

Copyright is owned by the Author of the thesis. Permission is given for a copy to be downloaded by an individual for the purpose of research and private study only. The thesis may not be reproduced elsewhere without the permission of the Author.

---

# Interactions of Neurotrophins and their Receptors

by

Allan Bates

A dissertation submitted in partial satisfaction of the requirements for the degree of

Doctor of Philosophy

in the

Institute of Molecular Biosciences

at

**MASSEY UNIVERSITY, NEW ZEALAND**

2003

---

---

## ABSTRACT

---

### Supervisors

Prof. Pat Sullivan, Ph.D. Massey University, Palmerston North, New Zealand.

Prof. Uri Saragovi, Ph.D. McGill University, Montreal, Canada.

Prof. E. N. Baker, Ph.D, Auckland University, Auckland, New Zealand.

Prof. William Mobley, MD., Ph.D., Stanford University, Palo Alto, California, U.S.A.

In order to investigate the interactions of neurotrophins with their receptors, a number of different domains of the extra-cellular regions of the TrkA, TrkB and TrkC receptors were expressed and the interactions of neurotrophins with these domains were investigated by biosensor. The entire extracellular domains of all three receptors were expressed in the yeast *Pichia pastoris*, while the leucine-rich regions and the immunoglobulin-like domains were expressed as MBP-fusion proteins in *E. coli*. Peptides representing the second leucine-rich regions and purported neurotrophin-binding domain of TrkA, TrkB and TrkC were synthesized. Proteins expressed in *Pichia pastoris* were purified by anion, cation and metal chelating columns; proteins expressed in *E. coli*, were purified on amylose columns. All recombinant Trk proteins were covalently attached, using EDC/NHS chemistry, to the methyl-dextran surface of a biosensor cuvette. Extensive kinetics measurements of the interactions of the neurotrophins with immobilized recombinant proteins established a difference in the binding interactions of NGF with TrkA compared with the interactions of BDNF with TrkB and NT-3 with TrkC. All NGF interactions with TrkA proteins showed biphasic kinetics. Interactions of BDNF and NT-3 with TrkB and TrkC showed monophasic kinetics. No interaction of NGF with the immunoglobulin-like domain of TrkA was observed for those proteins expressed in *E. coli*, however the interaction of BDNF was observed with the immunoglobulin-like domain of TrkB when expressed in *E. coli*. Interaction of NGF and BDNF was observed with the leucine-rich domain of TrkA and TrkB respectively. These results differ from previously reported studies, both *in vivo* and *in vitro*, of the interactions of the extra-cellular domains of the Trk receptors with neurotrophins. Previous studies have claimed to establish exclusive interaction of the neurotrophins with either the leucine-rich or immunoglobulin-like domains of the Trk receptors. The interaction studies reported here show a clear interaction of neurotrophins with both leucine-rich and immunoglobulin-like domains of the Trk receptors.

These interactions have similar affinity. This result suggests that the interactions of neurotrophin and receptor may be more complex than previously suggested. It is conceivable that neurotrophins bind initially to the leucine-rich domain of the receptor, followed by movement to the Immunoglobulin-like domain and the initiation of phosphorylation of the intra-cellular domains or internalization of receptor and bound neurotrophin. Kinetics studies of the synthetic peptides failed to show that these represent the exclusive neurotrophin-receptor interaction domain as previously reported. These results suggest that the development of small molecule mimetics of the neurotrophins as a therapy for Alzheimer's and other neurological diseases may be more complicated than previously envisioned.

---

## ACKNOWLEDGEMENTS

---

Rita Levi-Montalcini, the co-discoverer of the neurotrophin NGF and eventual Nobel Prize winner, began her studies of the protein shortly before the outbreak of the second-world war. She continued her studies in the confines of her home throughout the allied bombing campaign of Italy. After the war, the studies were continued in Europe, the United States and South America. It would be difficult to find a more inspiring story than the trials and difficulties of one scientist in the pursuit of knowledge, than that of Levi-Montalcini. My own project presented in this thesis, has taken me from the United States to Japan, New Zealand and Canada and often seemed so close to failure that it seemed prudent to abandon the entire study. I owe a great deal to those that encouraged me to continue.

An ancient Chinese myth relates the story of an emperor who commissions a noted sculptor to fashion the emperor's likeness out of a piece of jade from a nearby mountain. After many years, the time to present the sculpture to the emperor finally arrived. Upon revelation of the sculpture, the emperor was dismayed to see, not an image of himself, but a figurine of a flock of birds. Naturally the artist was called to account for the apparent lack of deference to the emperor and nature of the commission. He explained that he had not found the emperor's likeness within the jade but instead had found the image of birds. Pursuit of a scientific investigation is quite analogous to the myth.

The study for my thesis began with the idea that the receptor for NGF would be crystallized and its structure determined. It was quickly realized that in order to maximize the possibilities of success, other neurotrophin receptors and even neurotrophins should be crystallized. After the passage of more than 3 years the proteins had been expressed but no diffracting crystals for any receptor or neurotrophin had been obtained (this study is not contained within the following thesis). This situation caused many difficulties for me and came close to terminating the entire thesis. Fortunately a change of concept resulted in a new study, which has led me to a different interpretation of the interaction of neurotrophins and their receptors than that previously espoused. Hence, like the sculptor, I did not find the structure that was originally sought, but from the trials and tribulations of the study a different and in many ways an equally interesting (if not beautiful) result was obtained.

There have been many individuals with whom I have interacted and who have influenced me over the duration of this thesis study. Firstly I would like to thank my wife Siu Lan who has endured stoically these difficult years and who has been my greatest source of encouragement. Without her, this thesis study would have terminated, without success, years ago. I should also like to thank my mother, Eileen, for her support and whose suffering with Parkinson's disease provided me with the initial incentive to pursue development of molecular solutions for human diseases. Without attempting to apportion any degree of direct influence or contribution to the study, the following individuals are now acknowledged for their help and encouragement:

Chin Shou Huang, Connie Jimenez, Deb Hall, Ken Neet, Ljubica Ivanisevic, Louis Reichardt, Martin Spencer, Pat Sullivan, Peter Hwang, Rainer Marksteiner, Rainer Schneider, Robert Fletterick, Ted Baker, Uri Saragovi, Vladimir Basus and William Mobley. I should like to thank Ted for originating the study and looking at the early drafts I submitted. In particular I should like to thank Pat for carefully reading the final thesis submission and making appropriate comments. Uri has been very supportive of my efforts and has reviewed, prior to submission, the papers resulting from this study.

Finally I would like to thank David Zaring, Pangene Corporation, Eric Hnath and Affinity Sensors. Eric provided much information for the initial phases of the kinetics study and assistance in the checking of data. Affinity Sensors provided, without charge, the biosensor instrumentation necessary to conduct the kinetics study of the interactions of the neurotrophins and their receptors. David provided laboratory space and funding at Pangene Corporation to conduct the kinetics experiments and, in addition, dangled a salary and a fascinating scientific project in front of me, as an incentive to complete the thesis. I might mention that these past two years I have had the good fortune to begin an oncology project at Pangene. The project is, unlike the thesis, proceeding well and has influenced my concepts of small molecule mimetics of proteins, as well as serving to overcome some of the lingering negative effects of the thesis study. Many of the kinetics techniques learned in the thesis study now form the foundations for the development of small molecule inhibitors of DNA repair enzymes, the basis of Pangene's oncology program. Perhaps, in the end it has all been worthwhile.

---

## TABLE of CONTENTS

---

<b>Abstract</b> .....	i
<b>Acknowledgements</b> .....	iii
<b>Table of Contents</b> .....	v
<b>List of Figures</b> .....	viii
<b>List of Tables</b> .....	xi
<b>Appendix</b> .....	xii
<b>Abbreviations</b> .....	xiii

## TABLE OF CONTENTS

### CHAPTER 1 Neurotrophins and Their Receptors

1.1 Introduction	1
1.2 The Structure of NGF	2
1.3 Basic Residues	3
1.4 Aromatic Residues	4
1.5 Carboxyl Group Modifications	5
1.6 Summary of NGF Residues Implicated in Receptor Binding	5
1.7 Brain Derived Neurotrophic Factor (BDNF)	7
1.8 Neurotrophin-3 (NT-3)	7
1.9 The Trk Receptor	8
1.10 The Immunoglobulin Domains and NGF Binding	14
1.11 The Immunoglobulin-like Domains of TrkA, TrkB and TrkC	17
1.12 A Crystal Structure of NGF and the Immunoglobulin Domain of TrkA	18
1.13 The TrkB Receptor	21
1.14 The Leucine Rich Domain and BDNF Binding	22
1.15 The Immunoglobulin-Like Domains and Neurotrophin Binding	25
1.16 The TrkC Receptor	27
1.17 Neurotrophin Binding Domains of TrkC	27

1.18	The p75 Receptor	28
1.19	Rational for the Thesis Research	32

## **CHAPTER 2 Trk Receptors, Expression and Purification**

2.1	Introduction and Aims	34
2.2	Expression of Proteins in <i>P. pastoris</i> with the Vector pPICZαA	41
2.3	Construction of Expression Vectors	41
2.4	Cloning Techniques Following PCR	43
2.5	Transformation of <i>P. pastoris</i> strain X33 with trk/pPICZαA	49
2.6	Large Scale Protein Production and Purification	51
2.7	Purification by Ion Exchange Chromatography	53
2.8	Purification of Trk Protein	53
2.9	Protein Expression Results	59
2.10	Expression of Trk Proteins in <i>E. coli</i> .	61
2.11	Expression of MBP-fusion Proteins	61
2.12	Expression of Trk Protein Domains as His-tagged Proteins in <i>E. coli</i> .	65
2.13	Protein Expression	65
2.14	Protein Quantification	67
2.15	Identification of Recombinant Proteins	67
2.16	Mass Spectrometry	67
2.17	Western Blotting	71
2.18	Nerve Growth Factor Purification	79
2.19	Summary	80

## **CHAPTER 3 Ultracentrifuge Studies**

3.1	Introduction	82
3.2	Methods	85
3.3	Data Analysis	85
3.4	Results	86
3.5	Summary	92



## **CHAPTER 4 Kinetics**

4.1	Introduction	94
4.2	The IAsys Biosensor	94
4.3	Coupling of Trk Proteins to the IAsys Surface	98
4.4	Immobilization Chemistry of Trk Proteins to IAsys Biosensor Surface	100
4.5	Control of Non-Specific Interactions	101
4.6	Binding Data Collection	102
4.7	Definitions of Kinetics Terminology	104
4.8	Data Analysis and Error Propagation	106
4.9a	The Interactions of NGF with TrkA Proteins	113
4.9b	The Interactions of BDNF with TrkB Proteins	122
4.9c	The Interactions of NT-3 with TrkC Proteins	129
4.10	A Comparison of Neurotrophin-Receptor Interaction Studies	131
4.11	Summary	133

## **CHAPTER 5 Discussion**

5.1	Introduction	139
5.2	Recombinant Proteins, Native Proteins and Crystal Structures	140
5.3	Binding Studies and Controls	142
5.4	Binding Data and Trk Receptor Structure	143
5.5	Previous In Vivo and In Vitro Studies of Trk Receptors and Neurotrophins	144
5.6	Protein Immobilization and Kinetics Data	147
5.7	Biosensor Kinetics	148
5.8	Biphasic Kinetics of the NGF-TrkA Interaction	150
5.9	Previously Observed Biphasic Kinetics of the NGF-TrkA Interaction	151
5.10	Neurotrophins and Receptor Structure Influence Kinetics	151
5.11	Mass Transport and Biosensor Kinetics	153
5.12	Biphasic and Monophasic Kinetics Observed by Biosensor	154
5.13	Biosensor Kinetics and Accuracy of Data	154
5.14	The Second TrkA LRR Domain and Ligand-Binding	155
5.15	TrkB and TrkC Biosensor Kinetics	155
5.16	An Hypothesis of Receptor and Ligand Interaction	157

5.17 Multivalency and Structure Changes of Receptor and Ligand	158
5.18 The Effect of Charged Residues on Neurotrophin-Receptor Interactions	160
5.19 Interactions of Trk Receptors and Accessory Proteins	160
5.20 Trk Receptor Ligand-binding Domains; a Role for the LRR Domain	164
5.21 Summary	165

<b>REFERENCES</b>	170
-------------------	-----

<b>APPENDIX</b>	181
-----------------	-----

## LIST OF FIGURES

### CHAPTER 1

Figure 1.1 NGF Structure of NGF	6
Figure 1.2 Model of neurotrophin binding to Trk receptors	9
Figure 1.3 TrkA receptor subdomains	10
Figure 1.4 C-terminal immunoglobulin-like domains of the Trk proteins	17
Figure 1.5 The NGF binding domains of TrkA C-terminal domain	19
Figure 1.6 Interaction regions of NGF with d2 of TrkA	20
Figure 1.7 Interaction Regions of TrkA and NGF	21

### CHAPTER 2

Figure 2.1 Trk receptor structure	36
Figure 2.2 Map of pPICZαA	41
Figure 2.3 PCR products for human and rat TrkA extracellular domains	44
Figure 2.4 Agarose gels of the restriction enzyme digests of plasmid DNA	45
Figure 2.5 Gel showing digest of ligated DNA	46

Figure 2.6	Digests of ligated trkA and vector	47
Figure 2.7	Restriction digests of rat TrkA/pPICZαA	48
Figure 2.8	Restriction digests of human TrkA/pPICZαA plasmids	49
Figure 2.9	Expression of TrkA in <i>P. pastoris</i>	51
Figure 2.10	First purification step for Trk proteins expressed in yeast	56
Figure 2.11	Second purification step for Trk proteins expressed in yeast	57
Figure 2.12.	Third purification step for Trk proteins expressed in yeast	58
Figure 2.13	A coomassie stained SDS-gel of TrkA extracellular domain	60
Figure 2.14	A coomassie stained SDS-gel of the TrkB extracellular domain	60
Figure 2.15	A coomassie stained SDS-PAGE gel of TrkA MBP-fusion proteins	63
Figure 2.16	A coomassie stained SDS-PAGE gel of TrkB MBP-fusion proteins	63
Figure 2.17	Gel of His-tagged protein expression in <i>E. coli</i> .	66
Figure 2.18	MALDI-TOF analysis of an MBP-fusion protein	69
Figure 2.19	SDS-PAGE and electrotransfer for the Western Blots	72
Figure 2.20	Antibody control gels	73
Figure 2.21	Western blot of TrkA MBP-fusion proteins	74
Figure 2.22	Western blot of TrkA MBP-fusion proteins	74
Figure 2.23	Western blot of TrkA MBP-fusion proteins	75
Figure 2.24	Western blot of TrkB MBP-fusion proteins	75
Figure 2.25	Western blot of TrkB MBP-fusion proteins	76
Figure 2.26	Western blot of TrkB MBP-fusion proteins	76
Figure 2.27	Western blot of TrkC MBP-ED protein	77
Figure 2.28	Western blot of TrkA and TrkB proteins expressed in <i>P. pastoris</i>	77
Figure 2.29	A coomassie stained gel of purified NGF	80

### CHAPTER 3

Figure 3.1	Absorbance and statistical data TrkA ED	87
Figure 3.2	Absorbance and statistical data TrkA Ig-like Domain	87
Figure 3.3	Absorbance and statistical data TrkA Ig-like Domain	88

## CHAPTER 4

Figure 4.1	The arrangement of the IAsys	97
Figure 4.2	Immobilization chemistry	100
Figure 4.3	Buffer experiment	101
Figure 4.4	IAsys plot showing typical association/dissociation data	102
Figure 4.5	Association curves	106
Figure 4.6	Association rate	107
Figure 4.7	Binding rate fit	108
Figure 4.8	Dissociation rate fit	108
Figure 4.9	Association rate of neurotrophins for Trk proteins	111
Figure 4.10	Dissociation rate of neurotrophins	111
Figure 4.11	Bar plot of TrkA-NGF reaction data	119
Figure 4.12	Association rates of individual TrkA domains	120
Figure 4.13	Dissociation rates of individual TrkA domains	121
Figure 4.14	Bar plot of TrkB-BDNF reaction data	126
Figure 4.15	Association rates of individual TrkB domains	127
Figure 4.16	Dissociation rates of individual TrkB domains	128
Figure 4.17	Bar plot of TrkC-NT-3 reaction data	130

## CHAPTER 5

Figure 5.1	Hypothetical Model for Trk-Neurotrophin interactions	163
------------	--	-----

## LIST OF TABLES

### CHAPTER 1

Table 1.1	Binding data of NGF, BDNF and NT-3 binding to p75 receptor	29
-----------	--	----

### CHAPTER 2

Table 2.1	TrkA extracellular domains expressed as MBP-fusion proteins	37
Table 2.2	TrkA extracellular domains expressed as His-tagged proteins	37
Table 2.3	TrkA full-length extracellular domain expressed in yeast	38
Table 2.4	Synthetic human TrkA peptide	38
Table 2.5	TrkB extracellular domains expressed as MBP-fusion proteins	38
Table 2.6	TrkB extracellular domains expressed as His-tagged proteins	39
Table 2.7	TrkB full-length extracellular domain expressed in yeast	39
Table 2.8	Synthetic human TrkB peptide	39
Table 2.9	TrkC extracellular domains expressed as MBP-fusion proteins	40
Table 2.10	TrkB full-length extracellular domain expressed in yeast	40
Table 2.11	Synthetic human TrkB peptide	40
Table 2.12	Expression level of purified Trk proteins	59
Table 2.13	Approximate expression levels of the MBP-fusion proteins	64
Table 2.14	Mass Spectrometry of MBP-fusion protein tryptic digests	70

### CHAPTER 3

Table 3.1	Approximate molecular weights of Trk and neurotrophin proteins	89
Table 3.2	Protein solution stoichiometry	90

### CHAPTER 4

Table 4.1	Protein immobilization levels on the biosensor surface	99
Table 4.2	TrkA LRR2 synthetic peptide, association and dissociation rates	113

Table 4.3	TrkA MBP-LRR2, association and dissociation rates	113
Table 4.4	TrkA MBP-LRR, association and dissociation rates	114
Table 4.5	TrkA MBP-C1LRR12, association and dissociation rates	114
Table 4.6	TrkA MBP-LRR23C2, association and dissociation rates	114
Table 4.7	TrkA MBP-C1LRR, association and dissociation rates	115
Table 4.8	TrkA MBP-LRRC2, association and dissociation rates	115
Table 4.9	TrkA Ig-like domain, association and dissociation rates	116
Table 4.10	TrkA Ig-like domain, association and dissociation rates	116
Table 4.11	TrkA MBP-ED, association and dissociation rates	117
Table 4.12	TrkA ED ( <i>P. pastoris</i> ), association and dissociation rates	117
Table 4.13	TrkA ED (insect cells), association and dissociation rates	117
Table 4.14	Values of the equilibrium dissociation constant for TrkA proteins	118
Table 4.15	TrkB LRR2 synthetic peptide, association and dissociation rates	122
Table 4.16	TrkB MBP-C1LRR, association and dissociation rates	122
Table 4.17	TrkB MBP-LRRC2, association and dissociation rates	123
Table 4.18	TrkB MBP-C1LRRC2, association and dissociation rates	123
Table 4.19	TrkB MBP-Ig-like domain, association and dissociation rates	124
Table 4.20	TrkB MBP-ED, association and dissociation rates	124
Table 4.21	TrkB ED ( <i>P. pastoris</i> ), association and dissociation rates	125
Table 4.22	Values of the equilibrium dissociation constant for TrkB proteins	125
Table 4.23	TrkC LRR2 synthetic peptide, association and dissociation rates	129
Table 4.24	TrkC MBP-ED, association and dissociation rates	129
Table 4.25	Values of the equilibrium dissociation constant for TrkC proteins	129
Table 4.26	A comparison of the equilibrium binding constants	132

## APPENDIX

NGF sequence	181
BDNF sequence	181
TrkA (rat) sequence	182
TrkA (human) sequence	183
TrkB (rat) sequence	184
TrkB (mouse) sequence	185
TrkB (human) sequence	186
TrkC (rat) sequence	187

TrkC (human)	188
Neurotrophin Domains I-VII	190
Molecular Weights of MBP-fusion proteins	191
Charged Residues on the surfaces of neurotrophins	192

## ABBREVIATIONS

Relevant Abbreviations to material in the text are given within the body of the material and in footnotes.

$K_D$	Equilibrium Dissociation Constant
$\mu\text{M}$	Micromolar
mM	Millimolar
nM	Nanomolar
$K_D$	Equilibrium Dissociation Constant
$K_{\text{off}}$	Dissociation Rate
$K_{\text{on}}$	Association Rate
BDNF	Brain Derived Growth Factor
CNS	Central Nervous System
MBP	Maltose Binding Protein
NGF	Nerve Growth Factor
NTF	Neurotrophic Factor
NT-3	Neurotrophin-3
PNS	Peripheral Nervous System
Trk	Receptor for NTF's