-27.

Hermann, P.M., de Lange, R.P., Pieneman, A.W., ter Maat, A., & Jansen, R.F. (1997). Role of neuropeptides encoded on CDCH-1 gene in the organization of egg-laying behavior in the pond snail, Lymnaea stagnalis. Journal of Neurophysiology, 78, 2859-2869.

Hodgkin, A.L., & Huxley, A.F. (1939). Action potentials recorded from inside a nerve fiber. Nature, 144, 710-711.

Hodgkin, A.L., & Huxley, A.F. (1949). The effect of sodium ions on the electrical activity of the giant axon of the squid. Journal of Physiology, 108, 37-77.

Hodgkin, A.L. & Huxley, A.F. (1952). A quantitative description of membrane current and its application to conduction and excitation in nerve. Journal of Physiology, 117, 500-544.

Hubbard, S.R, & Till, J.H. (2000). Protein tyrosine kinase structure and function. Annual Review of Biochemistry, 69, 373-398.

Jaaro, H., Beck, G., Conticello, S.G., & Fainzilber, M. (2001). Evolving better brains: a need for neurotrophins? Trends in Neuroscience, 24, 79-85.

Jarvis, C.R., Xiong, Z.G., Plant, J.R., Churchill, D., Lu, W.Y., MacVicar. B,A, & MacDonald, J.F. (1997). Neurotrophin modulation of NMDA receptors in cultured murine and isolated rat neurons. Journal of Neurophysiology, 78, 2363-2371.

Kang, H., & Schuman, E.M. (1995). Long-lasting neurotrophin-induced enhancement of synaptic transmission in the adult hippocampus. Science, 267, 1658-1662.

Kang, H., Welcher, A.A., Shelton, D., & Schuman, E.M. (1997). Neurotrophins and time: different roles for TrkB signaling in hippocampal long-term potentiation. Neuron, 19, 653-664.

Kaplan, D.R., & Miller, F.D. (1997). Signal transduction by the neurotrophin receptors. Current Opinion in Cell Biology, 9, 213-221.

Kaplan, D.R., & Miller, F.D. (2000). Neurotrophin signal transduction in the nervous system. Current Opinion in Neurobiology, 10, 381-391.

Karchewski, L.A., Kim, F.A., Johnston, J., McKnight, R.M., & Verge, V.M. (1999). Anatomical evidence supporting the potential for modulation by multiple neurotrophins in the majority of adult lumbar sensory neurons. Journal of Comparative Neurology, 413, 327-341.

Kerkhoven, R.M., Van Minnen, J., & Boer, H.H. (1990). Neuron-specific monoclonal antibodies raised against the low molecular weight fraction of a brain homogenate of the pond snail Lymnaea stagnalis immunoreact with neurons in the central nervous system of the cockroach, the guppy, the wall lizard, the rat and man. Journal of Chemical Neuroanatomy, 3, 337-346.

Korte, M., Kang, H., Bonhoeffer, T., & Schuman, E. (1998). A role for BDNF in the late-phase of hippocampal long-term potentiation. Neuropharmacology, 37, 553-559.

Kreitman, M. (1983). Nucleotide polymorphism at the alcohol dehydrogenase locus of Drosophila melanogaster. Nature, 304, 412-417.

Kumar, S., Hedges, S.B. (1998). A molecular timescale for vertebrate evolution. Nature, 392, 917-920.

Lai KO, Fu WY, Ip FCF, Ip NY. (1998). Cloning and Expression of a Novel Neurotrophin, NT-7, from Carp. Molecular and Cellular Neuroscience, 11, 64-76.

Levi-Montalcini, R., & Hamburger, V. (1951). Selective growth stimulating effects of mouse sarcoma on the sensory and sympathetic nervous system of the chick embryo. Journal of Experimental Zoology, 116, 321-362.

Levi-Montalcini, R. (1966). The nerve growth factor: its mode of action on sensory and sympathetic nerve cells. Harvey Lectures, 60, 217-259.

Levi-Montalcini, R. (1987). The nerve growth factor 35 years later. Science, 237, 1154-1162.

Lohof, A.M., Ip, N.Y., & Poo, M.M. (1993). Potentiation of developing neuromuscular synapses by the neurotrophins NT-3 and BDNF. Nature, 363, 350-353.

Magoski, N.S., & Bulloch, A.G. (1999). Dopamine activates two different receptors to produce variability in sign at an identified synapse. Journal of Neurophysiology, 81, 1330-1340.

Magoski, N.S., & Bulloch, A.G. (1998). Trophic and contact conditions modulate synapse formation between identified neurons. Journal of Neurophysiology, 79, 3279-3283.

Maisonpierre, P.C., Belluscio, L., Friedman, B., Alderson, R.F., Wiegand, S.J., Furth, M.E., Lindsay, R.M., & Yancopoulos, G.D. (1990). NT-3, BDNF, and NGF in the developing rat nervous system: parallel as well as reciprocal patterns of expression. Neuron, 5, 501-509.

Mamounas, L.A., Altar, C.A., Blue, M.E., Kaplan, D.R., Tessarollo, L., & Lyons, W.E. (2000). BDNF promotes the regenerative sprouting, but not survival, of injured serotonergic axons in the adult rat brain. Journal of Neuroscience, 20, 771-782.

Mannion, R.J., Costigan, M., Decosterd, I., Amaya, F., Ma, Q.P., Holstege, J.C., Ji, R.R., Acheson, A., Lindsay, R.M., Wilkinson, G.A., & Woolf, C.J. (1999). Neurotrophins: peripherally and centrally acting modulators of tactile stimulus-induced inflammatory pain hypersensitivity. Proceedings of the National Academy of Sciences, USA, 96, 9385-9390.

Martin, S.C., Marazzi, G., Sandell, J.H., & Heinrich, G. (1995). Five Trk receptors in the zebrafish. Developmental Biology, 169, 745-758.

Martin, S.C., Sandell, J.H., & Heinrich, G. (1998). Zebrafish TrkC1 and TrkC2 receptors define two different cell populations in the nervous system during the period of axonogenesis. Developmental Biology, 195, 114-130.

Martin-Zanca, D., Oskam, R., Mitra, G., Copeland, T., & Barbacid, M. (1989). Molecular and biochemical characterization of the human trk proto-oncogene. Molecular and Cellular Biology, 9, 24-33.

Mauelshagen, J., Parker, G.R., & Carew, T.J. (1996). Dynamics of induction and expression of long-term synaptic facilitation in Aplysia. Journal of Neuroscience, 16, 7099-7108.

McKay, S.E., Purcell, A.L., & Carew, T.J. (1999). Regulation of synaptic function by neurotrophic factors in vertebrates and invertebrates: implications for development and learning. Learning and Memory, 6, 193-215.

Middlemas, D.S., Lindberg, R.A., & Hunter, T. (1991). trkB, a neural receptor protein-tyrosine kinase: evidence for a full-length and two truncated receptors. Molecular and Cellular Biology, 11, 143-153.

Ming, G., Song, H., Berninger, B., Inagaki, N., Tessier-Lavigne, M., & Poo, M. (1999). Phospholipase C-gamma and phosphoinositide 3-kinase mediate cytoplasmic signaling in nerve growth cone guidance. Neuron, 23, 139-148.

Moffett, S.B. (2000). Regeneration as an application of gastropod neural plasticity. Microscopy Research and Technique, 49, 579-588.

Montarolo, P.G., Goelet, P., Castellucci, V.F., Morgan, J., Kandel, E.R., & Schacher, S. (1986). A critical period for macromolecular synthesis in long-term heterosynaptic facilitation in Aplysia. Science, 234, 1249-1254.

Moreno, H., Nadal, M., Leznik, E., Sugimori, M., Lax, I., Schlessinger, J., & Llinas, R. (1998). Nerve growth factor acutely reduces chemical transmission by means of postsynaptic TrkA-like receptors in squid giant synapse. Proceedings of the National Academy of Sciences, USA, 95, 14997-15002.

Murphy, A.D., & Kater, S.B. (1980). Sprouting and functional regeneration of an identified neuron in Helisoma. Brain Research, 186, 251-272.

Myers, E.W., Sutton, G.G., Delcher, A.L., Dew, I.M., Fasulo, D.P., Flanigan ,M,J,, Kravitz, S.A., Mobarry, C.M., Reinert, K.H., Remington, K.A., Anson, E.L., Bolanos, R.A., Chou, H.H., Jordan, C.M., Halpern, A.L., Lonardi, S., Beasley, E.M., Brandon, R.C., Chen, L., Dunn, P.J., Lai, Z., Liang, Y., Nusskern, D.R., Zhan, M., Zhang, Q., Zheng, X., Rubin, G.M., Adams, M.D., & Venter, J.C. (2000). A whole-genome assembly of Drosophila. Science, 287, 2196-2204.

Novikova, L.N., Novikov, L.N., & Kellerth, J.O. (2000). Survival effects of BDNF and NT-3 on axotomized rubrospinal neurons depend on the temporal pattern of neurotrophin administration. European Journal of Neuroscience, 12, 776-780.

Nilsson AS, Fainzilber M, Falck P, Ibanez CF. (1998). Neurotrophin-7: a novel member of the neurotrophin family from the zebrafish. FEBS Letters, 424, 285-290.

Oudega, M., & Hagg, T. (1999). Neurotrophins promote regeneration of sensory axons in the adult rat spinal cord. Brain Research, 818, 431-438.

Peunova, N., & Enikolopov, G. (1995). Nitric oxide triggers a switch to growth arrest during differentiation of neuronal cells. Nature, 375, 68-73.

Pulido, D., Campuzano, S., Koda, T., Modolell, J., & Barbacid, M. (1992). Dtrk, a Drosophila gene related to the trk family of neurotrophin receptors, encodes a novel class of neural cell adhesion molecule. EMBO Journal, 11, 391-404.

Reichardt, L.F. & Farinas, I. (1997). Neurotrophic factors and their receptors: Roles in neuronal development and function. In W. M. Cowan, T.M. Jessell, & S.L. Zipursky (Eds.), Molecular and cellular approaches to neural development, (pp. 220-263). New York: Oxford.

Reynolds, A.J., Bartlett, S.E., & Hendry, I.A. (2000). Molecular mechanisms regulating the retrograde axonal transport of neurotrophins. Brain Research Reviews, 33, 169-178.

Ridgway, R.L., Syed, N.I., Lukowiak, K., & Bulloch, A.G. (1991). Nerve growth factor (NGF) induces sprouting of specific neurons of the snail, Lymnaea stagnalis. Journal of Neurobiology, 22, 377-390.

Ridgway, R.L., Syed, N.I., & Bulloch, A.G. (1993). In vitro evidence for multiple neuritogenic factors in the central nervous system of pulmonate molluscs. Acta Biologica Hungarica, 44, 109-113.

Rodriguez-Tebar, A., Dechant, G., Gotz, R., & Barde, Y.A. (1992). Binding of neurotrophin-3 to its neuronal receptors and interactions with nerve growth factor and brain-derived neurotrophic factor. EMBO Journal, 11, 917-922.

Sandell, J.H., Martin, S.C., & Heinrich, G. (1994). The development of neurotrophin receptor Trk immunoreactivity in the retina of the zebrafish (Brachydanio rerio). Developmental Brain Research, 81, 192-200.

Schacher, S., & Proshansky, E. (1983). Neurite regeneration by Aplysia neurons in dissociated cell culture: modulation by Aplysia hemolymph and the presence of the initial axonal segment. Journal of Neuroscience, 3, 2403-2413.

Stephens, R.M., Loeb, D.M., Copeland, T.D., Pawson, T., Greene, L.A., & Kaplan, D.R. (1994). Trk receptors use redundant signal transduction pathways involving SHC and PLC-gamma 1 to mediate NGF responses. Neuron, 12, 691-705.

Suneja, S.K., & Potashner, S.J. (1998). Quantification of a neurotrophin receptor from submilligram quantities of brain tissue using western blotting. Brain Research Protocols, 3, 88-93.

Syed, N.I., Ridgway, R.L., Lukowiak, K., & Bulloch, A.G. (1992). Transplantation and functional integration of an identified respiratory interneuron in Lymnaea stagnalis. Neuron, 8, 767-774.

Syed, N., Richardson, P., & Bulloch, A. (1996). Ciliary neurotrophic factor, unlike nerve growth factor, supports neurite outgrowth but not synapse formation by adult Lymnaea neurons. Journal of Neurobiology, 29, 293-303.

van Kesteren, R.E., Fainzilber, M., Hauser, G., van Minnen, J., Vreugdenhil, E., Smit, A.B., Ibanez, C.F., Geraerts, W.P., & Bulloch, A.G. (1998). Early evolutionary origin of the neurotrophin receptor family. EMBO Journal, 17, 2534-2542.

van Regenmortel, M.H., & Azimzadeh, A. (2000). Determination of antibody affinity. Journal of Immunoassay, 21, 211-234.

Verdi, J.M., Birren, S.J., Ibanez, C.F., Persson, H., Kaplan, D.R., Benedetti, M., Chao, M.V., & Anderson, D.J. (1994). p75LNGFR regulates Trk signal transduction and NGF-induced neuronal differentiation in MAH cells. Neuron, 12, 733-745.

Voronezhskaya, E.E., Hiripi, L., Elekes, K., & Croll, R.P. (1999). Development of catecholaminergic neurons in the pond snail, Lymnaea stagnalis: I. Embryonic development of dopamine-containing neurons and dopamine-dependent behaviors. Journal of Comparative Neurology, 404, 285-296.

Watson, F.L., Porcionatto, M.A., Bhattacharyya, A., Stiles, C.D., & Segal, R.A. (1999). TrkA glycosylation regulates receptor localization and activity. Journal of Neurobiology, 39, 323-336.

Wildering, W.C., Lodder, J.C., Kits, K.S., & Bulloch, A.G. (1995). Nerve growth factor (NGF) acutely enhances high-voltage-activated calcium currents in molluscan neurons. Journal of Neurophysiology, 74, 2778-2781.

Williams, J.A., Barrios, A., Gatchalian, C., Rubin, L., Wilson, S.W., Holder, N. (2000). Programmed cell death in zebrafish rohon beard neurons is influenced by TrkC1/NT-3 signaling. Developmental Biology, 226, 220-230.

Willingham MC. (1999). Conditional epitopes. is your antibody always specific? Journal of Histochemistry and Cytochemistry, 47, 1233-1236.

Wong, R.G., Hadley, R.D., Kater, S.B., & Hauser, G.C. (1981). Neurite outgrowth in molluscan organ and cell cultures: the role of conditioning factor(s). Journal of Neuroscience, 1, 1008-1021.

Wong, R.G., Barker, D.L., Kater, S.B., Bodnar, D.A. (1984). Nerve growth-promoting factor produced in culture media conditioned by specific CNS tissues of the snail Helisoma. Brain Research, 292, 81-91.

Wu, F., & Schacher, S. (1994). Pre- and postsynaptic changes mediated by two second messengers contribute to expression of Aplysia long-term heterosynaptic inhibition. Neuron, 12, 407-421.

Yano, H., & Chao, M.V. (2000). Neurotrophin receptor structure and interactions. Pharm Acta Helv, 74, 253-260.