THE ROLE OF NEU1 SIALIDASE IN Trk TYROSINE KINASE RECEPTOR ACTIVATION

by

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ABSTRACT

The signaling pathways of tyrosine kinase Trk receptors and their downstream biological effects are well known, but the parameters controlling the interactions between the receptors and their natural ligands still remain to be defined. Recent published reports from our laboratory indicate that nerve growth factor (NGF)-induced TrkA receptor activation is dependent on a membrane cellular sialidase. This sialidase activity specifically targets and hydrolyzes sialyl α-2, 3-linked β-galactosyl residues resulting in the desialylation and activation of the receptor. These findings support a novel hypothesis that places mammalian sialidase(s) in a cycle of activation of these receptors by their natural ligand. Taken together, they also predict a prerequisite desialylation of Trk receptors caused by a sialidase on the cell surface enabling the removal of a steric hindrance to receptor dimerization. Until now, the sialidase associated with neurotrophin-treated live Trk-expressing cells has not been identified. The molecular mechanism(s) of sialidase activation by neurotrophin factors binding to their receptors also remains unknown. In this thesis, the novel role of Neu1 sialidase in the activation of ligand-induced TrkA and TrkB receptors has been identified. It has been reported for the first time that Neu1 is already in complex with naïve and ligand-induced Trk receptors. In addition, a membrane sialidase mechanism initiated by NGF binding to TrkA has been indentified. It suggests a potentiation of GPCR-signaling via membrane Gαi subunit proteins and matrix metalloproteinase-9 (MMP-9) activation to induce Neu1 sialidase activation in live TrkA- and TrkB-expressing cells and primary neurons. These results establish a unique mode of regulation of Trk receptors by their natural ligand and define a
new function for Neu1 sialidase. Preliminary data indicate that members of the family of tyrosine kinase receptors like epidermal growth factor receptor (EGFR) and insulin receptor are also under the same regulatory control of Neu1 sialidase. Recent reports from the laboratory have indicated that ligand-induced activation of the highly glycosylated Toll-like receptors, TLR-2,-3 and -4 is also dependent on Neu1 sialidase on the cell surface. Taken all together, the findings in this thesis uncover a Neu1 and MMP-9 cross-talk on the cell surface which is critically essential for neurotrophin-induced Trk tyrosine kinase receptor activation and neuron function.
CO-AUTHORSHIP

CHAPTER 2 AND CHAPTER 3

Dr. Myron R Szewczuk and Preethi Jayanth wrote the papers, designed and performed the experiments. Schammim Ray Amith helped with the isolation of primary macrophages from mouse models. Katrina Gee performed the flow cytometry analyses. Dr. Myron R Szewczuk supervised the research design and the writing of the papers.
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# TABLE OF CONTENTS

Abstract .......................................................................................................................... ii  
Co-Authorship ................................................................................................................. iv  
Acknowledgements ........................................................................................................... v  
Table of Contents ............................................................................................................. vii  
List of Figures .................................................................................................................. xi  
List of Abbreviations ....................................................................................................... xv

## CHAPTER 1: General Introduction ............................................................................... 1  
Neurotrophins and their receptors .................................................................................. 1  
The Trk receptor tyrosine kinase ...................................................................................... 2  
Trk receptor structure and signaling ............................................................................... 6  
Trk receptor glycosylation ............................................................................................... 9  
Sialic acids and Sialidases .............................................................................................. 14  
Rationale and Hypothesis .............................................................................................. 18  
Objectives of the study ................................................................................................... 23

## CHAPTER 2: Neurotrophin-induced Trk receptor activation is dependent on Neu1 sialidase ......................................................................................................................... 25  
Abstract ......................................................................................................................... 26  
Introduction ...................................................................................................................... 27  
Materials and Methods ................................................................................................... 29  
Ligands and inhibitors ...................................................................................................... 29  
Antibodies ......................................................................................................................... 29  
Cell lines .......................................................................................................................... 30  
Primary cortical neurons ................................................................................................. 31  
Primary mouse bone marrow macrophage cells ............................................................... 31
Mouse models............................................................................................................ 32
Sialidase activity in viable cells................................................................................ 32
Immunocytochemistry of TrkA and TrkB receptors............................................... 33
Flow cytometry of cell surface Neu1 in live TrkA-PC12 cells............................. 34
Confocal colocalization....................................................................................... 34
Western blots and co-immunoprecipitation....................................................... 35
Neurite outgrowth............................................................................................... 36
Statistics.............................................................................................................. 36

Results.................................................................................................................... 37
Neu1 sialidase is induced in TrkA-expressing cell lines..................................... 37
Effect of neuraminidase inhibitors on NGF-induced sialidase........................... 48
Neu1 induces phosphorylation of TrkA and neuritogenesis............................. 54
Neu1 colocalizes with TrkA on the surface of TrkA-PC12 cells....................... 60
Neu1 sialidase induces TrkB receptor activation in primary neurons............. 68

Discussion............................................................................................................. 73

CHAPTER 3: Potential roles for G-protein coupled receptors and matrix
metalloproteinases in Neu1 activation............................................................... 78

Abstract............................................................................................................... 79

Introduction......................................................................................................... 81

Materials and methods....................................................................................... 88
Ligands and inhibitors....................................................................................... 88
Reagents............................................................................................................ 89
Cells lines and primary cortical neurons......................................................... 89
Sialidase activity in viable cells....................................................................... 90
Immunocytochemistry of pTrkA and pTrkB..................................................... 91
Flow cytometry of MMP-9 in live TrkA-PC12 cells....................................... 91
MMP-9 colocalization with Trk...................................................................... 92
MMP-9 colocalization with Neu1 .......................................................... 93
Co-immunoprecipitation........................................................................ 93
Neurite outgrowth ................................................................................ 94
Statistics ................................................................................................ 95
Results .................................................................................................. 96
Ligand-induced sialidase activity in inhibited by Suramin .................... 96
PTx blocks NGF-induced sialidase activation ......................................... 96
Sialidase activity is inhibited by Galardin and Piperazine ..................... 99
MMP-9 is involved in regulation of NGF-induced sialidase activity ....... 100
pTrkA is inhibited by PTx and MMP-9 inhibitor .................................... 105
GPCR ligand bombesin induces sialidase activity in TrkA-PC12 cells .... 108
Inhibition of bombesin-induced sialidase activity and pTrkA ............... 108
MMP-9 colocalizes with TrkA and Neu1 .............................................. 109
Gαi proteins and MMP-9 are involved in NGF-induced neuritogenesis ... 118
Neu1 and MMP-9 cross-talk is evident in primary neurons ............... 123
Discussion ............................................................................................ 129

CHAPTER 4: The role of Neu1 in Toll-like receptor activation and signaling .... 132
Abstract ............................................................................................... 133
Introduction .......................................................................................... 134
Materials and Methods ......................................................................... 137
Reagents ................................................................................................ 137
Cell lines ............................................................................................... 137
Nuclear extracts and EMSA ................................................................. 138
Co-immunoprecipitation and western blots .......................................... 139
Results .................................................................................................. 141
Neu1 sialidase co-immunoprecipitates with TLR2, TLR3 and TLR4 ..... 141
Tamiflu blocks LPS-induced NFkB activation ...................................... 149
Identification of sialyl residues involved in TLR activation ............... 156
Removal of α-2,3-sialyl residues by Neu1 is important for TLR4-MyD88 interaction……………………………………………………………………………….. 159
Discussion……………………………………………………………………………………………………….. 162

CHAPTER 5: Summary and Perspectives………………………………………………………….. 166
Role of Neu1 in Trk receptor activation…………………………………………………………….. 166
The mechanism of ligand-induced Neu1 sialidase activation……………………………………167
Neu1 sialidase-A master enzyme……………………………………………………………………174

REFERENCES…………………………………………………………………………………………180
# LIST OF FIGURES

## CHAPTER 1

<table>
<thead>
<tr>
<th>Figure</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.1</td>
<td>Neurotrophin binding affinities</td>
<td>4</td>
</tr>
<tr>
<td>1.2</td>
<td>Model of sialidase-induced TrkA activation</td>
<td>12</td>
</tr>
</tbody>
</table>

## CHAPTER 2

<table>
<thead>
<tr>
<th>Figure</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>2.1</td>
<td>NGF induces Neu1 sialidase in live TrkA-PC12 and 3T3-TrkA cells</td>
<td>38</td>
</tr>
<tr>
<td>2.2</td>
<td>Inhibition of sialidase activity induced by NGF in TrkA-PC12 cells by antibody against Neu1 sialidase</td>
<td>42</td>
</tr>
<tr>
<td>2.3</td>
<td>NGF-induced activation of sialidase was observed only in wild-type primary bone-marrow derived macrophages but not in Neu1 deficient macrophages</td>
<td>44</td>
</tr>
<tr>
<td>2.4</td>
<td>Sialidase activity is associated with neurotrophin-treated live cells specific for Trk receptors</td>
<td>46</td>
</tr>
<tr>
<td>2.5</td>
<td>Neuraminidase inhibitor Tamiflu inhibits sialidase activity in a dose dependent manner</td>
<td>50</td>
</tr>
<tr>
<td>2.6</td>
<td>Tamiflu, but not other neuraminidase inhibitors block NGF-induced sialidase activity in TrkA-PC12 cells</td>
<td>52</td>
</tr>
<tr>
<td>2.7</td>
<td>NGF-induced phosphorylation of TrkA (pTrkA) is inhibited by Tamiflu and anti-Neu1 antibody</td>
<td>56</td>
</tr>
<tr>
<td>2.8</td>
<td>Trans-sialidase or α-2,3,6 sialyl neuraminidase induce pTrkA in Neu1-deficient primary BM</td>
<td></td>
</tr>
</tbody>
</table>
macrophages

Figure 2.9 Neurite out-growth in TrkA-PC12 cells is inhibited by anti-Neu1 antibody

Figure 2.10A-B (A) Flow cytometry analysis of Neu1 expression (B) Neu1 colocalizes with TrkA

Figure 2.11A-B Co-immunoprecipitation (A) Neu1 co-immunoprecipitates with TrkA in cell lysates of naïve and NGF-induced TrkA-PC12 cells (B) Neu2, 3 and 4 do not co-immunoprecipitate with TrkA

Figure 2.12A-B (A) BDNF induces sialidase activity in primary neurons (B) BDNF induces phosphorylation of TrkB in primary neurons

Figure 2.13 Neu1 colocalizes with TrkB in naïve and BDNF-induced primary neurons

CHAPTER 3

Figure 3.1A-B Suramin inhibits NGF-induced sialidase activity in (A) TrkA-PC12 cells and (B) 3T3-TrkA cells

Figure 3.2A-B NGF-stimulated sialidase activity is blocked by broad range MMP inhibitors (A) Galardin (B) Piperazine

Figure 3.3 NGF-stimulated sialidase activity is blocked by GPCR Gαi protein inhibitor PTx and MMP-9 specific inhibitor.

Figure 3.4 NGF-induced phosphorylation of TrkA involves sialidase activity through the intermediate catalytic activity of MMP-9 and Gαi-proteins

Figure 3.5 GPCR agonist Bombesin induces sialidase activity in TrkA-PC12 cells and is inhibited by Tamiflu
Figure 3.6  Bombesin-induced sialidase activation in TrkA-PC12 cells is blocked by PTx and MMP-9 inhibitor .......................... 112
Figure 3.7  Bombesin-induced transactivation of pTrkA is dependent on activation of Ga\textsubscript{i} proteins and MMP-9……………….. 114
Figure 3.8A-C  (A) MMP-9 colocalizes with TrkA in naïve and NGF-stimulated TrkA-PC12 cells (B) Flow cytometry analysis of MMP-9 expression on the cell surface of live TrkA-PC12 cells (C) MMP-9 co-immunoprecipitates with TrkA............. 116
Figure 3.9A-B  (A) MMP-9 co-localizes with Neu1 on the surface of TrkA-PC12 in both naïve and NGF-stimulated cells (B) MMP-9 coimmunoprecipitates with Neu1.......... 119
Figure 3.10  Neurite out-growth in TrkA-PC12 cells is inhibited by MMP-9i and PTx .......................................................... 121
Figure 3.11A-B  (A) BDNF-induced sialidase activity in primary rat neurons is blocked by PTx and MMP-9 inhibitors (B) BDNF-induced pTrkB is blocked by PTx and MMP-9 inhibitors.................................................. 125
Figure 3.12A-B  MMP-9 colocalizes with (A) TrkB in primary neurons (B) Neu1 in primary neurons................................. 127

CHAPTER 4

Figure 4.1  Neu1 co-immunoprecipitates with TLR4....................... 143
Figure 4.2  Neu2, 3 and 4 do not co-immunoprecipitate with TLR4.......................................................... 145
Figure 4.3  Neu1 co-immunoprecipitates with TLR2 and -3........... 147
Figure 4.4  LPS induces phosphorylated NF\textkappa Bp65 pS529 (pNF\textkappa B) in TLR4-expressing cells.......................... 150
| Figure 4.5 | LPS induces NFκBp65 in TLR4-expressing cell line | 152 |
| Figure 4.6 | Supershift of nuclear NFκB | 154 |
| Figure 4.7 | MAL-2 lectin blot | 157 |
| Figure 4.8 | Tamiflu and MAL II inhibit LPS-induced TLR4/MyD88 complex formation | 160 |

---

**CHAPTER 5**

<p>| Figure 5.1 | Proposed model for NGF-induced TrkA activation | 172 |
| Figure 5.2 | EGF induces Neu1 sialidase activity in EGFR-expressing A431 cells | 175 |
| Figure 5.3 | Insulin induces Neu1 sialidase activity in Insulin-expressing A431 cells | 177 |</p>
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
</tr>
</thead>
<tbody>
<tr>
<td>4-MU</td>
<td>4-MUNANA, 2’-(4-methylumbelliferyl)-α-D-N-acetylneuraminic acid</td>
</tr>
<tr>
<td>AsialoGM1</td>
<td>Asialotetrahexosyl ganglioside</td>
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<tr>
<td>BDNF</td>
<td>Brain-derived neurotrophic factor</td>
</tr>
<tr>
<td>EBP</td>
<td>Elastin binding protein</td>
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<tr>
<td>EGFR</td>
<td>Epidermal growth factor receptor</td>
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<tr>
<td>GM1</td>
<td>Monosialotetrahexosyl ganglioside</td>
</tr>
<tr>
<td>GPCR</td>
<td>G-protein coupled receptor</td>
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<tr>
<td>G-protein</td>
<td>Guanine nucleotide binding protein</td>
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<td>IgG</td>
<td>Immunoglobulin G</td>
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<tr>
<td>IL-6</td>
<td>Interleukin-6</td>
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<td>LPS</td>
<td>Lipopolysaccharide</td>
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<tr>
<td>MAL-2</td>
<td>Maackia amurensis lectin 2</td>
</tr>
<tr>
<td>MAPK</td>
<td>Mitogen activated protein kinase</td>
</tr>
<tr>
<td>MD-2</td>
<td>Co-receptor for TLR4</td>
</tr>
<tr>
<td>MHC II</td>
<td>Major histocompatibility complex class</td>
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<tr>
<td>MMP</td>
<td>Matrix metalloproteinase</td>
</tr>
<tr>
<td>MyD88</td>
<td>Myeloid differentiation primary response gene 88</td>
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<tr>
<td>Neu1-4</td>
<td>Neuraminidase 1-4</td>
</tr>
<tr>
<td>NFXB</td>
<td>Nuclear transcription factor kappa B</td>
</tr>
<tr>
<td>NGF</td>
<td>Nerve growth factor</td>
</tr>
<tr>
<td>PACAP</td>
<td>Pituitary adenylate cyclase-activating peptide</td>
</tr>
<tr>
<td>PAMPs</td>
<td>Pathogen associated molecular pattern</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffered saline</td>
</tr>
<tr>
<td>PMGS</td>
<td>Plasma membrane ganglioside sialidase</td>
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<td>PTx</td>
<td>Pertussis toxin</td>
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<tr>
<td>RTK</td>
<td>Receptor tyrosine kinase</td>
</tr>
<tr>
<td>SNA</td>
<td>Sambucus nigra agglutinin (lectin)</td>
</tr>
<tr>
<td>TIMPs</td>
<td>Tissue inhibitors of metalloproteinases</td>
</tr>
<tr>
<td>TIR</td>
<td>Toll-Interleukin-1 receptor</td>
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<td>TLR2-4</td>
<td>Toll-like receptors 2-4</td>
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<td>TNF-α</td>
<td>Tumor necrosis factor alpha</td>
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<td>TrkA</td>
<td>Trk tyrosine kinase receptor A</td>
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<tr>
<td>TrkB</td>
<td>Trk tyrosine kinase receptor B</td>
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<td>TS</td>
<td>Trans-sialidase</td>
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</tbody>
</table>

xv
NEUROTROPHINS AND THEIR RECEPTORS

Neurotrophins are a highly homologous family of factors that are critical for the development and functioning of the nervous system. They elicit various biological effects ranging from proliferation to synaptic remodeling (Segal, 2003). Neurotrophins are produced as proforms of approximately 240 amino acids that are cleaved by proconvertases and furins to yield mature forms of 120 amino acids (Seidah et al., 1996). The mature proteins form stable, non-covalent dimers and are normally expressed at very low levels during development. The first neurotrophin identified was originally designated “the nerve growth factor” (NGF) and eventually the brain-derived neurotrophic factor was isolated from the brain (BDNF). In addition to NGF and BDNF, two more neurotrophin genes have been identified in mammals, neurotrophin-3 (NT-3) and neurotrophin-4/5 (NT-4/5) (Bibel and Barde, 2000). Different neurotrophins show binding specificities for certain receptors called the Trk receptors.
THE TRK RECEPTOR TYROSINE KINASE

Trk receptors belong to the family of receptor tyrosine kinases (RTKs). Receptor Tyrosine Kinases are the fundamental components of cell signaling networks and are high affinity receptors for various hormones, growth factors and cytokines. In addition to playing crucial roles in normal physiological processes, such as embryogenesis, cell proliferation and cell death (apoptosis), they are also involved in the development and progression of many types of cancer (Zwick et al., 2001). Epidermal growth factor receptors (EGFRs), insulin receptors and Trk receptors are some of the classes of RTKs. As mentioned above, the neuronal growth factors exhibit binding specificities for particular Trk receptors-(i) NGF preferentially binds to the TrkA receptors, (ii) BDNF and neurotrophin-4/5 bind to TrkB and (iii) neurotrophin-3 that binds to TrkC receptors (Figure 1.1). These interactions have been generally considered to be of high affinity but in reality NGF binding to TrkA, and of BDNF binding to TrkB is of intermediate affinity (Dechant et al., 1993; Mahadeo et al., 1994; Schropel et al., 1994) but it can be regulated by its association with p75NTR receptor (Arevalo et al., 2000; Esposito et al., 2001). The mature neurotrophin co-receptors consist of the Trk receptor tyrosine kinases and the p75NTR, wherein Trk transduces survival and differentiative signaling and p75NTR modulates the selectivity and affinity of Trk receptor activation. Two mechanisms for potential interactions between Trk receptors and p75NTR have been reported (Chao and Hempstead, 1995). The conformational model predicts that p75NTR changes the
conformation of the Trk receptor thus exposing more of the high-affinity binding sites. The ligand-presentation model assumes that p75NTR binds to the neurotrophins first and either increases the local concentration of the ligand, or transfers the neurotrophin to the Trk receptor. In the presence of p75NTR, NT-3 is much less effective in the activation of TrkA, and NT-3 and NT-4/5 are less potent in activating TrkB. Thus p75NTR enhances the specificity of TrkA receptors to their primary ligands. Although p75NTR and Trk receptors are co-expressed in many cells, independent expression of p75NTR and of individual members of the Trk family and their isoforms is also observed (Barbacid, 1994).
Figure 1.1. Neurotrophin binding affinities. Neurotrophin binding results in dimerization of each receptor. Neurotrophins bind selectively to specific Trk receptors, whereas all neurotrophins bind to p75. Trk receptors contain extracellular immunoglobulin G (IgG) domains for ligand binding and a catalytic tyrosine kinase sequence in the intracellular domain. Figure adapted from Chao MV, 2003.
Trk receptors are distributed on the plasma membrane of cells with the N-terminus located extracellularly. The domain organization of the extracellular portion of Trk receptors has been proposed based on the sequence information (Schneider and Schweiger, 1991). According to this proposal, the extracellular portion of the Trk receptors features five subdomains. Near the N-terminus there is a leucine-rich motif also known as D2 flanked by two cysteine-rich clusters, Cys-1 and Cys-2 also known as D1 and D3 respectively. Closer to the transmembrane region are two immunoglobulin-like subdomains termed Ig-C1 (D4) and Ig-C2 (D5). Neurotrophins transduce trophic signals by binding the extracellular domain of Trk and stabilizing receptor homodimerization (Mischel et al., 2002). It is postulated that a conformational change is induced upon ligand binding, which then activates the intrinsic intracellular tyrosine kinase catalytic activity (Miller and Kaplan, 2001). Analysis of a series of Trk receptor domain deletions and chimeras between the different members of the Trk family has shown that the second immunoglobulin-like domain of TrkC and TrkB confers specificity and affinity to their respective neurotrophins (Urfer et al., 1994; Wiesmann et al., 1999). The crystal structure of the complex between NGF and TrkA reveals the orientation of NGF in the signaling complex and elucidates the structural basis for binding in the Trk family (Wiesmann et al., 2000). The site of NGF binding has been located to the fifth extracellular domain (D5 domain) of the TrkA receptor with this region regulating both the specificity and affinity.
of the receptor. The leucine rich repeats have also been implicated to bind neurotrophins (Windisch et al., 1995a; Windisch et al., 1995b). Most of the NT-3 binding site on the TrkC receptor is contributed by residues in a loop connecting β-strands E and F at the membrane proximal portion of the binding domain. In contrast, while this same loop is important for TrkA binding of NGF, numerous residues outside the loop also contribute to binding suggesting a mechanism of specificity discrimination.

Both the intracellular and extracellular domains of p75 receptors display structural and sequence similarities to the tumor necrosis factor (TNF) family of receptors which also include Fas, CD40, CD30 and CD27 (Chao, 1994; Boldin et al., 1995). These receptors have four cysteine-rich ligand binding domains, a single transmembrane sequence and a noncatalytic cytoplasmic domain.

Activation of Trk receptors is typically initiated by ligand binding and this interaction induce activation, dimerization and internalization of the receptors. The receptor dimers phosphorylate each other in trans on tyrosine residues, thus resulting in the regulation of the catalytic and signaling activities of Trk-ligand complexes. The initial step of Trk signaling is the phosphorylation of tyrosine residues 674 and 675 at the catalytic activation loop of the receptor (Watson et al., 1999a). This leads to the phosphorylation of Tyr 490 that serves as a recognition site for the signaling protein Shc that binds to a Grb2-Sos complex. Sos is the nucleotide exchange factor that activates Ras by replacing
GDP for GTP, thus activating the serine-threonine kinase Raf that leads to the sequential activation of MEK/MAP. MAP kinase translocates to the nucleus and leads to the activation of various transcription factors resulting in neuronal differentiation. Two more effectors of TrkA activation are phospholipase C-gamma 1 (PLCγ1) and phosphoinositide-3 (PI-3) kinase, whose activation convey signals through PDK1 to AKT 1/2 and activates the phosphorylation of BAD. The phosphorylation of BAD upregulates antiapoptotic factors and prevents apoptosis and activation of NF-κB. NF-κB activation promotes survival of neuronal cells (Kaplan and Miller, 1997; Datta et al., 1997; Liu and Meakin, 2002).

In the absence of Trk receptors, the p75NTR receptors are capable of independent signaling that activates NF-κB or JNK activity (Hempstead, 2002; Roux and Barker, 2002). In contrast to Trk receptors which have a well-defined trophic role, p75NTR receptors have activities ranging from trophism to programmed cell death (Blochl and Blochl, 2007). Trophic and apoptotic activities of p75NTR are, of course, integrated with that of other receptors and mechanisms. Synergistic and antagonistic interactions of p75NTR and Trk receptors such as complex formation, mutual inhibition, or negative control of Trk effects by p75NTR have been investigated extensively (Dechant, 2001; Huang and Reichardt, 2003; Teng and Hempstead, 2004).
TRK RECEPTOR GLYCOSYLATION

The characteristic features of the Trk receptors also include a ligand binding domain rich in N-glycosylation sites. Sequence analysis revealed 11 to 15 potential N-glycosylation sites within various members of Trk receptors. Microscale deglycosylation assays showed that both the conserved and variable N-glycosylation sites on the NGF binding TrkA receptor played an important role in localizing the receptor to the cell surface where it can trigger the Ras/Raf/MAP kinase pathway (Watson et al., 1999b). Unglycosylated forms of TrkA are trapped intracellularly and are unable to activate kinases in the Ras-MAP kinase pathway. Studies have shown that ganglioside GM1, an endogenous regulator of Trk forms a complex with the Trk which is vital for the differentiation and normal development of neuronal cells. Unglycosylated Trk protein neither co-localizes nor associates with GM1 and this leads to unresponsiveness to NGF resulting in a non-functional receptor (Mutoh et al., 2000). However, glycosylation of TrkA receptors is also suggested to prevent autophosphorylation and function (Watson et al., 1999b). At present the precise role of glycosylation in Trk receptor activation has not been fully identified.

The common held theory is that when NGF binds with its receptor TrkA on a neuron, the retrograde survival signals involve the ligand–receptor complexes to be internalized into vesicles (Bhattacharyya et al., 1997; Grimes et al., 1996, 1997), and retrogradely
transported to the cell bodies providing survival signals to the neuron (Bhattacharyya et al., 1997; Riccio et al., 1997; Senger and Campenot 1997; Tsui-Pierchala and Ginty 1999; Watson et al., 1999a, 1999b; MacInnis and Campenot, 2002). Other studies show that neuronal survival signals can reach the cell bodies unaccompanied by the NGF that initiated it (MacInnis and Campenot, 2002; Senger and Campenot, 1997) and these retrograde survival signals do not depend on internalization and transport of NGF. These data led to the proposal that binding of NGF to TrkA receptors in nerve terminals results in the rapid propagation of a NGF-independent “wave” of TrkA receptor activation (MacInnis and Campenot 2002; Miller and Kaplan 2002; Campenot and MacInnis 2004). Based on these findings, the mechanism of NGF-independent TrkA receptor activation is unclear. Insight to the mechanism of Trk receptor activation came from the well-characterized model of TrkA family of RTK receptors used in our studies (Woronowicz et al., 2004; Woronowicz et al., 2007), demonstrating the important role of glycosylation in the activation of TrkA receptors. These findings not only indicated a novel mechanism for the control of Trk receptor signaling but also afforded an insight as to a possible function for the precise glycosylation of receptors and the role these sugars may play in receptor activation. Five important findings are presented in the studies and they substantiate the overall hypothesis for the present research proposal.

1. *T. cruzi* trans-sialidase (TS) treatment of TrkA-expressing PC12 cells led to TrkA activation sufficient to promote cell differentiation (neurite outgrowth) and this was independent of nerve growth factor (Woronowicz et al., 2004).
2. Discovered a membrane sialidase-controlling mechanism that depends on ligand binding to its receptor to induce sialidase activity which targets and desialylates the receptor and, consequently, induces receptor dimerization and activation (Woronowicz et al., 2007).

3. Identified a specific sialyl α-2,3-linked β-galactosyl sugar residue of TrkA tyrosine kinase receptor, which is rapidly targeted and hydrolyzed by the membrane sialidase induced by NGF binding TrkA (Woronowicz et al., 2007).

4. Sialidase activity induced by nerve growth factor (NGF) in TrkA-PC12 cells and by brain-derived neurotrophic factor (BDNF) in primary cortical neurons is blocked by the neuraminidase inhibitor Tamiflu.

5. *T. cruzi* trans-sialidase (TS) is capable of mimicking the activity of NGF and BDNF in mediating cell differentiation and neuronal cell survival responses.

These findings establish a novel mode of regulation of receptor activation by its natural ligand and defined a new function for cellular sialidases (Fig. 1.2).
Figure 1.2. Experimental model of sialidase-mediated Trk tyrosine kinase receptor activation. When NGF binds to TrkA receptors, there is an induction of an unknown cellular sialidase that targets and hydrolyzes α-2,3 sialic acids from the receptor, thus removing the steric hinderance and reshaping the receptors for dimerization and further activation. Woronowicz et al., 2004 has previously shown that Trypanosoma cruzi trans-sialidase and streptococcal α-2,3-neuraminidase induces Trk activation in the absence of nerve growth factor.
\[ T. cruzi \alpha-2,3\text{-trans-sialidase} \ (Woronowicz \ et \ al, \ 2004) \]

\[ S. pneumoniae \alpha-2,3\text{-neuraminidase} \ (Woronowicz \ et \ al, \ 2004) \]
SIALIC ACIDS AND SIALIDASES

Sialic Acids

Sialic acids are N- or O- substituted derivatives of the nine-carbon sugar neuraminic acid. These hydrophilic, electronegatively charged monosaccharides are widely distributed in the animal tissues, typically occupying the non-reductive terminal of the oligosaccharide chains that are linked to glycolipids and glycoproteins (Schauer, 1982). They are found to a lesser extent in other species ranging from bacteria and yeasts to fungi and plants. Sialic acids are implicated in a variety of important biological events including cell differentiation and embryogenesis and have the potential to inhibit many intercellular and intermolecular interactions because of their terminal location and negative charge (Fenderson BA et al., 1990; Feize T., 1991). They form α-2-3- and α-2-6- linkages to galactose and N-acetyl-galactosamine. In addition, they are also capable of forming homopolymers with α-2-8- or α-2-9-linkages called polysialic acids. Sialic acids also serve as surface components of certain pathogenic microorganisms, where the sialic acid functions as an anti-recognition molecule by allowing the sialylated microbe to masquerade as “self”. This modification helps the pathogen to evade the host immune mechanism that would otherwise rapidly clear the unsialylated strain. T. cruzi, expresses a trans-sialidase (TS) which transfers sialic acids from host glycoconjugates to β-galactose present in parasite mucins. This TS-mediated surface sialylation is necessary for the trypomastigote form of the parasite to attach to and enter the host cells (Burleigh and
Andrews, 1995). Sialylation of cell surface glycoproteins play a significant role in cell activation, which is likely mediated by cellular sialidase activity.

*Sialidases*

Cellular sialylation is metabolically regulated by sialidases. Sialidases are hydrolytic enzymes that influence cellular activity by cleaving the terminal sialic acids from glycoprotein and glycolipid conjugates. The four genetically characterized mammalian sialidases are Neu1, Neu2, Neu3 and Neu4 and they are distinct from each other based on their cellular localization. In addition to subcellular localization, these sialidases also differ in substrate specificites and required pH for optimum activity. All the four sialidases share conserved sequences with their viral and bacterial counterparts. They all have highly conserved active site residues, the F/YRIV/P motif in the N-terminal part and the Asp boxes (consensus S/ TXD(X)GXTW/F present as three to five repeats in the protein) (Roggentin et al., 1993).

Neu1, predominantly found in the lysosome has a catabolic activity of desialylating glycoproteins present in lysosomes. In the lysosome, Neu1 exists as a component of a multimeric complex consisting of the carboxypeptidase protective protein cathepsin A, β-galactosidase and N-acetyl-galactosamine-6-sulfate sulfatase (Pshezhetsky and Ashmarina, 2001). Recently, it has been shown that Neu1 is also found in extralysosomal locations such as the periphery of activated lymphocytes (Lukong et al., 2001). Elastin
binding protein that plays a major role in elastic fibre deposition, has been also been reported to form a complex with Neu1/cathepsin A on the cell surface (Hinek et al., 2006). More recent data further show that Neu1 is up-regulated during monocyte differentiation, and is trafficked to the membrane via MHC II-positive vesicles. Furthermore, recent data reveal that cell-surface Neu1 activates phagocytosis in macrophages and dendritic cells through desialylation (Seyrantepe et al., 2010). Reports also exist that myeloid differentiation is also regulated by the induction of cell-surface Neu1 (Ghadoum and Sackstein, 2008). Inherited mutations in NEU1 gene result in the autosomal recessive disorder sialidosis, which is characterized by the progressive lysosomal storage of sialylate glycopeptides and oligosaccharides (Seyrantepe et al., 2003).

Cytosolic Neu2 is usually expressed in low levels in cells, but highly expressed in skeletal muscle (Sato and Miyagi, 1996), the liver (Miyagi and Tsuiki, 1985), and the thymus (Kotani et al., 2001), as well as playing potential role in myoblast differentiation (Fanzani et al., 2008). The crystal structure of human Neu2 in its free form as well as in complex with the neuraminidase inhibitor 2-deoxy-2, 3-dehydro-N-acetylneuraminic acid (DANA) has been resolved by Chavas et al.

Neu3 is also referred to as plasma membrane ganglioside sialidase (PMGS) and is a key enzyme for ganglioside degradation and preferentially targets GM1 gangliosides. The
over-expression of Neu3 in mice has been implicated in the development of severe insulin-resistant diabetes, while more recent reports have shown that Neu3 may be an important regulator of insulin sensitivity and glucose tolerance (Yoshizumi et al., 2007). Neu3 is also upregulated in human cancers resulting in the suppression of apoptosis, thus serving as an essential enzyme in cancer cell survival (Wada et al., 2007).

Neu4 is a lysosomal lumen sialidase as well as a mitochondrial sialidase encoded by the NEU4 gene on human chromosome-2. With regard to the human ortholog, NEU4, it appears to consist of two isoforms differing in their possession of 12 N-terminal amino acid residues within the mitochondria. These Neu4 isoforms are also differentially expressed in a tissue-specific manner where in the brain, the muscle and the kidney they contain both the long and short forms, while in the liver and the colon they are predominantly the short form as revealed by RT-PCR. Both isoforms of Neu4 possess broad substrate specificity, including activity towards mucin. In addition, Neu4 may be involved in cell apoptosis or neural differentiation. Neu4 is also highly expressed in the mucosal surfaces of colon and this expression was markedly reduced in colon cancer, suggesting a role for Neu4 in the maintenance of normal colon mucosa (Yamanami et al., 2007). Recent studies suggest that mouse Neu4 plays an important regulatory role in neurite formation, possibly through desialylation of glycoproteins (Shiozaki et al., 2009).
RATIONALE AND HYPOTHESIS

Recent published reports from our laboratory have provided evidence that Neu1 sialidase may be an important intermediate link in the initial process of ligand induced Toll-like receptor (TLR) activation and subsequent cell function (Amith et al., 2009). An initial rapid activation of Neu1 activity is only induced by TLR ligand binding. Activated Neu1 targets the α-2,3 sialyl sugars linked to the β-galactosyl residues on the TLR ectodomains to generate a functional receptor. The removal of these sugars facilitates TLR dimerization, MyD88/TLR formation and NFκB activation which is indicative of TLR activation (Amith et al., 2010). Central to this process is that Neu1 and not the other three mammalian sialidases forms a complex with TLR-2, -3 and -4 receptors in naïve macrophage cells. The findings in these reports suggest that Neu1 sialidase may be a key regulator of pathogen-molecule induced TLR activation to generate a functional receptor.

Platelet derived growth factor-BB (PDGF-BB) and insulin growth factor-2 (IGF-2) whose receptors also belong to the receptor tyrosine kinase family, are potent stimulants of cellular proliferation (Raines, 2004; Tallquist and Kazlauskas, 2004) that have been implicated in the development of hyperproliferative vascular diseases in humans (Bayes-Genis et al., 2000; Zaina and Nilsson., 2003; Raines, 2004). There is direct evidence that Neu1 desialylates both PDGF and IGF-1 receptors and diminish the intracellular signals induced by the mitogenic ligands PDGF-BB and IGF-2 (Hinek et al., 2008).
Recent studies have also revealed that the desialylation of insulin receptors and insulin growth factor receptors by Neu1 neuraminidase has an effect on the proliferative response of skeletal myoblasts to insulin (Arabkhari et al., 2010). They led us to postulate that perhaps the Trk tyrosine kinase receptors maybe also under the regulatory control of Neu1 sialidase.

For the nerve growth factor receptor TrkA, glycosylation is required to localize the receptor to the cell surface but glycosylation is suggested to prevent autophosphorylation. At present, the precise role of glycosylation in Trk receptor activation has not been fully identified. Insight for the role of glycosylation in Trk receptor activation came from our reported findings on the model of TrkA family of tyrosine-protein kinase demonstrating the importance of the removal of sialyl α-2,3-linked β-galactosyl residues of the Trk receptor by sialidase for receptor dimerization and subsequent activation (Woronowicz et al., 2007). For TrkA receptors, dimerization is a prerequisite for the activation of the kinase. In conjunction with the dimerization and kinase activation, the receptor molecules undergo conformational changes following ligand binding, which allow a basal kinase activity to phosphorylate a critical tyrosine residue, thereby releasing the kinase and leading to full enzymatic activity directed toward other tyrosine residues in the receptor molecules as well as other substrates for the kinase. These observations led us to our proposed study to identify the sialidase induced when ligand binds to the Trk receptor.
and to elucidate the molecular mechanism(s) of the activation of the sialidase which targets the sialyl residues of glycosylated Trk receptors.

Insight for the mechanism of sialidase activation came from reports regarding the transactivation of many mitogenic growth factor receptors in response to G-protein receptor signaling. For example, receptors of epidermal growth factor and insulin-like growth factor-1 can be transactivated by ligand-induced G-protein coupled receptors (GPCRs) (Evaul and Hammes, 2008). Ligands for GPCRs are capable of activating the mitogen-activated protein (MAP) kinase signaling pathway, in addition to classic G protein-dependent signaling pathways involving adenylyl cyclase and phospholipase (Daub et al., 1996; Castellino and Chao, 1996). Induction of mitogenic receptor tyrosine kinase phosphorylation also occurs through signaling from several GPCRs (Luttrell et al., 1999). In particular, receptors for epidermal growth factor, platelet-derived growth factor, and insulin-like growth factor 1 can be transactivated by GPCRs (Linseman et al., 1995; Rao et al., 1995; Daub et al., 1996). There is evidence that activation of the Trk receptors also can occur via a GPCR mechanism independent of neurotrophins (Lee and Chao, 2001). These studies indicate that there are alternative modes of activating Trk receptor tyrosine kinases in the absence of neurotrophin binding at the cell surface and that receptor signaling may occur and persist inside of neuronal cells (Rajagopal R et al., 2004). Adenosine, a neuromodulator is known to activate Trk tyrosine kinase receptors by binding to the seven- membrane spanning adenosine receptor. Similarly, pituitary-
adenylate cyclase activating polypeptide (PACAP) is also shown to activate Trk receptors resulting in the survival of neural cells (Lee et al., 2002). Studies reveal that the glycosylated platelet-derived growth factor-β (PDGFβ) receptor is tethered to a sphingosine-1-phosphate GPCR (Alderton et al., 2001) and constitutively active LPA1 GPCR receptor enables Gβγ subunit proteins for use by the TrkA receptor (Moughal et al., 2006). Taken together, we propose that GPCRs might be involved in activation of sialidase associated with ligand stimulation on the cell membrane.

In addition, matrix metalloproteinases (MMPs) have also been implicated in the transactivation of epidermal growth factor receptor (EGFR) by GPCR agonists. There are over 25 known MMPs and most of them exhibit overlaps in substrate specificities and are regulated by tissue inhibitors of metalloproteinases (TIMPs) (Chakraborti et al., 2003). MMPs are a family of proteolytic enzymes that regulate cell-matrix composition and have various substrate specificities (including collagenases, gelatinases and elastases) and function in normal and pathological processes, including multiple roles in the normal immune response to infection (Chakraborti et al., 2003; Elkington et al., 2005). Preliminary data revealed that the sialidase being induced upon ligand binding to Trk-expressing live cells is the Neu1 sialidase on the cell surface. Central to this process is that Neu1 exists as a complex with cathepsin A and elastin binding protein (EBP) forming the elastin receptor complex on the cell surface. The catalytic activity of Neu1 facilitates elastic fibre assembly (Hinek et al., 2006), although the exact molecular
mechanism is unknown. We propose that ligand-induced Neu1 sialidase activation requires EBP to be cleaved from the Neu1/cathepsin A/EBP complex. This removal of EBP is likely mediated by an enzyme with elastase substrate specificity, like an MMP. Evidence exists that NGF-induced activation of the TrkA receptor induces MMP-9 expression in both primary cultured rat aortic smooth muscle cells and in a smooth muscle cell line genetically manipulated to express TrkA (Khan et al., 2002). The response to NGF was specific for MMP-9 expression, as the expression of MMP-2, MMP-3, or the tissue inhibitor of metalloproteinase-2 was not changed. MMP-9 is also involved in controlling Schwann cell proliferation via IGF-1 and ErbB receptor-mediated activation (Chattopadhyay and Shubayev, 2009). The role of MMPs is myriad from degrading components of the extracellular matrix allowing for the resolution of inflammation and wound healing, to modulating cytokine and chemokine activity (Elkington et al., 2005). Here we focus on a more upstream function for MMP, either at the cell surface or in the cytosol, in very close proximity to the membrane. Chapter 3 of this thesis outlines the proposed mechanism(s) of regulation of Neu1 sialidase activation involving GPCRs and MMPs.
OVERALL HYPOTHESIS

Neu1 sialidase is activated upon neurotrophic factors binding to Trk receptors through the catalytic activity of MMP-9 mediated by GPCR-signaling via membrane Gαi subunit.

OBJECTIVES OF THIS STUDY

Based on our hypothesis, the overall objectives of this study are:

- To identify the cellular sialidase involved in ligand-induced Trk activation
- To elucidate the mechanism of neurotrophic factor-induced sialidase activation on the cell surface of Trk receptors

BIOLOGICAL SIGNIFICANCE

There is evidence that neurotrophic signaling pathways play a role in regulating respiratory motor plasticity and inhibition of the Trk receptor. Trk receptor inhibition on hypoglossal motor neurons prevents long-term enhancement of genioglossus muscle tone triggered by repeated airway obstructions in rats (Tadjalli and Peever, 2010). The Trk receptors are also emerging as key players in carcinogenic progression in non-neuronal tissues. Lagadec et al showed that breast tumors present high levels of TrkA and phospho-TrkA compared to normal breast tissues. TrkA overexpression enhanced tumor
growth, angiogenesis and metastasis of xenografted breast cancer cells in immunodeficient mice (Lagadec et al., 2009). Trk receptor alterations have also been reported in Alzheimer’s disease. Truncated TrkB receptors were found in high levels in senile plaques, while the full-length receptor was expressed in glial-like cells in the hippocampus of Alzheimer's disease brains (Connor et al., 1996). We hypothesize that Neu1 sialidase is involved in the regulation of Trk receptor activation along with other key players such as MMP-9 and GPCRs and perhaps targeting any one of these vital components could be a potential therapeutic strategy for RTK-related diseases.
CHAPTER 2

NEUROTROPHIN-INDUCED Trk RECEPTOR ACTIVATION IS DEPENDENT ON NEU1 SIALIDASE

The data from this chapter were included in the following publication

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Author contributions
PJ performed all the experiments reported in this chapter. SRA helped with the isolation of primary mouse macrophages. KG helped with the flow cytometry analysis. MRS supervised the research design and writing of the paper.
Neurotrophin-induced Trk tyrosine kinase receptor activation and neuronal cell survival responses have recently been reported to be under the control of a membrane associated sialidase. Here, we uncovered another unprecedented membrane mechanism that is initiated by nerve growth factor (NGF) binding to TrkA to induce Neu1 sialidase activation within minutes in live primary neurons and TrkA- and TrkB-expressing cell lines. Central to this process is that Neu1 is in complex with TrkA on the cell surface of naïve primary neurons and TrkA-expressing cells. The neuraminidase inhibitor Tamiflu (oseltamivir phosphate) completely blocks this sialidase activity in live TrkA-PC12 cells treated with NGF with an IC$_{50}$ of 3.876 μM with subsequent inhibition of Trk activation in primary neurons and neurite outgrowth in TrkA-PC12 cells respectively. Our findings establish a novel mechanism for neurotrophic factor-induced Trk tyrosine kinase receptor activation and neuron function, which is tightly regulated by Neu1 sialidase.
CHAPTER 2

NEUROTROPHIN-INDUCED TrK RECEPTOR ACTIVATION IS DEPENDENT ON NEU1 SIALIDASE

INTRODUCTION

Neuronal survival signals can actually reach the neuron cell bodies unaccompanied by the nerve growth factor (NGF) that initiated it (Senger and Campenot, 1997; MacInnis and Campenot, 2002). They do not depend on the internalization and transport of NGF. These data led to the proposal that binding of NGF to TrkA receptors in nerve terminals results in the rapid propagation of a NGF-independent activation of TrkA receptors (MacInnis et al., 2002; Miller and Kaplan, 2002; Campenot and MacInnis, 2004). This proposal is consistent with other observations of a ligand-independent activation process for epidermal growth factor (EGF) receptors originating from EGF stimulation (Verveer et al., 2000). To date, the precise mechanism(s) of NGF-induced TrkA receptor activation still remains unclear (Miller et al., 2002).

An insight for this atypical neuronal signal by NGF came from our recent report demonstrating that NGF binding to TrkA induces a cellular sialidase(s) that specifically targets and hydrolyzes sialyl α-2-3-linked β-galactosyl residues of TrkA receptors.
(Woronowicz et al., 2007a). This desialylation process was proposed to be an initial step for receptor dimerization, internalization, and subsequent activation in Trk expressing cells and primary cortical neurons (Woronowicz et al., 2007a). It was also sufficient for the subsequent development of neurite outgrowth in these cells and cell survival responses against cell death caused by oxidative stress, hypoxia-induced neurite retraction and serum/glucose deprivation (Woronowicz et al., 2007b). Taken together, these findings predict a prerequisite desialylation of Trk receptors caused by a cellular sialidase enabling the removal of a steric hinderance to receptor association. Until now, the cellular sialidase associated with NGF treated TrkA-expressing cells and neurotrophin factor treated neurons has not been identified.

This report reveals for the first time that Neu1 forms a complex with TrkA and TrkB receptors on the cell membrane thus resulting in the rapid activation of neurotrophin-induced Neu1 sialidase activity in live Trk-expressing cells and primary neurons. According to our study, the induction of Neu1 sialidase activity appears to be a critical step in Trk receptor activation, which may provide important pioneering approaches to neurological disease intervention strategies.
MATERIALS AND METHODS

Ligands and Inhibitors

Nerve growth factor (NGF; 250 ng/mL) and brain-derived neurotrophic factor (BDNF; 200 ng/mL) (Sigma, St. Louis, MO) were used at predetermined optimal dosage. Tamiflu (99% pure oseltamivir phosphate, Hoffmann-La Roche Ltd., Mississauga, Ontario, Lot # BS00060168) was used at the indicated concentrations. Pure compounds, zanamivir and oseltamivir carboxylate were kindly provided by Prof. Mark von Itzstein, Griffith University Institute for Glycomics, Queensland). The optimal dose of 200 ng/mL of recombinant T. cruzi trans-sialidase (TS) or pure C. perfringens neuraminidase (Sigma) was used as predetermined elsewhere (Woronowicz et al., 2004).

The sialidase substrate, 2’-(4-methylumbelliferyl)-α-D-N-acetylneuraminic acid (98% pure, 4-MUNANA, Biosynth International Inc., Itasca, IL, USA) was used at optimal concentration of 0.318 mM for the live cell sialidase assay as predetermined elsewhere (Woronowicz et al., 2004).

Antibodies

Rabbit anti-human Neu1 IgG, mouse anti-human Neu2 IgG and rabbit polyclonal anti-MMP9, were all acquired from Santa Cruz Biotechnology, Santa Cruz, CA, USA. Other antibodies were mouse anti-human Neu3 IgG (Medical & Biological Laboratories Co.,
Ltd., Japan) and rabbit anti-human Neu4 polyclonal antibody (ProteinTech Group Inc., Chicago, IL, USA). AlexaFluor-labeled secondary antibodies included F(ab′)2 goat anti-rabbit AlexaFluor 488 (Molecular Probes, Eugene, OR, USA), F(ab′)2 goat anti-rabbit AlexaFluor 594 (Molecular Probes), goat anti-mouse AlexaFluor 594 (Invitrogen, Corp.) were used at predetermined optimal concentrations in these studies. Horse radish peroxidase-labeled goat anti-rabbit antibody was obtained from Santa Cruz Biotechnology.

**Cell lines**

TrkA-PC12 cell line (over expressing human TrkA receptors) (Hempstead et al., 1992), was used in these studies as previously described (Woronowicz et al., 2004). The 3T3-TrkA and 3T3-p75NTR cells are NIH-3T3 cells stably transfected to express human TrkA and p75NTR receptors (kindly provided by Dr Uri Saragovi, Lady Davis Institute for Medical Research, 3755 Côte Ste-Catherine Road, Montreal, Quebec H3T 1E2, Canada). All cell lines were grown at 37°C in 5% CO2 in culture medium containing DMEM (Gibco, Rockville, MD) supplemented with 10% horse serum (Gibco) and 5% fetal calf serum (HyClone, Logan, Utah, USA) and selection in 0.4 mg/mL G418 (for 3T3-TrkA) and 0.2 mg/mL Zeocin (Cedarlane Laboratories Limited, Hornby, Ontario L0P 1E0) (for 3T3-p75NTR)
**Primary cortical neurons**

Primary cultures of rat cortical neurons were prepared as described previously (Brewer, 1995; Brewer, 1997). GIBCO® Primary Rat Cortex Neurons (Invitrogen Canada Inc. Burlington, ON, L7L 5Z1) were isolated from day-18 Fisher 344 rat embryos and cryopreserved in liquid nitrogen. The neurons were grown on poly-D lysine coated 12mm circular glass slides in Neurobasal media supplemented with 0.5mM GLUTAMAX and 2% B27 at 37°C in a humidified atmosphere of 5% CO2. The cells were grown for 7 days before conducting an experiment. The cultures consist of 99% neurons.

**Primary mouse bone marrow macrophage cells**

Primary BM macrophages were derived from wild-type mice (WT), hypomorphic cathepsin A mice with the secondary ~90% reduction of the Neu1 activity (Neu1-CathA KD) (Seyrantepe et al., 2008b), and mice with normal Neu1sialidase bound to inactive cathepsin A Ser190Ala mutant (Neu1-CathAKI) (Seyrantepe et al., 2008b). Bone marrow (BM) cells were flushed from femurs and tibias of mice with sterile Tris-buffered saline (TBS) solution. The cell suspension was centrifuged for 3 min at 900 rpm, and the cell pellet resuspended in red cell lysis buffer for 5 min. The remaining cells were washed once with sterile TBS, and then resuspended in RPMI conditioned medium supplemented with 10% FBS and 20% (v/v) of L929 cell supernatant as a source of monocyte colony-stimulating factor (M-CSF) according to Alatery and Basta (Alatery and Basta, 2008) and 1x L-glutamine-penicillin-streptomycin (Sigma-Aldrich Canada Ltd., Oakville, Ontario)
sterile solution. The primary BM macrophages were grown on 12mm circular glass slides in RPMI conditioned medium for 7-8 days in a humidified incubator at 37°C and 5% CO₂. Primary BM macrophage cells by day 7 are more than 95% positive for macrophage marker F4/80 molecule as detected by flow cytometry.

**Mouse Models**

Wild-type (WT) and Neu4 KO (Neu4 knockout) (Seyrantepe et al., 2008a) were obtained from Dr. Alexey Pshezhetsky’s laboratory. The Neu1-CathA KD (Neu1 deficient and cathepsin A deficient) mice have a hypomorphic cathepsin A phenotype with a secondary ~90% reduction of the Neu1 activity (Seyrantepe et al., 2008b) and CathA KI (normal Neu1 sialidase bound to inactive cathepsin A Ser190Ala mutant) mice (Seyrantepe et al., 2008b).

**Sialidase activity in viable cells**

Primary bone marrow macrophages and Trk-expressing cells were grown on 12 mm circular glass slides in conditioned medium as described above. After removing medium, 0.318 mM 4-MUNANA (4-MU) substrate [2’-(4-methylumbelliferyl)-α-D-N-acetyleneuraminic acid] (Biosynth International Inc., Itasca, IL, USA) in Tris buffered saline pH 7.4 was added to each well alone (control), with predetermined dose of NGF, BDNF, GM1, asialo-GM1 or in combination of neurotrophin and indicated inhibitors. The substrate is hydrolyzed by sialidase to give free 4-methylumbelliferone which has a
fluorescence emission at 450 nm (blue color) following an excitation at 365 nm. Fluorescent images were taken after 1-2 minutes using epi-fluorescent microscopy (40x objective).

**Immunocytochemistry of pTrkA or pTrkB**

Trk-expressing cells were pretreated with pure Tamiflu, anti-Neu1 at indicated concentrations for 30 mins followed with NGF for 15 min. Primary BM macrophages were treated with either NGF, *T. cruzi* trans-sialidase or neuraminidase (*C. perfringens*) for 30 min. Primary cortical neurons were pretreated with anti-Neu1 for 30 mins followed with BDNF for 15 min. Cells were fixed with 4% paraformaldehyde, permeabilized with Triton-X100 and immunostained with rabbit anti-pTyr490 of Trk as indicated followed simultaneously with Alexa Fluor 568 anti-rabbit IgG (Molecular Probes, Inc., Eugene, OR). Stained cells were visualized by epi-fluorescence microscopy using a 40x objective. Quantitative analysis was done by assessing the density of cultured cell staining corrected for background in each panel using Corel Photo Paint 8.0 software. Each bar in the figures represents the mean corrected density of staining ± SEM for cultured cells (n) within the respective images. The cultures in each panel are of equal cell density. *P* values represent significant differences at 95% confidence using Dunnett’s Multiple Comparison Test compared to control (Ctrl) in each group.
Flow cytometry of cell surface Neu1 in live TrkA-PC12 cells

Cells were grown in 25-cm² flasks at 90% confluence. For cell surface staining, live cells in serum free cold phosphate-buffered saline (PBS) were stained with rabbit anti-Neu1 antibody (H-300, Santa Cruz Biotechnology, Inc. California, US) for 15–20min at 4°C, washed and followed with Alexa Fluor488 F(ab’')2 goat anti-rabbit IgG. After washing with cold PBS buffer, the cells were prepared for flow cytometry analysis. 40000 cells were acquired on a Beckman Coulter (Miami, FL) Epics XL-MCL flow cytometer and analyzed with Expo32 ADC software (Beckman Coulter). For overlay histograms, control cells treated with Alexa Fluor488 F(ab’')2 goat anti-rabbit IgG alone are represented by the unfilled histogram with the dashed line. Cells treated with anti-Neu1 antibody together with Alexa Fluor488 F(ab’')2 secondary antibody are depicted by the unfilled histogram with the black line. Auto-fluorescence of untreated cells is depicted by gray-filled histogram. The mean fluorescence for each histogram is indicated for 80% gated cells.

Confocal Colocalization

TrkA-PC12 cells and primary cortical neurons cells were cultured on circular glass slides in 24 well tissue culture plates as described above. TrkA-PC12 cells or primary neurons were stimulated with 250 ng/mL NGF or 200 ng/mL BDNF, respectively for 15 min or left untreated as no ligand controls. Cells were fixed, permeabilized, and immunostained with anti-Neu1, anti-pan TrkA or anti-pan TrkB as indicated followed with Alexa
Fluor594 goat anti-rabbit IgG. Stained cells were visualized using a confocal inverted microscope (Leica TCS SP2 MP inverted Confocal Microscope) with a 100x (oil) objective. Images were captured using a z-stage of 8–10 images per cell at 0.5-mm steps.

**Western blots and co-immunoprecipitation**

TrkA-PC12 cells are left cultured in media, pretreated with 200 μM Tamiflu for 30 min followed with 250 ng/mL NGF for 15 min or in media containing 250 ng/mL NGF for 15 min. Cells are pelleted and lysed in lysis buffer (50mM Tris, pH 8, 150 mM NaCl, 1% NP-40, 0.2mg/mL leupeptin, 1% β-mercaptoethanol, and 1mM phenylmethanesulfonyl fluoride (PMSF)). Cell lysates are immunoprecipitated with 2 μg of goat anti-pan TrkA, goat anti-pTrkA, or rabbit anti-Neu1 antibodies for 18 hrs. Following immunoprecipitation, complexes are isolated using protein G magnetic beads, washed 3x in buffer (10mM Tris, pH8, 1mM EDTA, 1mM EGTA, 150mM NaCl, 1% Triton X-100 and 0.2mM sodium orthovanadate) and resolved by 8% gel electrophoresis (SDS-PAGE). Proteins are transferred to polyvinylidene fluoride (PVDF) transfer membrane blot. The blot is probed for either Neu1 (45.5 kDa) anti-Neu1 (Abcam), Neu2 (42 kDa) with anti-Neu2 (Santa Cruz Biotechnology Inc.), Neu3 (48 kDa) with anti-Neu3 (MBL Medical and Biological Laboratories Co., Ltd. Japan), Neu4 (53 kDa) with anti-Neu4 (Protein Tech Group, Inc., Chicago, USA), or TrkA with anti-pan TrkA antibodies followed by HRP conjugated secondary IgG antibodies and Western Lightning Chemiluminescence Reagent Plus. The initial IP blot is stripped and further probed for described proteins. The
chemiluminescence reaction is analyzed with either a Fluorochem HD2 Imaging System (Alpha Immunotech, San Leandro, CA, USA) or x-ray film. Sample concentration for gel loading was determined by Bradford reagent.

**Neurite outgrowth**

TrkA-PC12 cells were grown in a 24-well culture plate in DMEM media containing 5% FCS. The cells were starved for 4 hours prior to treatments. Cells were then pre-treated with 100 μg/mL of anti-Neu1 followed with 250 ng/mL NGF or are left untreated as control. The live cells were observed after 2 days using an AMG/Westover Scientific Digital USB2 Microscope and the images were recorded using the Micron Imaging Software.

**Statistics**

Comparisons between two groups were made by one-way ANOVA at 95% confidence using unpaired t-test and Bonferroni’s Multiple Comparison Test or Dunnett’s Multiple Comparison Test for comparisons among more than two groups.
RESULTS

**Neu1 sialidase activity is associated with NGF- treated TrkA-expressing cells**

To identify the mammalian cellular sialidase associated with NGF-treated TrkA-expressing cells, we used a recently developed assay to detect sialidase activity on the surface of viable cells (Woronowicz *et al.*, 2007b; Amith *et al.*, 2009). This sialidase activity is revealed in the periphery surrounding the cells using a fluorogenic sialidase specific substrate, 4-MUNANA [2’-(4-methylyumbelliferyl)-α-D-N-acetylneuraminic acid], which fluoresces at 450nm caused by the emission of 4-methylumbelliferone. Firstly, we used specific antibodies against the four mammalian sialidases (Miyagi *et al.*, 1990) known as lysosomal Neu1 (Lukong *et al.*, 2000; Feng *et al.*, 2006; Hinek *et al.*, 2006; Nan *et al.*, 2007; Duca *et al.*, 2007; Yogalingam *et al.*, 2008; Starcher *et al.*, 2008) cytosolic Neu2, the plasma membrane bound Neu3 (Rodriguez *et al.*, 2001b; Sasaki *et al.*, 2003b; Papini *et al.*, 2004) and the fourth sialidase, Neu4, localized to either the mitochondrial (Yamaguchi *et al.*, 2005) compartment or the lysosomal lumen (Seyrantepe *et al.*, 2003). As shown in **Figure 2.1**, Neu1 antibody as well as the neuraminidase inhibitor Tamiflu blocked NGF-induced sialidase activity in live TrkA-PC12 and 3T3-TrkA cells compared to the NGF positive controls. The TrkA-PC12 cell line was used because it over expresses the human TrkA receptors in the NGF responsive PC12 rat pheochromocytoma cell line (Hempstead *et al.*, 1992).
Figure 2.1. NGF induces Neu1 sialidase activity in live TrkA-PC12 and 3T3-TrkA cells. TrkA-PC12 (PC-12 Rat pheochromocytoma cells transfected with TrkA receptors)umcells and 3T3-TrkA (Mouse fibroblasts transfected with TrkA receptors) cells were allowed to adhere on poly-D lysine coated 12mm circular glass slides in media containing 5% fetal bovine serum for 24h at 37°C. After removing media, 0.318 mM 4-MUNANA (4-MU) substrate [2’-(4-methyumbelliferyl)-α-D-N-acetylneuraminic acid] (Sigma) in Tris buffered saline pH 7.4 was added to cells alone (Control) with NGF (160 ng/mL) or with NGF in combination either with 250 µg/mL Tamiflu (broad range Neuraminidase inhibitor), or 250 µg/mL Anti-Neu1 (Neutralizing antibody against Neu1 sialidase). Sialidase activity is inhibited by the neutralizing effect of Anti-Neu1 antibody indicating the activation of Neu1 sialidase upon ligand stimulation. The substrate is hydrolysed by sialidase to give free 4-methylumbelliferone which has a fluorescence emission at 450nm (blue color) following excitation at 365nm. Fluorescent images were taken at 1 min after adding substrate using epi-fluorescent microscopy (40x objective). The mean fluorescence surrounding the cells for n ≥ 50 replicates in each of the images was measured using Image J Version 1.43 Software (NIH). The data are a representation of one out of five independent experiments showing similar results.
The anti-Neu1 antibody is specific for the epitope corresponding to amino acids 116-415 mapping at the C-terminus of Neu1 of human origin. It also detects Neu1 of mouse, rat and human origin. In contrast, antibodies against the other three human sialidases had no blocking effect on NGF-induced sialidase activity in TrkA-PC12 cells (Figure 2.2). It has been reported that murine macrophages actually express NGF and its receptors TrkA and p75NTR (Barouch et al., 2001) as well TrkB and TrkC receptors but not BDNF, NT-3 or NT-4 (Barouch et al., 2001). To further confirm our results, we used this Neu1 deficient mouse model of TrkA expression in macrophages. We obtained primary bone marrow (BM) macrophages derived from the hypomorphic cathepsin A mice with the secondary ~90% reduction of the Neu1 activity (Neu1-CathA KD) (Seyrantepe et al., 2008b). For controls, we also used primary BM macrophages derived from wild-type (WT) mice, CathA KI mice (normal Neu1 sialidase bound to inactive cathepsin A Ser190Ala mutant) (Seyrantepe et al., 2008b) and Neu4 KO mice (Neu4 knockout) (Seyrantepe et al., 2008a). After 7 days in culture with conditioned medium containing monocyte colony stimulating factor (M-CSF) as previously described (Alatery et al., 2008), the primary macrophage cells were stimulated with NGF to induce sialidase activity. The data indicated that NGF induces a cell surface sialidase activity within 1 min in WT, CathA KI and Neu4 KO primary BM macrophages derived from these mice (Figure 2.3). Furthermore, this sialidase activity associated with NGF treated live primary macrophage cells was blocked by Tamiflu. In contrast, primary BM macrophage cells derived from the Neu1-deficient mice (Neu1-CathA KD) exhibited little NGF induced sialidase
activity. These latter data provide clear evidence that Neu1 is involved in the NGF induced sialidase activity. If neurotrophins are involved in the process of activating Neu1 sialidase, we should not expect to see this activity in ligand-treated Trk-deficient 3T3 mouse embryonic fibroblast cell line compared to 3T3 cells stably transfected with TrkA or with the p75 neurotrophin receptor (p75\textsuperscript{NTR}). The data showed this to be the case (Figure 2.4). Only neurotrophins specific for their receptors induced sialidase activity. Neurotrophins can also activate two types of cell surface receptors, Trk receptors and the shared p75\textsuperscript{NTR} (He and Garcia, 2004). Surprisingly, Neu1 sialidase activity was also associated with NGF and BDNF treated live 3T3-p75\textsuperscript{NTR} cells, and this activity was blocked by Tamiflu and anti-Neu1 antibody (Figure 2.4). Several lines of evidence have suggested that ganglioside GM1 stimulates neuronal sprouting and enhances the action of NGF, but its precise mechanism is unknown. Others have reported that GM1 directly and tightly associates with TrkA (Mutoh et al., 1995), and strongly enhances TrkA activation, neurite outgrowth and neurofilament expression in rat PC12 cells (Ferrari et al., 1995; Duchemin et al., 1998; Tatsuro Mutoh et al., 2000; Duchemin et al., 2002; Duchemin et al., 2008). If GM1 tightly binds to TrkA similarly like NGF, we would predict that it might induce sialidase activity. The data in Figure 2.4 support this prediction indicating that sialidase activity is associated with GM1 and asialo-GM1 treated live 3T3-TrkA and 3T3-p75\textsuperscript{NTR} cells.
Figure 2.2. Inhibition of sialidase activity induced by NGF in live TrkA-PC12 cells by antibody against human Neu1 sialidase. After removing medium, 0.318 mM 4-MU substrate in Tris buffered saline pH 7.4 was added to cells alone (Control), with 160 ng/mL NGF, antibody alone or with anti-Neu1 (33 μg/mL), anti-Neu2 (33 μg/mL), anti-Neu3 (33 μg/mL), or anti-Neu4 (33 μg/mL) neutralizing antibodies in combination with NGF NGF- induced sialidase activity is blocked when TrkA-PC12 cells are treated with Anti-Neu1 antibody. This effect was not observed when cells were treated Anti-Neu2, Anti-Neu3 and Anti-Neu4 antibodies suggesting that Neu1 is activated on the surface and hence neutralized by Anti-Neu1. These observations also suggest that Neu2, Neu3 and Neu4 are not activated when the cells are stimulated with NGF. Fluorescent images were taken at 1 min after adding substrate using epi-fluorescent microscopy (40x objective).
Figure 2.3. NGF-induced activation of sialidase was observed only in wild-type primary bone-marrow derived macrophages but not in Neu1-deficient macrophages. Primary bone marrow macrophage cells were derived from normal wild type (WT), Neu1- CathA KD (Neu1 and cathepsin A deficient), CathA KI (normal Neu1 bound to inactive cathepsin A) and Neu4 KO (Neu4 knockout) mice and these primary cells were grown in conditioned RPMI media supplemented with 20% (v/v) M-CSF, 10% FBS and 1x Pen/Strep/ Glut for 7 days on circular glass slides in 24 well tissue culture plates. After removing media, 0.318 mM 4-MU substrate was added to cells as in A. NGF was added in combination with 400μM Tamiflu. The data are a representation of one out of two independent experiments showing similar results.
**Primary MΦ**  |  **Phase**  |  **Control**  |  **NGF**  |  **Tamiflu + NGF**
--- | --- | --- | --- | ---
WT  |  |  |  |  |
Neu1-CathA KD  |  |  |  |  |
CathA KI  |  |  |  |  |
Neu4 KO  |  |  |  |  |

**Sialidase assay - Primary macrophages**

![Sialidase assay graph]

- Control
- NGF
- Tamiflu + NGF
Figure 2.4. Sialidase activity is associated with neurotrophin-treated live cells specific for Trk receptors. 3T3-TrkA, 3T3-p75NTR, NIH-3T3 or TrkB-nnr5 cells were allowed to adhere on 12mm circular glass slides and sialidase assay was conducted. Ganglioside GM1 and asialo-GM1 were used in addition to the neurotrophins NGF and BDNF. After removing media, 0.318mM 4-MU in Tris buffered saline pH 7.4 was added to cells alone (Control) or with the indicated ligands or in combination with ligand and Tamiflu or anti-Neu1 antibody. Fluorescent images were taken at 2 min after adding substrate using epi-fluorescent microscopy (40x objective). The data are a representation of one out of three independent experiments showing similar results.
<table>
<thead>
<tr>
<th>Phase</th>
<th>Control</th>
<th>NGF</th>
<th>BDNF</th>
<th>Tamiflu + ligand</th>
<th>Anti-Neu1 + ligand</th>
<th>GM1</th>
<th>Asialo-GM1</th>
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<tr>
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<tr>
<td>TrkB-nnr5</td>
<td>Phase</td>
<td>Control</td>
<td>BDNF</td>
<td>Tamiflu + BDNF</td>
<td></td>
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</tbody>
</table>
As seen in the figures described so far, the sialidase activity as revealed by a fluorescence (λem 450 nm) surrounding the cells was variable from being largely cell associated for NGF binding to TrkA-PC12 cells to nearly totally diffused for 3T3-TrkA cells. These observations could indicate that the relative density of TrkA expression on the cell surface might be associated with sialidase inducing its activity. We have previously reported that the fluorescence associated with ligand treated cells as seen in figures was not due to a form of secreted or shed sialidase from the cells (Woronowicz et al., 2007b; Amith et al., 2009). In addition, it is noteworthy that live cells treated with the substrate alone did not show any fluorescence surrounding the cells. Instead, they revealed an internal blue fluorescence indicating substrate internalization by the cell revealing internal sialidase activity (Figures 2.1 to 2.4). Taken together, these results suggest that the diffuse fluorescence associated with ligand-treated cells is due to an activation of a cellular sialidase on the cell surface.

**Effect of neuraminidase inhibitors on NGF-induced sialidase activity in live TrkA-PC12 cells**

Next, we tested whether neuraminidase inhibitors would inhibit sialidase activity associated with NGF-treated TrkA-PC12 cells. It was not surprising that this NGF induced sialidase activity was completely blocked by the neuraminidase inhibitor Tamiflu (pure oseltamivir phosphate) at 400 µM as shown in Figures 2.1, 2.3 and 2.4 but also in a dose-dependent range of 0.025-250 µg/mL (Figure 2.5). To further elucidate the
inhibitory capacity of Tamiflu, the 50% inhibitory concentration (IC\textsubscript{50}) of the compound was determined by plotting the decrease in sialidase activity against the log of the agent concentration. As shown in Figure 2.5, Tamiflu had an IC\textsubscript{50} of 3.876 μM which is comparable to a reported IC\textsubscript{50} of 1.175 μM for TLR4 LPS induced sialidase activity in BMC-2 macrophage cells (Amith et al., 2009). In addition, other purified neuraminidase inhibitors such as zanamivir (4-guanidino-Neu5Ac2en) and oseltamivir carboxylate had a limited inhibition of NGF-induced sialidase activity in live TrkA-PC12 cells at 1-2 mM compared to the NGF positive control (Figure 2.6). Other studies using recombinant soluble human sialidases have shown that oseltamivir carboxylate scarcely inhibited the activities of the four human sialidases even at 1 mM (Hata et al., 2008), while zanamivir significantly inhibited the human Neu2 and Neu3 sialidases in the micromolar range. Furthermore, Nan et al using lysates from mature dendritic cells have found that zanamivir completely inhibited Neu1 and Neu3 sialidase activity at 2 mM (Nan et al., 2007).
Figure 2.5. Neuraminidase inhibitor Tamiflu inhibits sialidase activity in a dose dependent manner. TrkA-PC12 cells were treated with different concentrations of the broadrange neuraminidase inhibitor Tamiflu along with 125 ng/mL NGF. 2mM of 4-MUNANA substrate was added and images were taken at 1 min after adding the substrate using epi-fluorescent microscopy (40x objective). The mean fluorescence surrounding the cells for each group was measured using the Image J software. The 50% inhibitory concentration was determined by plotting the decrease in sialidase activity against the log of the inhibitor Tamiflu concentration.
Tamiflu (IC$_{50}$ = 3.876μM)

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<td><strong>Microscopy</strong></td>
<td>![Control]</td>
<td>![NGF]</td>
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<td>![Tamiflu (2.5 μg/ml) + NGF]</td>
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<td>![Tamiflu (25 ng/ml) + NGF]</td>
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</table>

**Graph:**

Tamiflu (IC$_{50}$ = 3.876μM)

- **Mean Fluorescence**
- **Inhibitor (log$_{10}$ μg/mL)**
Figure 2.6. Tamiflu, (Oseltamivir phosphate) but not other neuraminidase inhibitors block NGF-induced sialidase activity in TrkA-PC12 cells. NGF-induced sialidase activity in live TrkA-PC12 cells was measured after adding 0.318 mM 4-MU substrate together with NGF (160 ng/mL) and 50 µg/mL of neuraminidase inhibitors (O. phosphate, oseltamivir phosphate; O. carboxylate, oseltamivir carboxylate; zanamivir) using epi-fluorescent microscopy (40x objective). The mean fluorescence surrounding the cells for each of the images was measured using Image J Software. The data are a representation of one out of three independent experiments showing similar results.
<table>
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<tr>
<th>Phase</th>
<th>Control</th>
<th>NGF</th>
<th>O. Phosphate + NGF</th>
<th>O. Carboxylate + NGF</th>
<th>Zanamivir + NGF</th>
</tr>
</thead>
<tbody>
<tr>
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</tbody>
</table>

![Bar Graph](image)

Mean Fluorescence

- Control
- NGF
- O. Phosphate
- O. Carboxylate
- Zanamivir


**Neu1 sialidase activity is associated with NGF-induced phosphorylation of TrkA and neuritogenesis**

If NGF-induced TrkA activation is dependent on Neu1 sialidase activity, then a neuraminidase inhibitor like Tamiflu should have an inhibitory effect on NGF-induced phosphorylation of TrkA (pTrkA) in TrkA-expressing cells. Immunocytochemistry analyses shown here demonstrate that Tamiflu significantly inhibited NGF-induced pTrkA in TrkA-PC12 and 3T3-TrkA cells (**Figure 2.7**) and in WT primary BM macrophages (**Figure 2.8**) compared to the NGF treated controls. These results are consistent with our previous report using flow cytometry for pTrkA expression and western blot analyses that Tamiflu significantly inhibited NGF-induced pTrkA compared to NGF treated cells (Woronowicz *et al.*, 2007b). To confirm that Neu1 is associated with NGF induced pTrkA, we used neutralizing antibodies against Neu1 and the plasma membrane bound Neu3 (Rodriguez *et al.*, 2001b; Sasaki *et al.*, 2003a; Papini *et al.*, 2004). As shown in **Figure 2.7**, anti-Neu1 antibody blocked NGF-induced pTrkA in TrkA-PC12 and 3T3-TrkA cells compared with the NGF positive control. These latter data signify that Tamiflu and anti-Neu1 antibodies may have a direct effect on the Neu1 sialidase associated with NGF treated cells. These results are consistent with our other report indicating that Tamiflu is not only a potent inhibitor (IC$_{50}$ of 1.175 μM) of Neu1 sialidase associated with TLR ligand treated live macrophage cells but also significantly inhibits TLR4 ligand LPS- or TLR2 ligand zymosan-induced NFκB activation in primary
macrophage cells, LPS-induced pro-inflammatory IL-6 and TNFα cytokines, and LPS-induced nitric oxide production (Amith et al., 2009).

To confirm that Neu1 is involved, we used primary BM macrophages derived from the hypomorphic cathepsin A mice with the secondary ~90% reduction of the Neu1 activity (Neu1-CathA KD) (Seyrantepe et al., 2008b). After 7 days in culture with conditioned medium containing monocyte colony stimulating factor (M-CSF) (Alatery et al., 2008), the primary macrophage cells were stimulated with NGF to induce pTrkA. The data indicated that NGF was unable to induce pTrkA in primary BM macrophage cells derived from these mice (Figure 2.8) compared to primary BM macrophage cells derived from WT mice. These latter data provide evidence that Neu1 is involved in the NGF induced pTrkA. Previously, we have shown that T. cruzi trans-sialidase (TS) targets TrkA receptors and specifically hydrolyses sialyl α-2,3-linked β-galactosyl residues (Woronowicz et al., 2004). When Neu1 deficient (Neu1-CathA KD) primary BM macrophages were similarly treated with T. cruzi TS or C. perfringens neuraminidase, they exhibited pTrkA (Figure 2.8). Our mutant TS∆Asp98-Glu (Laroy and Contreras, 2000) has a point mutation in the catalytic domain of TS reducing the sialidase and sialyltransferase activities to approximately 3.6% and 4.5%, respectively, of the wild type TS (Woronowicz et al., 2004). It is interesting to note here that exogenous α2,3-sialyl specific T. cruzi TS but not the catalytically inactive mutant TS can override the Neu1-deficiency to activate pTrkA in primary BM macrophage cells.

Figure 2.7. NGF-induced phosphorylation of TrkA (pTrkA) is inhibited by broad range neuraminidase inhibitor Tamiflu and anti-Neu1 antibody. TrkA-PC12 and NIH-3T3 TrkA cells were allowed to adhere on 12mm circular glass slides in medium containing 5% calf sera for 24h. They were pretreated with either 400 μM Tamiflu, 100 μg/mL anti-Neu1 antibodies for 30 mins followed with 250 ng/mL NGF for 15 min or left untreated as controls. Cells were fixed with 4% paraformaldehyde, permeabilized with Triton-X100 and immunostained with goat anti-pTyr490 of Trk followed with Alexa Fluor594 rabbit anti-goat IgG. Stained cells were visualized by epi-fluorescence microscopy using a 40x objective. No ligand images had no primary and secondary antibodies with no treatment. Quantitative analysis was done by assessing the density of cell staining corrected for background in each panel image using Corel Photo Paint 8.0 software. Each bar in the figures represents the mean corrected density of culture cell staining±SEM for equal cell density (5x10^5 cells) within the respective images. The data are a representation of one out of three independent experiments showing similar results.
Figure 2.8. Purified recombinant *T. cruzi* trans-sialidase (TS) or α-2,3,6 sialyl neuraminidase (*C. perfringens*) but not the catalytically inactive mutant TSΔAsp98-Glu (mutant TS) induce pTrkA in Neu1 deficient (Neu1-CathA KD) primary BM macrophages independent of NGF. Primary bone marrow macrophages were derived from normal Wild type (WT) and Neu1-CathA Knockdown (Neu1 and Cathepsin A deficient) mice and these cells were grown in RPMI medium supplemented with 20% M-CSF, 10% FBS and 1xPen/Strep/Glut The WT BM derived macrophages were pretreated with 400 uM Tamiflu followed with 250 ng/mL NGF or treated with 250 ng/mL NGF alone for 15 min. The BM derived macrophages from Neu1- CathA Knockdown mice were treated with either 250 ng/mL NGF, 50 mU/mL α-2,3,6 sialyl neuraminidase (*C. perfringens*), 200 ng/mL recombinant *T. cruzi* trans-sialidase (TS) or 200 ng/mL mutant TSΔAsp98-Glu (Mutant TS) for 30 min or left untreated as no ligand control. Cells were fixed, permeabilized, and immunostained with anti-pTyr490 of Trk followed with Alexa Fluor594 rabbit anti-goat IgG. Stained cells were visualized by epi-fluorescence microscopy using a 40x objective. Phosphorylation of TrkA was absent in the cells derived from Neu1-CathA Knockdown mice suggesting that activation of Neu1 is required for activation of the TrkA.
We have also shown that Tamiflu inhibits neurite outgrowth in NGF-stimulated TrkA-PC12 cells and BDNF-stimulated TrkB-nnr5 cells (Woronowicz et al., 2007b). To test our hypothesis, we treated TrkA-PC12 cells with anti-Neu1 neutralizing antibodies followed with NGF stimulation, and asked whether these cells would exhibit neurite outgrowth. The data in Figure 2.9 indicate that anti-Neu1 antibody blocked NGF-induced neurite outgrowth in these live cells compared with NGF positive control. These latter data provide evidence that Neu1 is involved in NGF-induced pTrkA and subsequent neuritogenesis.

**Neu1 sialidase colocalizes with TrkA receptors on the cell surface in naïve TrkA-PC12 cells**

Since Neu1 sialidase is a lysosomal storage enzyme and it is induced within a minute on the cell surface membrane by neurotrophin ligands, we asked how Neu1 can rapidly associate with Trk receptors on the cell surface. The immunolocalization of Neu1 to the cell surface was firstly confirmed by flow cytometry using live TrkA-PC12 cells immunostained with anti-Neu1 antibody and Alexa488 conjugated secondary F(ab’)_2 antibody. The data shown in Figure 2.10A clearly indicate that 40000 acquired live cells showed significant immunostaining for Neu1 on the cell surface. Pshezhetsky and colleagues have also shown that during the differentiation of monocytes into macrophages, the majority of Neu1 relocalizes from the lysosomes to the cell surface while the other cellular sialidases Neu2, Neu3 and Neu4, whose expression either remain
unchanged or are downregulated (Feng et al., 2006). The lysosomal carboxypeptidase, cathepsin A, which forms a complex with and activates Neu1 in the lysosome, is also sorted to the plasma membrane of the differentiating cells. We have also shown that Neu1 and not Neu2, -3 and -4 forms a complex with TLR-2, -3 and -4 on the cell surface of naïve macrophage cells (Amith et al., 2009).

If Neu1 is localized to the cell surface of TrkA-expressing cells, we asked whether or not it is associated with Trk receptors since sialidase activity is associated with NGF treated live TrkA-expressing cells. Confocal microscopy validated the predicted association of Neu1 with TrkA receptors in naïve and NGF-treated fixed, permeabilized TrkA-PC12 cells (Figure 2.10B).

Co-immunoprecipitation experiments using TrkA-PC12 cells further demonstrated that Neu1 (Figure 2.11A) but not Neu2, -3 or -4 (Figure 2.11B) forms a complex with naïve or NGF-stimulated TrkA receptors. Conversely, TrkA receptors co-immunoprecipitated with Neu1 in cell lysates from naïve, NGF-treated or Tamiflu and NGF-treated TrkA-PC12 cells (Figure 2.11A). Surprisingly, Neu1 also co-immunoprecipitated with pTrkA receptors in NGF-treated TrkA-PC12 cells but not in naïve or Tamiflu treated cells (Figure 2.11A). Taken together, these results indicate that Neu1/TrkA complexes are present on the cell membrane prior to ligand binding, which has not been previously observed.
Figure 2.9. Neurite out-growth in TrkA-PC12 cells is inhibited by anti-Neu1 antibody. TrkA-PC12 cells were grown in a 24-well culture plate in DMEM media containing 5% FBS. The cells were starved for 4 hours prior to treatments. Cells were pretreated with 100 μg/mL of anti-Neu1 followed with 250 ng/mL NGF. Cells left untreated served as no ligand controls. The cells were observed after 2 days using an AMG/Westover Scientific Digital USB2 Microscope and the images were recorded using the Micron Imaging Software.
No ligand

NGF

Anti-Neu1 +NGF
Figure 2.10. (A) Flow cytometry analysis of Neu1 expression on the cell surface of live TrkA-PC12 cells. Histogram shows staining with rabbit anti-Neu1 antibodies after incubation on ice for 15 min and followed with Alexa488 conjugated F(ab’)2 secondary antibody for additional 15 min on ice. Control cells were stained with Alexa488 conjugated secondary F(ab’)2 antibody for 15 min on ice. Cells were analyzed by Beckman Coulter Epics XL-MCL flow cytometry and Expo32 ADC software (Beckman Coulter). Overlay histograms are displayed. Control Alexa488 secondary antibody treated cells are represented by the unfilled histogram with the gray line. Live cells stained with anti-Neu1 antibody are depicted by the unfilled histogram with the black line. Auto-fluorescence of the cells is depicted by the gray-filled histogram. The mean fluorescence for each histogram is indicated for 40000 acquired cells (80% gated). The data are a representation of one out of two experiments showing similar results.

(B) Neu1 colocalizes with TrkA. TrkA-PC12 cells were left untreated as no ligand control or stimulated with 250 ng/mL NGF for 15 min. Cells were fixed with 4% paraformaldehyde, permeabilized and immunostained with rabbit anti-Neu1 antibodies and FITC-conjugated goat anti-pan TrkA antibodies followed with Alexa594 anti-rabbit IgG conjugated secondary antibody. Stained cells were visualized using a confocal inverted microscope (Leica TCS SP2 MP inverted Confocal Microscope) with a 100x objective (oil). The data are a representation of one out of three independent experiments showing similar results.
A

![Graph showing relative cell numbers vs. Neu1 expression](image)

B

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</tbody>
</table>
Figure 2.11. (A) Neu1 co-immunoprecipitates with TrkA in cell lysates of both naïve and NGF-stimulated TrkA -PC12 cells. TrkA-PC12 cells were pretreated with 400 μM Tamiflu for 30 min followed with 250 ng/mL NGF for 15 min or left untreated as control (media). Cells were pelleted, lysed in lysis buffer and the cell lysates were immunoprecipitated with antibodies against the indicated proteins for 18 hrs. Immunocomplexes were isolated using protein G magnetic beads, resolved by SDS-PAGE and the blot probed with antibodies against the indicated proteins. The data are a representation of one out of two independent experiments showing similar results. (B) Neu2, 3 and 4 do not co-immunoprecipitate with TrkA. TrkA-PC12 cells were used as described in (A).
**A**

- IP: anti-TrkA
  - Blot: anti-Neu1
  - MW kDa: 46
  - media: [image]
  - NGF: [image]
  - NGF + Tamiflu: [image]
  - Neu1 (45.5 kDa)

- IP: anti-Neu1
  - Blot: anti-TrkA
  - MW kDa: 170
  - media: [image]
  - NGF: [image]
  - Neu1 (45.5 kDa)

- IP: anti-pTrkA
  - Blot: anti-Neu1
  - MW kDa: 46
  - media: [image]
  - NGF: [image]
  - Neu1 (45.5 kDa)

- IP: anti-TrkA
  - Blot: anti-TrkA
  - MW kDa: 170
  - media: [image]
  - NGF: [image]
  - TrkA (140 kDa)

**B**

- IP: anti-TrkA
  - Blot: anti-Neu2
  - MW kDa: 46
  - media: [image]
  - NGF: [image]

- IP: anti-TrkA
  - Blot: anti-Neu3
  - MW kDa: 46
  - media: [image]
  - NGF: [image]

- IP: anti-TrkA
  - Blot: anti-Neu4
  - MW kDa: 46
  - media: [image]
  - NGF: [image]
**Neu1 sialidase is also involved in BDNF-induced TrkB receptor activation and colocalizes with TrkB receptors on the surface of primary cortical neurons**

To validate our hypothesis, we also used primary rat cortical neurons. They were grown on poly-D lysine coated 12mm circular glass slides in neurobasal media supplemented with 0.5mM glutamax and 2% B27 at 37°C in a humidified atmosphere of 5% CO2. After 7 days, the cells were treated with the anti-Neu1 antibodies followed by stimulation with brain derived neurotrophic factor (BDNF). The anti-Neu1 antibody is a rabbit polyclonal antibody against amino acids 116-415 mapping at the C-terminus of Neu1 human origin. This antibody detects Neu1 of mouse, rat and human origin by Western blotting. The data shown in Figure 2.12 confirms the results for TrkA-expressing cell lines showing that antibody against Neu1 completely blocked BDNF-induced Neu1 sialidase activity (Figure 2.12A) in primary cortical neurons as well as significantly inhibiting BDNF-induced pTrkB in these neurons (Figure 2.12B).

Confocal microscopy also revealed the cell-surface colocalization of Neu1 and MMP-9 with TrkB in naïve and BDNF-treated primary cortical neurons as shown in Figure 2.13. These findings suggest that Neu1 may be a common requisite intermediate in regulating neurotrophin- induced Trk receptors.
Figure 2.12. (A) BDNF-induced sialidase activity in primary rat cortical neurons is blocked by anti-Neu1. GIBCO® primary rat cortex neurons were grown on poly-D lysine coated 12mm circular glass slides in Neurobasal media supplemented with 0.5 mM GLUTAMAX and 2% B27 at 37°C in a humidified atmosphere of 5% CO2. The cells were grown for 7 days before conducting an experiment. The cultures consist of 99% neurons. After removing media, 2 mM 4-MUNANA substrate [2’-(4-methlyumbelliferyl)-α-D-N-acetylneuraminic acid] in Tris buffered saline pH 7.4 was added to cells alone (Control), with 200 ng/mL BDNF alone or with BDNF in combination with 100 ug/mL anti-Neu1 antibody. The substrate is hydrolysed by sialidase to give free 4-methylumbelliferone which has a fluorescence emission at 450nm (blue color) following excitation at 365nm. Fluorescent images were taken at 2 min after adding substrate using epi-fluorescent microscopy (40x objective). The mean fluorescence surrounding the cells for n ≥ 50 replicates in each of the images was measured using Image J Version 1.43 Software (NIH). The data are a representation of one out of five independent experiments showing similar results. (B) BDNF-induced pTrkB in primary rat cortical neurons is blocked by anti-Neu1 antibody. Primary neurons were pretreated with anti-Neu1 for 30 min followed with 200 ng/mL BDNF for 15 min. Cells were fixed, permeabilized, and immunostained with FITC conjugated goat anti-Tyr490 of Trk. Stained cells were visualized by epi-fluorescence microscopy using a 40x objective. Quantitative analysis was done by assessing the density of cell staining corrected for background in each panel image using Corel Photo Paint 8.0 software.
Figure 2.13. Neu1 colocalizes with TrkB in naïve and BDNF-stimulated primary cortical neurons. Primary neurons were left untreated as no ligand control or stimulated with 200 ng/mL BDNF for 15 min. The cells were fixed, permeabilized and immunostained with FITC conjugated goat anti-pan TrkB and rabbit anti-Neu1 antibodies followed by Alexa Fluor594 conjugated goat anti-rabbit IgG. Stained cells were visualized using a confocal inverted microscope (Leica TCS SP2 MP inverted Confocal Microscope) with a 100x objective (oil).
No ligand

BDNF

TrkB      Neu1      Merge      % Overlay

15.4%

38.3%
DISCUSSION

The molecular mechanism(s) by which Trk tyrosine kinase receptors become activated or even inhibited are not well understood. The data presented in this report provide evidence that Neu1 sialidase is an important intermediate link in the initial process of neurotrophin induced Trk activation and subsequent cell function. They indicate an initial rapid activation of Neu1 activity which is only induced by neurotrophin binding to the receptor. Central to this process is that Neu1 and not the other three mammalian sialidases forms a complex with TrkA and TrkB receptors in naïve TrkA-expressing cells or primary cortical neurons. This would actually make Neu1 complexed with Trk receptors readily available to be induced upon neurotrophin binding. Our data support this premise because the sialidase activity induced by neurotrophin treated live cells occurs within a minute. Others have shown that p75NTR binds along the homodimeric interface of NGF, which disables NGF’s symmetry-related second p75 binding site through an allosteric conformational change (He and Garcia, 2004). Surprisingly, we have shown in this report that NGF or BDNF binding to p75NTR in 3T3 cells stably expressing p75NTR induces sialidase activity which is inhibited by Tamiflu and by anti-Neu1 antibodies (Figure 2.4). It appears that Neu1 is an intermediate link in the initial process of neurotrophin induced p75NTR activation, which has not been previously observed. It is interesting to note here that oseltamivir phosphate (Tamiflu) was found to be more potent than the other neuraminidase inhibitors in inhibiting the sialidase activity associated with NGF-treated
live TrkA-PC12 cells. Tamiflu is the ethyl ester pro-drug of the anti-influenza drug oseltamivir carboxylate, which is converted to this biologically active form \textit{in vivo} (Mendel \textit{et al.}, 1998). Although Tamiflu is known to be ineffective \textit{in vitro} because its antiviral activity is achieved by its hydrolytic metabolite oseltamivir carboxylate (Shi \textit{et al.}, 2006), we have actually observed the opposite effect in our live cell assay system. The reason for this inhibitory potency of Tamiflu on Neu1 sialidase activity is unknown. The antiplatelet agent clopidogrel has been previously determined to completely inhibit the hydrolysis of oseltamivir phosphate by carboxylesterase as much as 90% (Shi \textit{et al.}, 2006). In a recent report, we had shown that the anticarboxylesterase agent clopidogrel had no effect on Tamiflu’s capacity to inhibit ligand induced sialidase activity (Amith \textit{et al.}, 2009). Together, these results suggest that Tamiflu is a potent inhibitor of the sialidase associated with ligand NGF-treated live cells. However, it may be due to a unique orientation of Neu1 with the molecular multi-enzymatic complex that contains β-galactosidase and cathepsin A (Lukong \textit{et al.}, 2000) and elastin-binding protein (EBP) (Hinek \textit{et al.}, 2006), the complex of which would be associated within the ectodomain of Trk receptors. Stomatos and colleagues have also shown that Neu1 on the cell surface is tightly associated with a subunit of cathepsin A and the resulting complex influences cell surface sialic acid in activated cells and the production of IFN\(\gamma\) (Nan \textit{et al.}, 2007). It has also been shown using Neu1-deficient mice that they produce markedly less IgE and IgG1 antibodies following immunization with protein antigens, which may be the result of their failure to produce IL-4 cytokine (Chen \textit{et al.}, 1997). Another possibility may
involve Tamiflu’s direct effect on Neu1 sialidase with specificity for sialyl α-2,3-linked β-galactosyl residues of Trk receptors. We have recently reported that Neu1 desialylation of α-2,3-sialyl residues of TLR receptors enables in removing a steric hinderance to receptor association for TOLL-like receptor (TLR) activation and cellular signaling (Amith et al., 2010). The report showed that TLR ligand-induced NFκB responses were not observed in TLR deficient HEK293 cells, but were re-established in HEK293 cells stably transfected with TLR4/MD2, and were significantly inhibited by α-2,3-sialyl specific *Maackia amurensis* (MAL-2) lectin, α-2,3-sialyl specific galectin-1 and neuraminidase inhibitor Tamiflu but not by α-2,6-sialyl specific *Sambucus nigra* lectin (SNA). Also, Tamiflu inhibited LPS-induced sialidase activity in live BMC-2 cells with an IC_{50} of 1.2μM compared to an IC_{50} of 1015μM for its hydrolytic metabolite oseltamivir carboxylate (Amith et al., 2009). Tamiflu blockage of LPS-induced Neu1 sialidase activity was not affected in BMC-2 macrophage cells pretreated with anticarboxylesterase agent, clopidogrel. In another study, Neu1 was found to negatively regulate lysosomal exocytosis in hematopoietic cells where it processes the sialic acids on the lysosomal membrane protein LAMP-1 (Yogalingam et al., 2008). On the cell surface, Seyrantepe et al. have recently shown that Neu1 can actually activate phagocytosis in macrophages and dendritic cells through the desialylation of surface receptors, including Fc receptors for immunoglobulin G (FcγR) (Seyrantepe et al., 2010).
The data presented in this report further signify an important role of Neu1 sialidase as an intermediate link in the initial process of ligand induced Trk tyrosine kinase receptor activation and subsequent cellular function. The premise is that Neu1 forms a complex with glycosylated Trk receptors within the ectodomain, which is consistent with our previous report with TLR receptors (Amith et al., 2009). Secondly, Neu1 may be a requisite intermediate in regulating Trk activation following neurotrophin binding to the receptor. Thirdly, activated Neu1 by neurotrophin binding to the receptor predicts a rapid removal of α-2,3-sialyl residues linked to β-galactosides on Trk ectodomains to generate a functional Trk receptor based on our previous report (Amith et al., 2010). Although there are four identified human sialidases classified according to their subcellular localization (Miyagi et al., 1990), the sialidases classified as cytosolic (Neu2), plasma membrane bound (Neu3) (Rodriguez et al., 2001a; Sasaki et al., 2003a; Papini et al., 2004) and Neu4 (Seyrantepe et al., 2003; Yamaguchi et al., 2005) are not involved in the sialidase activity associated with neurotrophin treated live Trk-expressing cells and primary cortical neurons. The importance of sialyl α-2,3-linked β-galactosyl residues involvement in receptor activation was further emphasized by exogenous α2,3-sialyl specific neuraminidases. T. cruzi trans-sialidase (TS) has been previously reported to be a novel ligand of nerve growth factor TrkA receptors (Woronowicz et al., 2004; Chuenkova and Pereira Perrin, 2004) and brain-derived neurotrophin factor TrkB (Woronowicz et al., 2007a). The catalytic sialidase activity of TS specific for α2,3-sialyl residues is the central process involved in the activation of these Trk receptors, and not the physical
binding of TS to these cells (Woronowicz et al., 2004). In the present studies, primary bone marrow macrophages derived from Neu1-deficient mice treated with a purified recombinant neuraminidase (C. perfringens) or TS but not the mutant TSAsp98-Glu induced pTrkA activation. These results are consistent with our previous reports (Woronowicz et al., 2004; Woronowicz et al., 2007a; Amith et al., 2010). This model corroborates the importance of sialyl α-2,3-linked β-galactosyl residues of Trk in the initial stages of neurotrophin induced receptor activation.

In conclusion, the data presented in this report suggest that at least for TrkA, TrkB and p75NTR receptors the initial mechanism for receptor activation and subsequent cell function is dependent on Neu1 sialidase activity.
POTENTIAL ROLES FOR G-PROTEIN COUPLED RECEPTORS AND MATRIX METALLPROTEINASES IN NEU1 SIALIDASE ACTIVATION

The data from this chapter were included in the following publication

Jayanth, P., Amith, S.R., Gee, K. and Szewczuk, M.R. Neu1 sialidase and matrix metalloproteinase-9 cross-talk is essential for neurotrophin activation of Trk receptors and cellular signaling. Cellular Signaling, 2010 (Accepted for publication)

Author contributions
PJ performed all the experiments reported in this chapter. KG helped with the flow cytometry analysis. MRS supervised the research design and writing of the paper.
ABSTRACT

We have shown that a membrane Neu1 sialidase is activated upon neurotrophin binding to Trk receptors. The exact mechanism of Neu1 activation which is initiated by ligand binding is not well understood. Our recent discovery that Neu1 forms a complex with TrkA and TrkB receptors provided us a clue to the mechanism of Neu1 induction. There are reports that G-protein coupled receptors can transactivate receptor tyrosine kinases such as epidermal growth factor and insulin-like growth factor-1 receptors. Matrix metalloproteinases (MMPs) have also been implicated in the transactivation of epidermal growth factor receptors (EGFRs) by G-protein coupled receptor (GPCR) agonists. Our data show that pertussis toxin Ptx (inhibitor of GPCR Gαi proteins) and Galardin (GM 6001), a broad range inhibitor of MMPs completely blocks the activation of the Neu1 sialidase associated with ligand-induced Trk receptor activation. These findings identify an unprecedented membrane sialidase mechanism that is initiated by nerve growth factor (NGF) binding to TrkA to potentiate GPCR-signaling via membrane Gαi subunit proteins and matrix metalloproteinase-9 (MMP-9) activation to induce Neu1 sialidase activation within minutes in live primary neurons and TrkA- and TrkB-expressing cell lines. Central to this process is that Neu1 and MMP-9 are in complex with each other and with TrkA on the cell surface of naïve primary neurons and TrkA-expressing cells. Our findings
establish a novel mechanism for neurotrophin-induced Trk tyrosine kinase receptor activation and neuron function, which is critically dependent on Neu1 and MMP-9 cross-talk on the cell surface.
CHAPTER 3
POTENTIAL ROLES FOR G-PROTEIN COUPLED RECEPTORS AND MATRIX METALLPROTEINASES IN NEU1 SIALIDASE ACTIVATION

INTRODUCTION

G protein coupled receptors (GPCR) are the largest family of cell surface receptors and indeed of all protein families. In mammalian cells, they are activated by a multitude of ligands such as odors, pheromones and also a range of hormones and neurotransmitters. GPCRs sense molecules outside the cell and transduce signals resulting in cellular responses. Their signature motif consists of seven helices that transverse the membrane, thus dividing the proteins into extracellular, transmembrane and cytoplasmic domains. The extracellular domains of the GPCRs can be glycosylated. GPCRs are involved in many diseases and hence exhibit great pharmacological importance. Upon ligand binding, GPCRs undergo a conformational change resulting in the activation of GTP-binding G proteins (guanine nucleotide-binding proteins) and their further effect depends on the type of G protein being activated. G proteins composed of α-, β- and γ-subunits are termed heterotrimeric G proteins and they act as molecular switches by alternating from inactive GDP to active GTP upon ligand binding. In the traditional view of heterotrimeric protein activation, exchange of GDP for GTP triggers the dissociation of the Gα subunit,
bound to GTP from the Gβγ dimer and the receptor. Both Gα-GTP and Gβγ can then activate different signaling cascades and effector proteins, while the receptor is able to activate the next G protein (Marinissen and Gutkind, 2001; Gurevich and Gurevich, 2008). There are four classes of Gα subunits: Gαs, Gαi, Gαq/11, and Gα12/13. They behave differently in the recognition of the effector, but share a similar mechanism of activation. As a result of cross-talk between various intracellular signaling networks, there is accumulating evidence that GPCRs are able to signal without G proteins.

The processes by which Trk-mediated cellular responses are regulated are not clearly understood. Recently, we have shown that Trk activation upon ligand binding is mediated by the rapid induction of Neu1 sialidase, which desialylates the receptor, potentially allowing for its dimerization and subsequent activation. Sialidase specific inhibitor Tamiflu (oseltamivir phosphate) and anti-Neu1 antibodies were shown to inhibit phosphorylation of Trk in both TrkA- and TrkB-expressing cells as well as primary neurons.

There is evidence that GPCRs can activate Tyrosine kinase receptors without the involvement of neurotrophins. Two GPCR ligands, adenosine and pituitary adenylate cyclase-activating polypeptide (PACAP), can activate Trk receptor activity to increase the survival of neural cells through stimulation of Akt activity (Rajagopal R et al., 2004). The exact mechanism of Trk receptor transactivation by GPCR, however, has not been
elucidated. PACAP acts through the GPCR VPAC-1 and exerts trophic effects by transactivating the RTK TrkA receptor in neuronal cells. Studies show that NF-κB activation occur through transactivation mechanisms involving VPAC-1, NGF and TrkA-associated tyrosine kinase activity in PACAP-activated monocytes (El Zein N et al., 2007).

Cross-communication between GPCRs and receptor tyrosine kinases (RTKs) is a complex process utilizing signaling molecules that are determined by cell context and types of receptors activated. Studies reveal that the differential involvement of RTKs and downstream signaling pathways activated in response to GPCR-mediated stimulation elicits a variety of cellular effects during development, proliferation, differentiation, survival, repair and synaptic transmission in the CNS (Shah and Catt, 2003). There is also mounting evidence of cross-talk between GPCRs and epidermal growth factor receptors (EGFRs) (Evaul and Hammes, 2008) and as mentioned earlier the mechanism involving this cross-talk varies depending on the GPCR and the cell type. Spingosine 1-phosphate (S1P) and lysophosphatidic acid (LPA) are bioactive lipid phosphates that bind to cell surface GPCRs and exhibit intracellular actions. There are reports that the constitutively active LPA1 GPCR receptor enables Gβγ subunit proteins for use by the TrkA receptor (Moughal et al., 2006). These Gβγ subunits were found to enhance the ability of NGF to promote TrkA signaling and subsequently regulate p42/p44 MAPK signaling pathway in PC12 cells (Moughal et al., 2004). Indeed, LPA1 GPCR was found to co-
immunoprecipitate with naïve and NGF-treated TrkA receptors in cell lysates, suggesting that LPA1 GPCR forms a complex with TrkA. In addition, their results indicated that NGF-induced stimulation of p42/p44 MAPK through the TrkA-LPA1 receptor complex appears to be dependent on Gβγ subunit proteins, while the activation of the p42/p44 MAPK cascade in response to LPA may involve predominantly Gia2 subunit protein. However, the LPA-induced stimulation of p42/p44 MAPK was also found to be independent of the TrkA receptor. For the sphingosine 1-phosphate (S1P) model, PDGF and S1P induce the recruitment of the proto-oncogenic tyrosine kinase c-Src to the PDGFh-S1P1 receptor complex (Waters et al., 2005). This led to a G protein/c-Src-dependent tyrosine phosphorylation of Gab1 and accumulation of dynamin II at the plasma membrane, a step required for endocytosis of the PDGFh receptor–GPCR complex. In addition, Rakhit et al have shown that the G protein-coupled receptor kinase 2 (GRK2) is constitutively bound with the TrkA receptor, and that NGF stimulates the pertussis toxin sensitive binding of β-arrestin I to the TrkA-GRK2 complex. Both GRK2 and β-arrestin I are involved in the clathrin-mediated endocytic signaling to p42/p44 MAPK. Taken all together, these findings provide evidence for the first time that TrkA receptors utilize a classical GPCR signaling pathway to promote differentiation of PC12 cells, and thus establishes a molecular organizational platform of a novel tyrosine kinase receptor and GPCR signal cross-talk in mammalian cells.

An important functional interaction between these lipid phosphate GPCRs and receptor tyrosine kinases has been eloquently summarized (Pyne and Pyne, 2008). They describe a
model that enables the formation of functional complexes between the S1P1 receptor and PDGFβ receptor. This molecular model may effectively re-programme PDGF from a mitogenic to a migratory stimulus. This is achieved by the integration of the PDGFβ- and GPCR-specific signals enabling a spatial regulation of a cytoplasmic retained pool of extracellular signal regulated kinase-1/2 linked to myosin light chain kinase, myosin light chain phosphorylation and migration. It is interesting to note here that the up-regulation of proMMP-9 was found to be mediated through the c-Src-dependent PDGFR/PI3K/Akt/p300 cascade in IL-1β stimulation.

In COS cells, β2-adrenergic receptor activation triggers activation of the EGFR in an intracellular, ligand-independent, process (Daaka et al., 1997; Maudsley et al., 2000) whereas the β2-adrenergic receptor activates the EGFR in an extracellular, ligand-dependent, fashion in cardiac fibroblasts by stimulating matrix metalloproteinases (MMPs) to cleave membrane-bound EGFR ligand ectodomains (Kim et al., 2002). In another example, lysophosphatidic acid trans-activates the EGFR via MMPs and ectodomain shedding in COS cells (Drube et al., 2006) but through intracellular mechanisms in adrenal glomerulosa cells (Shah et al., 2005). Based on these reports, we hypothesize a potential role of MMPs in Trk-mediated cellular regulation. MMPs are a highly conserved family of proteolytic enzymes that regulate cell matrix composition. There are around 28 known MMPs and most of them exhibit overlaps in substrate specificites and are all regulated by the secretion of specific tissue inhibitors of
metalloproteinases (TIMPs). Recently, it was shown that the elastin receptor complex was able to transduce signals through the catalytic activity of its Neu-1 subunit (Duca et al., 2007), and so we think that there might be a Neu1 connection with a MMP. In the lysosome, Neu1 sialidase is associated with a serine carboxypeptidase (protective protein cathepsin A), β-galactoside and N-acetyl-galactosamine-6-sulphate sulphatase (Feng et al., 2006). Cathepsin A is sorted to the plasma membrane of differentiating monocytes similarly to Neu1 and is required for normal Neu1 enzymatic activity. Cell surface Neu1/cathepsin A can also associate with elastin-binding protein (EBP) forming the elastin receptor complex, where catalytic activity of Neu1 facilitates elastic fibre assembly (Hinek et al., 2006). Based on these reports, we propose that when neurotrophin binds to Trk receptor, the EBP must be removed from the Neu1/cathepsin A complex in order for the Neu1 to become activated and this removal is likely catalyzed by an enzyme with elastase substrate specificity like an MMP. Reports indicated that NGF-induced activation of the TrkA receptor induces MMP-9 expression in both primary cultured rat aortic smooth muscle cells and in a smooth muscle cell line genetically manipulated to express TrkA (Khan et al., 2002). The response to NGF was specific for MMP-9 expression, as the expression of MMP-2, MMP-3, or the tissue inhibitor of metalloproteinase-2 was not changed. MMP-9 is also involved in controlling Schwann cell proliferation via IGF-1 and ErbB receptor-mediated activation (Chattopadhyay and Shubayev, 2009).
Taken all together, we propose that NGF-induced TrkA activation is tightly controlled by Neu1 sialidase in alliance with Gαi proteins and MMP-9.
MATERIALS AND METHODS

Ligands and Inhibitors

Nerve growth factor (NGF; 250 ng/mL) and brain-derived neurotrophic factor (BDNF; 200 ng/mL) (Sigma, St. Louis, MO) were used at predetermined optimal dosage. GPCR ligand Bombesin (bombesin acetate salt hydrate, Sigma Aldrich) is a neuropeptide that activates the Gαq/11 pathway in GPCR-mediated signaling.

Tamiflu (pure oseltamivir phosphate, Hoffmann-La Roche Ltd., Mississauga, Ontario, Lot # BS00060168) were used at indicated concentrations. Pertussis toxin from Bordetella pertussis (in buffered aqueous glycerol solution, Sigma Aldrich) catalyzes the ADP-ribosylation of the α subunits of the heterotrimeric guanine nucleotide regulatory proteins G1, Go, and Gt. This prevents the G protein heterotrimers from interacting with receptors, thus blocking their coupling and activation. Since the Gα subunits remain in their GDP-bound, inactive state, they are unable to inactivate adenylyl cyclase or open K+ channels. Galardin (GM 6001; molecular formula C20H28N4O4 N-[(2R) 2-(Hydroxamidocarbonylmethyl)-4-methylpentanoyl]-L-tryptophan Methylamide; Calbiochem-EMD Chemicals Inc., Darmstadt, Germany) is a potent broad-spectrum hydroxamic acid inhibitor of matrix metalloproteinases (MMPs); IC50 = 400 pM for skin fibroblast collagenase MMP-1; IC50 = 500 pM for gelatinase A MMP-2; IC50= 27 nM for stromelysin MMP-3; IC50 = 100 pM for neutrophil collagenase MMP-8; and IC50 =
200 pM for gelatinase B MMP-9. Piperazine (N-Hydroxy-1,3-di-(4-methoxybenzenesulphonyl)-5,5-dimethyl-[1,3]-piperazine-2-carboxamide; Calbiochem-EMD Chemicals Inc., Darmstadt, Germany) is a potent, reversible, broad-range inhibitor of matrix metalloproteinases that inhibits MMP-1 (IC$_{50}$ = 24 nM), MMP-3 (IC$_{50}$ = 18.4 nM), MMP-7 (IC$_{50}$ = 30 nM), and MMP-9 (IC$_{50}$ = 2.7 nM). MMP-3 inhibitor (Ac-RCGVPD-NH$_2$, Stromelysin-1 Inhibitor; molecular formula C$_{27}$H$_{46}$N$_{10}$O$_9$S; Calbiochem-EMD Chemicals Inc., Darmstadt, Germany) inhibits the matrix metalloproteinase MMP-3 (IC$_{50}$ = 5 µM). MMP-9 inhibitor (molecular formula C$_{27}$H$_{33}$N$_3$O$_5$S; Calbiochem-EMD Chemicals Inc., Darmstadt, Germany) is a cell-permeable, potent, selective, and reversible MMP-9 Inhibitor (IC$_{50}$ = 5 nM). It inhibits MMP-1 (IC$_{50}$ = 1.05 µM) and MMP-13 (IC$_{50}$ = 113 nM) only at much higher concentrations.

**Reagents**

The sialidase substrate, 2’-(4-methylumbelliferyl)-α-D-N-acetylneuraminic acid (98% pure, 4-MUNANA or 4-MU, Biosynth International Inc., Itasca, IL, USA) was used at optimal concentration of 0.318 mM for the live cell sialidase assay.

**Cell lines and primary cortical neurons**

TrkA-PC12 cell line (overly expressing human TrkA receptors) (Hempstead et al., 1992), was used in these studies and these were grown at 37°C in 5% CO$_2$ in culture media containing DMEM (Gibco, Rockville, MD) supplemented with 5% fetal calf serum.
(FBS) (HyClone, Logan, Utah, USA). The 3T3-TrkA cells are NIH-3T3 cells stably transfected to express human TrkA receptors (kindly provided by Dr Uri Saragovi, Lady Davis Institute for Medical Research, 3755 Côte Ste-Catherine Road, Montreal, Quebec H3T 1E2, Canada) and these were grown in 5% CO₂ in culture media containing RPMI (Gibco) supplemented with 5% fetal calf serum (HyClone, Logan, Utah, USA), 1x Penicillin/Streptomycin/Glutamine, 1 mM HEPES and 0.1 mg/mL G418. Primary cultures of rat cortical neurons were prepared as described previously (Brewer, 1995; Brewer, 1997). GIBCO® Primary Rat Cortex Neurons (Invitrogen Canada Inc. Burlington, ON, L7L 5Z1) were isolated from day-18 Fisher 344 rat embryos and cryopreserved in liquid nitrogen. The neurons were grown on poly-D lysine coated 12mm circular glass slides in Neurobasal media supplemented with 0.5mM GLUTAMAX and 2% B27 at 37°C in a humidified atmosphere of 5% CO₂. The cells were grown for 7 days before conducting an experiment. The cultures consist of 99% neurons.

Sialidase activity in viable cells

Primary bone marrow macrophages and Trk-expressing cells were grown on 12 mm circular glass slides in conditioned medium as described above. After removing medium, 0.318 mM 4-MUNANA (4-MU) substrate [2’-(4-methylumbelliferyl)-α-D-N-acetylneuraminic acid] (Biosynth International Inc., Itasca, IL, USA) in Tris buffered saline pH 7.4 was added to each well alone (control), with predetermined dose of NGF, or in combination of NGF and indicated inhibitors. The substrate is hydrolyzed by
sialidase to give free 4-methylumbelliferone which has a fluorescence emission at 450 nm (blue color) following an excitation at 365 nm. Fluorescent images were taken after 1-2 minutes using epi-fluorescent microscopy (40x objective).

**Immunocytochemistry of pTrkA and pTrkB**

Trk-expressing TrkA-PC12 cells were pretreated with inhibitors at indicated concentrations for 30 mins followed with NGF for 15 min. Primary cortical neurons were pretreated with the indicated inhibitors for 30 mins followed with BDNF for 15 min. Cells were fixed with 4% paraformaldehyde, permeabilized with Triton-X100 and immunostained with FITC-conjugated goat anti-pTyr490 of Trk as indicated. Stained cells were visualized by epi-fluorescence microscopy using a 40x objective. Quantitative analysis was done by assessing the density of cultured cell staining corrected for background in each panel using Corel Photo Paint 8.0 software. Each bar in the figures represents the mean corrected density of staining ± SEM for cultured cells (n) within the respective images. The cultures in each panel are of equal cell density. P values represent significant differences at 95% confidence using Dunnett’s Multiple Comparison Test compared to control (Ctrl) in each group.

**Flow cytometry of cell surface MMP-9 in live TrkA-PC12 cells**

Cells were grown in 25-cm2 flasks at 90% confluence. For cell surface staining, live cells in serum free cold phosphate-buffered saline (PBS) were stained with rabbit anti-MMP-9
antibody (H-300, Santa Cruz Biotechnology, Inc. California, US) for 15–20 min at 4°C, washed and followed with Alexa Fluor488 F(ab’)2 goat anti-rabbit IgG. After washing with cold PBS buffer, the cells were prepared for flow cytometry analysis. 40000 cells were acquired on a Beckman Coulter (Miami, FL) Epics XL-MCL flow cytometer and analyzed with Expo32 ADC software (Beckman Coulter). For overlay histograms, control cells treated with Alexa Fluor488 F(ab’)2 goat anti-rabbit IgG alone are represented by the unfilled histogram with the dashed line. Cells treated with anti-MMP-9 antibody together with Alexa Fluor488 F(ab’)2 secondary antibody are depicted by the unfilled histogram with the black line. Auto-fluorescence of untreated cells is depicted by gray-filled histogram. The mean fluorescence for each histogram is indicated for 80% gated cells.

**MMP-9 co-localization with Trk**

TrkA-PC12 cells were grown for 24 hours on poly D lysine coated circular glass slides in 24 well tissue culture plates. Primary neuronal cells were grown for 7 days in their neurobasal media supplemented with Glutamax and B27 supplement on poly D lysine coated circular glass slides in 24 well tissue culture plates. The cells were either left untreated as control or stimulated with 250 ng/mL NGF (for TrkA-PC12 cells) or 200 ng/mL BDNF (for primary neuronal cells) for 15 mins. The cells were fixed, permeabilized and immunostained with FITC conjugated antibodies against Trk and rabbit anti-MMP-9 (H-129: sc-10737, Santa Cruz Biotech) followed by Alexa Flour594 goat
anti-rabbit IgG. Stained cells were visualized using a confocal inverted microscope (Leica TCS SP2 MP inverted Confocal Microscope) with a 100x objective (oil).

**MMP-9 co-localization with Neu1**

TrkA-PC12 cells and primary neuronal cells were grown in their respective media and treatments were carried out as mentioned previously. The cells were fixed, permeabilized and immunostained with goat anti-MMP-9 (C-20: sc-6840, Santa Cruz Biotech) against MMP-9 and rabbit anti-Neu1 (H-300: sc-32936, Santa Cruz Biotech) against Neu1 followed by Alexa Fluor568 rabbit anti-goat IgG and Alexa Fluor488 donkey anti-rabbit IgG respectively. Stained cells were visualized using a confocal inverted microscope (Leica TCS SP2 MP inverted Confocal Microscope) with a 100x objective (oil).

**Co-immunoprecipitation**

TrkA-PC12 cells are left cultured in media, pretreated with 200 μM Tamiflu for 30 min followed with 250 ng/mL NGF for 15 min or in media containing 250 ng/mL NGF for 15 min. Cells are pelleted and lysed in lysis buffer (50mM Tris, pH 8, 150 mM NaCl, 1% NP-40, 0.2mg/mL leupeptin, 1% β-mercaptoethanol, and 1mM phenylmethanesulfonyl fluoride (PMSF)). Cell lysates are immunoprecipitated with 2 μg of goat anti-pan TrkA, goat anti-pTrkA, rabbit anti-Neu1 or rabbit anti-MMP-9 antibodies for 18 hrs. Following immunoprecipitation, complexes are isolated using protein A magnetic beads, washed 3x in buffer (10mM Tris, pH8, 1mM EDTA, 1mM EGTA, 150mM NaCl, 1% Triton X-100.
and 0.2mM sodium orthovanadate) and resolved by 8% gel electrophoresis (SDS-PAGE). Proteins are transferred to polyvinylidene fluoride (PVDF) transfer membrane blot. The blot is probed for either Neu1 (45.5 kDa) with anti-Neu1 (Abcam), Proteins are transferred to polyvinylidene fluoride (PVDF) transfer membrane blot. The blot is probed for either Neu1 (45.5 kDa) anti-Neu1 (Santacruz Biotech Inc), MMP-9 (92 kDa) with anti-MMP-9 (Santa-cruz Biotech Inc), or TrkA with anti-pan TrkA antibodies followed by HRP conjugated secondary IgG antibodies and Western Lightning Chemiluminescence Reagent Plus. The initial IP blot is stripped and further probed for described proteins. The chemiluminescence reaction is analyzed with either a Fluorochem HD2 Imaging System (Alpha Immunotech, San Leandro, CA, USA) or x-ray film. Sample concentration for gel loading was determined by Bradford reagent.

**Neurite outgrowth**

TrkA-PC12 cells were grown in a 24-well culture plate in DMEM media containing 5% FCS. The cells were starved for 4 hours prior to treatments. Cells were then pre-treated with 100 μg/mL of the inhibitor followed with 250 ng/mL NGF or are left untreated as control. The live cells were observed after 2 days using a AMG/Westover Scientific Digital USB2 Microscope and the images were recorded using the Micron Imaging Software.
Statistics

Comparisons between two groups were made by one-way ANOVA at 95% confidence using unpaired t-test and Bonferroni’s Multiple Comparison Test or Dunnett’s Multiple Comparison Test for comparisons among more than two groups.
RESULTS

*Ligand-induced sialidase activity in TrkA-expressing cells is inhibited by GPCR inhibitor Suramin*

If G-protein coupled receptors (GPCRs) are involved in the transactivation of Trk receptors, then using a broad range GPCR inhibitor should result in inhibition of ligand-induced sialidase activity. We found that when NGF was added to live TrkA-expressing TrkA-PC12 and 3T3-TrkA cells, sialidase activity was induced on the cell surface and this activity was inhibited in the presence of the broad range GPCR inhibitor Suramin (*Figure 3.1A and 3.1B*). Suramin uncouples the G-proteins from GPCRs by blocking their interaction with intracellular receptor domains; also inhibiting GDP-GTP exchange, the rate limiting step in the activation of G\(_\alpha\) subunits.

*Pertussis toxin, a Gai protein inhibitor blocks NGF-induced sialidase activation*

Since the broad range GPCR inhibitor Suramin blocked sialidase activity in live TrkA-expressing cells, we used a more specific inhibitor to identify the specific G protein subunit involved in the regulation of sialidase activation. Using the sialidase assay described previously, we show that NGF-induced sialidase activity in live TrkA-PC12 cells is inhibited by pertussis toxin PTx (*Figure 3.2*) thus providing evidence that the G proteins involved in sialidase activation are G\(_{ai}\) proteins.
Figure 3.1. Suramin inhibits NGF-induced sialidase activity in TrkA-PC12 cells and 3T3-TrkA cells. (A)TrkA-expressing PC-12 and (B) 3T3-TrkA cells were allowed to adhere on 12mm circular glass slides (coated with poly-D-lysine) overnight in media supplemented with 5% fetal calf serum at 37°C. After removing media, 0.318 mM 4-MUNANA (4-MU) substrate [2’-(4-methyumbelliferyl)-α-D-N-acetylneuraminic acid] (Sigma) in Tris buffered saline pH 7.4 was added to the cells alone (Control), with 160 ng/mL NGF, or to NGF in combination with 200 µM Suramin. The substrate is hydrolysed by sialidase to give free 4-methylumbelliferone which has a fluorescence emission at 450nm (blue color) following excitation at 365nm. Fluorescent images were taken at different time intervals after adding substrate using epi-fluorescent microscopy (40x objective). The mean fluorescence surrounding the cells for n=50 replicates in each of the images was measured using Image J Version 1.43 Software (NIH). The data are a representation of one out of five independent experiments showing similar results.
A

Phase contrast

TrkA-PC12 cells

3T3-TrkA cells

Control Suramin+NGF NGF

Phase contrast Control NGF Suramin+NGF

Control NGF Suramin+NGF

Control NGF Suramin+NGF

Corrected Density

Corrected Density

Corrected Density

98
Sialidase activity in TrkA-expressing TrkA-PC12 cells is inhibited by broad range MMP inhibitors Galardin and Piperazine

Studies reveal that agonist-bound GPCRs have been shown to activate numerous matrix metalloproteinases (MMPs) (Fischer et al., 2006), including MMP-3 (Lee and Murphy, 2004), MMPs 2 and 9 (Murasawa et al., 1998; Le Gall et al., 2003). Hinek and colleagues have shown that protective protein cathepsin A is sorted to the plasma membrane of differentiating monocytes similarly to Neu1 and is required for normal Neu1 enzymatic activity. Cell surface Neu1/cathepsin A complex can also associate with elastin-binding protein (EBP) forming the elastin receptor complex, where catalytic activity of Neu1 facilitates elastic fibre assembly. Based on these reports, we propose that when neurotrophin binds to the Trk receptor, the EBP must be removed from the Neu1/cathepsin A complex in order for the Neu1 to become activated and this removal is likely catalyzed by an enzyme with elastase substrate specificity like an MMP. This led us to hypothesize that MMPs play a potential role in sialidase activation associated with ligand-binding Trk receptors. When the broad range MMP inhibitor Galardin was used in the sialidase assay, there was an inhibition of sialidase activity as shown in Figure 3.2A, suggesting a role of MMP in regulation of sialidase activation in NGF-induced TrkA-PC12 cells.
MMP-9 is potentially involved in the regulation of NGF-induced sialidase activation in live TrkA-PC12 cells

Inhibition of sialidase activation with broadrange MMP inhibitors Galardin helped us deduce that a specific MMP that might be playing a role in activating the sialidase in NGF-stimulated TrkA-PC12 cells. Based on the inhibitory specificities of Galardin and Piperazine, we propose that MMP-1, MMP-3 or MMP-9 might be involved in this process (Figure 3.2A and B). Galardin inhibits MMP-1, MMP-2, MMP-3, MMP-8 and MMP-9. Piperazine inhibits MMP-1, MMP-3, MMP-7 and MMP-9. When the MMP-3 specific inhibitor was used we did not see any inhibition of NGF-induced sialidase activity in live TrkA-PC12 cells whereas MMP-9 specific inhibitor blocked this activity (Figure 3.3). These data suggest a role for MMP-9 in ligand-induced activation of sialidase.
Figure 3.2. (A) NGF-stimulated sialidase activity in blocked by broadrange matrix metalloproteinase (MMP) inhibitor Galardin and GPCR Gαi protein inhibitor Pertussis toxin (PTx). TrkA-PC12 cells were allowed to adhere on poly-D lysine coated 12mm circular glass slides in media containing 5% fetal bovine sera for 24h at 37°C. After removing media, 0.138 mM 4-MUNANA (4-MU) substrate [2’-(4-methylumbelliferyl)-α-D-N-acetylneuraminic acid] (Sigma) in Tris buffered saline pH 7.4 was added to cells alone (Control); and with 160 ng/mL NGF alone or in combination either with 250 µg/mL Tamiflu (broad range Neuraminidase inhibitor), 250 ug/mL Galardin or 25 ng/mL Pertussis toxin (PTx). Fluorescent images were taken at 1 min after adding substrate using epi-fluorescent microscopy (40x objective). The data are a representation of one out of five independent experiments showing similar results. (B) NGF-stimulated sialidase activity in blocked by matrix metalloproteinase (MMP) inhibitor Piperazine. Sialidase assay was carried out as described in A. The data are a representation of one out of three independent experiments showing similar results.
A

Phase contrast
Control
NGF
Tamiflu+NGF
Galardin+NGF
PTx+NGF

B

Phase contrast
Control
NGF
Tamiflu+NGF
Piperazine+NGF

TrkA-PC12 cells

Sialidase assay-TrkA-PC12 cells
Figure 3.3. NGF-stimulated sialidase activity in blocked by GPCR Ga\textsubscript{i} protein inhibitor Pertussis toxin (PTx) and MMP-9 specific inhibitor indicating the involvement of Ga\textsubscript{i} proteins and MMP-9 in sialidase activation. Sialidase assay was conducted on TrkA- PC12 cells by adding the fluorogenic substrate 4-MUNANA (4-MU) substrate [2’-(4-methlyumbelliferyl)-\textalpha-D-N-acetylneuraminic acid] (Sigma) in Tris buffered saline pH 7.4 along with 160 ng/mL NGF alone or in combination either with 50 µg/mL Tamiflu (broad range Neuraminidase inhibitor), 50 µg/mL MMP-9i (inhibitor specific for MMP-9), 50 µg/mL MMP-3i (inhibitor against MMP-3) or 25 ng/mL Pertussis toxin (PTx) which is an inhibitor specific for GPCR Ga\textsubscript{i} proteins. NGF-induced sialidase activity was blocked by MMP-9i and PTx indicating significant involvement of Ga\textsubscript{i} proteins and MMP-9 in sialidase activation. This data is a representation of one out of three independent experiments.
Phosphorylation of TrkA in TrkA-expressing TrkA-PC12 cells is inhibited by Pertussis toxin and MMP-9 inhibitor

Trk phosphorylation is the initial event of receptor activation when the receptors are bound by their natural ligands. Previously, we have shown that Tamiflu, a broadrange neuraminidase inhibitor blocks not only sialidase activation but also pTrkA activation upon NGF binding to TrkA-expressing cells (Woronowicz et al., 2004). When we used pertussis toxin PTx a Gαi sensitive protein inhibitor and MMP-9 specific inhibitor in live cell sialidase assay, we observed inhibition of NGF induces sialidase activity in TrkA-PC12 cells. To test if these inhibitors were capable of blocking phosphorylation of TrkA in TrkA-PC12 cells, receptor activation in Trk-expressing cells was determined by immunocytochemistry (ICC) and visualized by epi-fluorescent microscopy. The TrkA-PC12 cells were treated with NGF, in combination of NGF and PTx or left untreated as controls. Cells were fixed and permeabilized prior to adding primary antibodies against phosphorylated Trk (pTyr490). The secondary antibody is a fluorescein (FITC) conjugated goat anti mouse antibody. The results were observed as an immunofluorescent signal using the epifluorescent microscope (objective 40x). The data in Figure 3.4 show that cells pretreated with PTx and MMP-9 inhibitors (MMP-9i) significantly blocked NGF-induced pTrkA while the MMP-3 inhibitor (MMP-3i) has limited inhibition. These results confirm that MMP-9 is involved in the Neu1 sialidase activity associated with NGF treated live TrkA expressing cells.
Figure 3.4. NGF-induced phosphorylation of TrkA involves sialidase activity through the intermediate catalytic activity of MMP-9 mediated by GPCR-signaling via Gαi-proteins. TrkA-PC12 were pretreated with either 400 µM Tamiflu, 100 µg/mL of MMP-9i, Anti-MMP-9, MMP3i or 100 ng/mL Ptx for 30 min followed with 250 ng/mL NGF for 15 min. Cells were also left untreated as control. Cells were fixed, permeabilized, and immunostained with FITC conjugated goat-anti pTrkA. Stained cells were visualized by epi-fluorescence microscopy using a 40x objective. Quantitative analysis was done by assessing the density of cell staining corrected for background in each panel image using Corel Photo Paint 8.0 software. The data is a representation of one out of three independent experiments.
**GPCR ligand Bombesin induces sialidase activity in live TrkA-PC12 cells**

Data reveals that neurotrophin-induced Trk activation is regulated by GPCRs and based on these results we hypothesize that a GPCR ligand should be sufficient enough to trigger sialidase activity in the absence of Trk-specific neurotrophic ligands. We used the neuropeptide Bombesin to test our hypothesis. Bombesin is a 14 amino acid peptide purified originally from amphibian skin (Anastasi *et al.*, 1971). Its human homologues include the gastrin releasing peptide and neuromedin B. Bombesin-like peptides are widely distributed in the central nervous system and also the gastrointestinal tract. They bind to GPCRs on the surface to elicit their effects and those GPCRs that are categorized as Bombesin receptors include BB1 and BB2, which couple primarily to the \( G_{q11} \) family of G proteins. BB1 and BB2 receptor-mediated activation results in various effector functions such as stimulation of tissue growth, exocrine and endocrine processes and many other secretory processes (Alexander *et al.*, 2008). Using the sialidase assay, we observed a strong sialidase activity associated with bombesin treatment of live TrkA-PC12 cells comparable to cells treated with NGF (*Figure 3.5*). These results signify a connection between GPCRs and Neu1 sialidase activity.

**Bombesin-induced sialidase activity and Bombesin-induced phosphorylation of Trk are inhibited by Pertussis toxin and MMP-9 inhibitor**

Our next step was to test if Gai protein sensitive specific inhibitor PTx and MMP-9 inhibitor block bombesin-induced sialidase activity in live TrkA-expressing cells. PTx
and MMP-9i not only blocked bombesin-induced sialidase activity (Figure 3.6) but also bombesin-induced phosphorylation of TrkA as shown in Figure 3.7 These data suggest that bombesin-induced phosphorylation of TrkA in live TrkA-expressing cells involves sialidase activity through the intermediate catalytic activity of MMP-9 mediated by GPCR-signaling via Gαi-proteins.

**MMP-9 colocalizes with TrkA and Neu1 in live TrkA-PC12 cells**

Since most studies describing the expression of MMP-9 on the cell surface use growth factors or phorbol esters, we questioned whether or not MMP-9 is expressed on the cell surface in live, naïve and untreated TrkA-PC12 cells. Using confocal fluorescence microscopy, MMP-9 was found to colocalize with TrkA on the cell surface in naïve and NGF-stimulated cells (Figure 3.8A). The immunolocalization of MMP-9 on the cell surface of these cells was confirmed by flow cytometry using live cells immunostained with anti-MMP-9 antibodies followed with Alexa Fluor596 conjugated F(ab’)2 secondary antibody. The data shown in Figure 3.8B clearly indicate that 40000 acquired live, untreated TrkA-PC12 cells showed significant immunostaining for MMP-9 expression on the cell surface. Co-immunoprecipitation experiments using cell lysates from TrkA-PC12 cells further confirms that MMP-9 forms a complex with naïve TrkA receptors but less so with NGF-stimulated receptors (Figure 3.8C).
Figure 3.5. GPCR agonist Bombesin induces sialidase activity in TrkA-PC12 cells in the absence of NGF and bombesin-induced sialidase activity is inhibited by broadrange sialidase inhibitor Tamiflu. TrkA-PC12 cells were allowed to adhere on poly-D lysine coated 12mm circular glass slides in media containing 5% fetal bovine sera for 24h at 37°C. After removing media, 0.138 mM 4-MUNANA (4-MU) substrate [2’-(4-methyumbelliferyl)-α-D-N-acetylneuraminic acid] (Sigma) in Tris buffered saline pH 7.4 was added to cells alone as untreated control; with 125 ng/mL NGF; with 250 µg/mL Bombesin or Bombesin in combination with 250 µg/mL Tamiflu (broad range neuraminidase inhibitor). Fluorescent images were taken at 1 min after adding substrate using epi-fluorescent microscopy (40x objective). The data is a representation of one out of five independent experiments.
Figure 3.6. Bombesin-induced sialidase activation in TrkA-PC12 cells is blocked by pertussis toxin and MMP-9 inhibitor. Sialidase assay was conducted on TrkA-PC12 cells by adding the fluorogenic substrate 4-MUNANA (4-MU) substrate [2’-(4-methlyumbelliferyl)-α-D-N-acetylneuraminic acid] (Sigma) in Tris buffered saline pH 7.4 along with Bombesin (250 μg/mL) alone or in combination with 50 μg/mL indicated inhibitor. Epi-fluorescent images (40x objective) taken at 1 min after adding substrate revealed that bombesin-induced sialidase activity was blocked by MMP-9i and PTX. The data is a representation of one of three independent experiments.
Control
Bombesin
Anti-MMP-9+
Bombesin
MMP-9i+
Bombesin
MMP-3i+
Bombesin
Galardin+
Bombesin
Piperazine+
Bombesin

Phase contrast
NGF
Bombesin
PTx+
Bombesin

Sialidase assay-TrkA-PC12 cells

Mean Fluorescence
Figure 3.7. Bombesin-induced transactivation of pTrkA is dependent on activation of Gai proteins and MMP-9. TrkA-PC12 cells were grown on poly-D lysine coated 12mm circular glass slides in DMEM media supplemented with 5% FBS for 24 hrs. Cells were treated with the indicated inhibitors for 30 mins followed by stimulation with 250 μg/mL Bombesin for 15 min. The treated cells were then fixed, permeabilized and immunostained with FITC conjugated pTrk antibody. The stained cells were then visualized under the 40x objective of the epi-fluorescent microscope. Analysis was carried out by assessing the density of the cell staining corrected for background using Corel Photo Paint 8.0 software. The data is a representation of one of three independent experiments.
Figure 3.8. (A) MMP-9 colocalizes with TrkA in naïve and NGF-stimulated TrkA-PC12 cells. Cells were left untreated as no ligand control or stimulated with 250 ng/mL with NGF for 15 mins. The cells were fixed, permeabilized and immunostained with FITC conjugated goat anti-pan TrkA and rabbit anti-MMP-9 antibodies followed by Alexa Fluor594 conjugated goat anti-rabbit IgG. Stained cells were visualized using a confocal inverted microscope (Leica TCS SP2 MP inverted Confocal Microscope) with a 100x objective (oil). TrkA-PC12 cells were left untreated as control or stimulated with 250 ng/mL with NGF for 15 mins. The cells were fixed, permeabilized and immunostained with antibodies against both TrkA and MMP-9 followed by Alexa Flour conjugated antibodies. Stained cells were visualized using a confocal inverted microscope (Leica TCS SP2 MP inverted Confocal Microscope) with a 100x objective (oil).

(B) Flow cytometry analysis of MMP-9 expression on the cell surface of live TrkA-PC12 cells. Histogram shows staining with rabbit anti-MMP-9 antibodies after incubation on ice for 15 min and followed with Alexa488 conjugated F(ab’ )2 secondary antibody for additional 15 min on ice. Control cells were stained with Alexa488 conjugated secondary F(ab’ )2 antibody for 15 min on ice. Cells were analyzed by Beckman Coulter Epics XL-MCL flow cytometry and Expo32 ADC software (Beckman Coulter). Overlay histograms are displayed. Control Alexa488 secondary antibody treated cells are represented by the unfilled histogram with the gray line. Live cells stained with anti-MMP-9 antibody are depicted by the unfilled histogram with the black line. Auto-fluorescence of the cells is depicted by the gray-filled histogram. The mean fluorescence for each histogram is indicated for 40000 acquired cells (80% gated). The data are a representation of one out of two experiments showing similar results.

(C) MMP-9 co-immunoprecipitates with TrkA. TrkA-PC12 cells were treated with 250 ng/mL NGF for 15 min or left untreated as control (media). Cells were pelleted, lysed in lysis buffer and the cell lysates were immunoprecipitated with antibodies against the indicated proteins for 18 hrs. Immunocomplexes were isolated using protein A magnetic beads, resolved by SDS-PAGE and the blot probed with antibodies against the indicated proteins. The data are a representation of one out of two independent experiments showing similar results.
No ligand

NGF

A

TrkA
MMP-9
Merge

% Overlay

37.2%
25.9%

B

IP: anti-TrkA
Blot: anti-MMP-9

C

MW kDa
media NGF

IP: anti-TrkA
Blot: anti-MMP-9

80 ➔ MMP-9 (92 kDa)

IP: anti-TrkA
Blot: anti-TrkA

➔ TrkA (140 kDa)
Based on our previous results, the data suggest that Neu1 sialidase is activated upon ligand binding to Trk receptors. In addition, Neu1 forms a complex with TrkA receptors in naive and NGF stimulated TrkA-PC12 cells. If Neu1 and MMP-9 are localized on the cell surface in regulating Trk activation, they should be associated with each other in alliance with Trk receptors. Confocal microscopy revealed the cell-surface colocalization of Neu1 and MMP-9 in naïve and NGF-treated TrkA-PC12 cells (Figure 3.9A). Co-immunoprecipitation experiments using cell lysates from TrkA-PC12 cells further demonstrated that MMP-9 forms a complex with Neu1 sialidase (Figure 3.9B). Together, the additional intracellular and cell-surface co-localization of Neu1 and MMP-9 validated the predicted cross-talk between Neu1 and MMP-9 in alliance with TrkA receptors.

**Gαi-sensitive pertussis toxin and MMP-9 specific inhibitor block NGF-induced neuritogenesis in TrkA-PC12 cells**

Previously, we have shown that Tamiflu inhibits neurite outgrowth in NGF-stimulated TrkA-PC12 cells and BDNF-stimulated TrkB-nmr5 cells (Woronowicz et al., 2007b). We have also shown anti-Neu1 antibody blocked NGF-induced neurite outgrowth in live TrkA-PC12 cells compared with NGF positive control. Here we test if inhibitors of GPCRs and MMPs can block NGF-induced neurite outgrowth. The data show that Gαi protein specific inhibitor PTx and MMP-9 specific inhibitor block neurite outgrowth in TrkA-PC12 cells whereas MMP-3 inhibitor had no effect (Figure.3.10).
Figure 3.9. (A) MMP-9 co-localizes with Neu1 on the surface of TrkA-PC12 in both naïve and NGF-stimulated cells. TrkA-PC12 cells were left untreated as control or stimulated with 250 ng/mL with NGF for 15 mins. The cells were fixed, permeabilized and immunostained with antibodies against both Neu1 and MMP-9 followed by Alexa Flour conjugated antibodies. Stained cells were visualized using a confocal inverted microscope (Leica TCS SP2 MP inverted Confocal Microscope) with a 100x objective (oil). (B) MMP-9 coimmunoprecipitates with Neu1. TrkA-PC12 cells were pelleted and then lysed in lysis buffer to obtain protein lysates which were then immunoprecipitated with antibodies against the indicated proteins for 18 hours. Complexes were then isolated using Protein A beads and resolved by SDS-PAGE. The blots were then probed with antibodies against the indicated proteins. The data is a representation of one out of two independent experiments.
A

No ligand

NGF

% Overlay

25%

34%

B

IP: anti-MMP-9
Blot: anti-Neu1

Neu1 (45.5 kDa)

IP: anti-Neu1
Blot: anti-MMP-9

MMP-9 (92 kDa)

IP: anti-MMP-9
Blot: anti-TrkA

TrkA (140 kDa)
Figure 3.10. Neurite out-growth in TrkA-PC12 cells is inhibited by MMP-9i and PTx. TrkA-PC12 cells were grown in a 24-well culture plate in DMEM media containing 5% FBS. The cells were starved for 4 hours prior to treatments. Cells were pretreated with either 100 μg/mL of MMP-9i, MMP-3i or 100 ng/mL PTx followed with 250 ng/mL NGF. Cells left untreated served as no ligand controls. The cells were observed after 2 days using an AMG/Westover Scientific Digital USB2 Microscope and the images were recorded using the Micron Imaging Software.
No ligand | NGF | MMP3i+NGF
--- | --- | ---
[Image] | [Image] | [Image]
MMP9i+NGF | PTx+NGF
[Image] | [Image]
Neu1 and MMP-9 cross-talk is evident in ligand-induced TrkB-expressing primary neurons

To validate our hypothesis, we also used primary rat cortical neurons. They were grown on poly-D lysine coated 12mm circular glass slides in neurobasal media supplemented with 0.5mM glutamax and 2% B27 at 37°C in a humidified atmosphere of 5% CO2. After 7 days, the cells were treated with the anti-Neu1 antibodies, MMP-9i specific inhibitor, anti-MMP-9 antibodies or pertussis toxin (PTx) followed by stimulation with brain-derived neurotrophic factor (BDNF). The anti-Neu1 antibody is a rabbit polyclonal antibody against amino acids 116-415 mapping at the C-terminus of Neu1 human origin. This antibody detects Neu1 of mouse, rat and human origin by Western blotting. The anti-MMP-9 antibody is a rabbit polyclonal antibody against amino acids 459-587 of MMP-9 human origin. This antibody also detects MMP-9 of mouse, rat and human origin by Western blotting. Our data confirms the results for TrkB-expressing cell line showing that these specific inhibitors completely blocked BDNF-induced Neu1 sialidase activity (Figure 3.11A) in live primary cortical neurons as well as significantly inhibiting BDNF-induced pTrkB in these neurons (Figure 3.11B).

Confocal microscopy also revealed the cell-surface colocalization of MMP-9 with TrkB in naïve and BDNF-treated primary cortical neurons (Figure 3.12A). If Neu1 and MMP-9 cross-talk is localized to the cell surface in regulating TrkB activation in primary cortical neurons, they should also be associated with each other in alliance with TrkB
receptors. Confocal microscopy revealed the cell-surface colocalization of Neu1 and MMP-9 in naïve and BDNF-treated primary neurons (Figure 3.12B).
Figure 3.11 (A). BDNF-induced sialidase activity in primary rat cortical neurons is blocked by anti-MMP-9 antibodies, MMP-9i specific inhibitor and GPCR Gαi protein inhibitor pertussis toxin (PTx). GIBCO® primary rat cortex neurons were grown on poly-D lysine coated 12mm circular glass slides in Neurobasal media supplemented with 0.5mM GLUTAMAX and 2% B27 at 37°C in a humidified atmosphere of 5% CO2. The cells were grown for 7 days before conducting an experiment. The cultures consist of 99% neurons. After removing media, 0.138 mM 4-MUNANA substrate [2’-(4-methylumbelliferyl)-α-D-N-acetylneuraminic acid] in Tris buffered saline pH 7.4 was added to cells alone (Control), with 200 ng/mL BDNF alone or with BNDF in combination with either 100 μg/mL MMP-9i (specific inhibitor for MMP-9), 100 μg/mL anti-MMP-9 antibody or 25 ng/mL PTx (specific inhibitor for GPCR Gαi protein). The substrate is hydrolysed by sialidase to give free 4-methylumbelliferone which has a fluorescence emission at 450nm (blue color) following excitation at 365nm. Fluorescent images were taken at 2 min after adding substrate using epi-fluorescent microscopy (40x objective). The mean fluorescence surrounding the cells for n ≥ 50 replicates in each of the images was measured using Image J Version 1.43 Software (NIH). The data are a representation of one out of three independent experiments showing similar results. (B) BDNF-induced pTrkB in primary rat cortical neurons is blocked by MMP-9i specific inhibitor, and PTX. Primary neurons were pretreated with the indicated inhibitors as described in (A) for 30 min followed with 200 ng/mL BDNF for 15 min. Cells were fixed, permeabilized, and immunostained with FITC conjugated goat anti-Tyr490 of Trk. Stained cells were visualized by epi-fluorescence microscopy using a 40x objective. Quantitative analysis was done by assessing the density of cell staining corrected for background in each panel image using Corel Photo Paint 8.0 software.
A

Control  BDNF  MMP-9i+BDNF  Anti-MMP9+BDNF  PTx+BDNF

B

Phase contrast  No ligand  BDNF  MMP-9i+BDNF  PTx+BDNF
Figure 3.12 (A) MMP-9 colocalizes with TrkB in naïve and BDNF-stimulated primary cortical neurons. Primary neurons were left untreated as no ligand control or stimulated with 200 ng/mL with BDNF for 15 min. The cells were fixed, permeabilized and immunostained with FITC conjugated goat anti-pan TrkB and rabbit anti-MMP-9 antibodies followed by Alexa Fluor594 conjugated goat anti-rabbit IgG. Stained cells were visualized using a confocal inverted microscope (Leica TCS SP2 MP inverted Confocal Microscope) with a 100x objective (oil). (B) MMP-9 colocalizes with Neu1 in naïve and BDNF-stimulated primary cortical neurons. Primary neurons were treated and analyzed as described in (A). The cells were fixed, permeabilized and immunostained with rabbit anti-Neu1 and goat anti-MMP-9 antibodies followed by Alexa Fluor488 donkey anti-rabbit IgG and Alexa Fluor568 goat anti-rabbit IgG, respectively. Stained cells were visualized using a confocal inverted microscope (Leica TCS SP2 MP inverted Confocal Microscope) with a 100x objective (oil).
DISCUSSION

Previously, we have shown that Neu1 sialidase is activated when neurotrophin binds to Trk receptor. The findings in this thesis also provide evidence for the potentiation of G-protein coupled receptor (GPCR)-signaling via membrane targeting of Gαi subunit proteins and matrix metalloproteinase-9 (MMP-9) activation in inducing Neu1 sialidase on the cell surface. Neu1 in alliance with GPCR-signaling Gαi subunit proteins and MMP-9 is expressed on the cell surface of TrkA-expressing cells. This tripartite alliance would actually make Neu1 readily available to be induced by neurotrophin binding to the receptor. Our data support this premise. How Neu1 sialidase is rapidly induced by MMP-9 together with GPCR Gαi subunit proteins remains unknown. We propose that MMP-9 activation is required to remove elastin-binding protein (EBP) complexed to Neu1 and cathepsin A (Hinek et al., 2006). Perhaps, it can be speculated that neurotrophin binding to the receptor on the cell surface initiates GPCR-signaling via GPCR Gαi subunit proteins to activate MMP.

In our experimental model, we propose that when neurotrophin binds to Trk receptor, it triggers a conformational change in Trk receptor resulting in the activation of a nearby GPCR, either independently or via heterotrimeric Gα protein. Activated GPCR then subsequently activates an MMP, most likely an MMP with elastase activity (MMP-9) which can cleave the elastin binding protein from the Neu1/Cathepsin A/EBP complex on
the plasma membrane. This removal of EBP catalytically activates Neu1 and allows for desialylation of Trk, thus priming the receptor for dimerization, internalization and subsequent activation. It is well known that agonist-bound GPCRs have been shown to activate numerous MMPs (Fischer et al., 2006), including MMP-3 (Lee et al., 2004), MMPs 2 and 9 (Murasawa et al., 1998; Le Gall et al., 2003), as well as members of the ADAM family of metalloproteases (Prenzel et al., 1999; Gooz et al., 2006). Using the well established sialidase assay we have shown that inhibitors of Gαi subunit proteins and MMP-9 inhibit both NGF-induced sialidase activation and Trk activation. The receptor signaling paradigm in our studies further signifies a GPCR-signaling via Gαi-proteins and MMP-9 activation in inducing Neu1 sialidase, all of which form a tripartite complex with TrkA at the ectodomain on the cell surface. Using confocal microscopy on permeabilized naïve and neurotrophin-treated cells and co-immunoprecipitation experiments, the additional intracellular and cell surface co-localization of Neu1, MMP-9 and TrkA validated the predicted association of MMP-9 with Neu1 in alliance with Trk receptors.

In conclusion, neurotrophin binding to its receptor may induce allosteric conformational changes in the receptor, which in turn potentiates GPCR-signaling and MMP-9 activation to induce Neu1 sialidase. Central to this process is that Neu1/MMP-9 complex in alliance with Trk receptors is expressed on the cell surface of Trk-expressing cells and primary cortical neurons. This tripartite alliance would actually make Neu1 readily available to be induced by neurotrophin binding to the receptor. The findings in this thesis suggest that
Neu1 sialidase and MMP-9 cross-talk in alliance with Trk receptors may be the key regulators of neurotrophin induced Trk activation to generate a functional receptor.
PJ performed all the experiments reported in this chapter; these data were included in the following publications:


ABSTRACT

The signaling pathways of mammalian Toll-like receptors (TLR) are well characterized, but the initial molecular mechanisms activated following ligand interactions with the receptors remain poorly defined. Here, we show that Neu1 sialidase and not Neu2,-3 and-4 forms a complex with TLR-2,-3 and-4 receptors on the cell surface membrane of naïve and activated macrophage cells. Activation of Neu1 is induced by TLR ligands binding to their respective receptors. We also show that endotoxin lipopolysaccharide (LPS)-induced MyD88/TLR4 complex formation and subsequent NFκB activation is dependent on the removal of α-2,3-sialyl residue linked to β-galactoside of TLR4 by the Neu1 activity associated with LPS-stimulated live primary macrophage cells, macrophage and dendritic cell lines but not with primary Neu1-deficient macrophage cells. Taken together, the findings suggest that Neu1 desialylation of α-2,3-sialyl residues of TLR receptors enables in the removal of steric hindrance to receptor association for TLR activation and cellular signaling.
INTRODUCTION

Mammalian Toll-like receptors (TLRs) are a family of receptors that recognize pathogen-associated molecular patterns (PAMPS). They play key roles in activating immune responses during infection by linking the host’s innate and adaptive immune responses against pathogen infections. Studies of the crystal structure at 2.1 angstroms of human TLR3 ectodomain (ECD) reveal a large horseshoe-shaped solenoid which is highly glycosylated with several N-linked glycosylation sites contributing significantly to the additional mass of TLR3 ECD (Choe et al., 2005). Structural studies of TLR ECD have also revealed a single sterically unhindered sugar-free face, which may be involved in the formation of the dimerization interface. Glycosylation has been shown to be a common property of a number of TLRs, including TLR-2, -3, and -4 (da Silva et al., 2002; Weber et al., 2004; Sun et al., 2006). Lack of glycosylation or mutation of glycosylated residues has been shown to negatively affect not only the receptor’s ability to transduce signals, but also maintenance of receptor complexes (da Silva et al., 2002; Weber et al., 2004; Sun et al., 2006). Taken together, these findings suggest that glycosylation of TLRs may play a critical role in receptor activation and its modification may be a cellular mechanism of
controlling TLR activation in promoting dimerization. To date, the precise role of N-linked glycosylation in TLR receptor activation and cell function following their pathogen-molecule interactions has not been defined.

Insight for the role of glycosylation in TLR activation came from the well-characterized model of TrkA tyrosine-protein kinase receptor family, which functions as signaling receptors for the neurotrophin family of molecules of nerve growth factor (NGF). For NGF TrkA receptors, glycosylation of the receptor is required to localize the receptors to the cell surface where it prevents ligand-independent activation of receptors (Watson et al., 1999). Recently, we discovered a membrane sialidase-controlling mechanism that depends on neurotrophin growth factors binding to their specific TrkA and TrkB receptors to induce sialidase activity (Woronowicz et al., 2007a; Woronowicz et al., 2007b). The sialidase targets and desialylates Trk and, consequently causes the induction of receptor dimerization and activation. We also identified a specific sialyl α-2,3-linked β-galactosyl sugar residue of TrkA, which was rapidly targeted and hydrolyzed by the activated sialidase (Woronowicz et al., 2007b).

Here, we report that Neu1 sialidase and not Neu2, -3 and -4 forms a complex with TLR-2, -3 and -4 receptors. Neu1 is expressed on the cell surface membrane of primary macrophage cells and macrophage cell lines. Activation of Neu1 is induced by TLR ligands binding to their respective receptors. The neuraminidase inhibitor Tamiflu
(oseltamivir phosphate) completely inhibits Neu1 sialidase activity associated with the TLR ligand treated live macrophage cells. Also, Tamiflu significantly inhibits endotoxin LPS induced NFκB activation in primary or macrophage cell lines. These findings reveal an unprecedented mechanism of pathogen-molecule induced TLR activation and cell function. Neu1 sialidase may be a key regulator of TLR activation to generate a functional receptor.
MATERIALS AND METHODS

Reagents

TLR4 ligand lipopolysaccharide (LPS 3 µg/mL, from Serratia marcescens and purified by phenol extraction; Sigma, St. Louis, MO), TLR2 ligand Zymosan A (200 µg/mL, from Saccharomyces cerevisiae; Sigma, St. Louis, MO) TLR3 ligand polyinosinic-polycytidylic acid (poly I:C, 20 µg/mL; Sigma) were used at the indicated optimal dosage. optimal concentration of 0.318 mM for the live cell sialidase assay. Tamiflu (99% pure oseltamivir phosphate, Hoffmann-La Roche Ltd., Mississauga, Ontario, Lot # BS00060168) was used at indicated concentrations as well. Maackia amurensis lectin 2 (MAL-2) (Vector Laboratories Inc., Burlington, Ont., Canada) which binds specifically to α-2,3 sialic acid linked to terminal galactose was used in these studies at predetermined optimal dosage.

Cell lines

Stable HEK-TLR cells were obtained by calcium phosphate transfection of a pCDNA3 expression vector for a specific chimeric TLR with an in frame C-terminal YFP and selection in 0.4 µg/mL G418. The HEK-TLR4/MD2 cell line was generated by additional co-transfection of an expression plasmid for human MD2. All cells were grown at 37°C in 5% CO₂ in culture media containing DMEM (Gibco, Rockville, MD) supplemented
with 5% horse serum (Gibco) and 3% fetal calf serum (FBS) (HyClone, Logan, Utah, USA).

**Nuclear extracts and electrophoretic mobility shift (EMSA)**

Nuclear extracts of HEK-TLR4 cells were prepared by harvesting the cells before and after treatment with LPS and Tamiflu as described. Cells were lysed in a buffer consisting of 100 mM HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid), 100 mM KCl, 100 EDTA (ethylenediamine tetraacetic acid), 100 mM DTT (dithiothreitol), protease inhibitors and 10% NP-40 (Nonidet-P40). The lysates were incubated on ice for 10 min and centrifuged at 13,000 rpm for 3 min. The pellets were resuspended in 100 mM Hepes, 2 M NaCl, 5 mM EDTA and 50% glycerol, incubated on ice again for 2h and then centrifuged at 13,000 rpm for 5 min. The supernatant containing the nuclear extracts was stored at −80 °C. Electrophoretic mobility shift assay (EMSA) detects NF-κB DNA binding activity using a 3′ biotinylated double-stranded oligonucleotide sequence 5′ AGT TGA GGG GAC TTT CCC AGG C 3′ (Operon Biotechnologies Inc., Huntsville, AL) against the NF-κB binding site. The reaction volumes were 20 μl comprising of 20 μg of nuclear extract incubated at room temperature for 30min with 5 ng of the biotinylated oligonucleotide probe in binding buffer (Pierce Lightshift Chemiluminescent EMSA kit, Rockford, USA). The NF-κB bound to oligonucleotide was resolved on a 5% polyacrylamide gel in Tris/ Glycine running buffer at 20 mA constant current. The gel was transferred onto a PVDF
membrane and cross-linked by UV radiation for 3 min. The blot was probed with streptavidin-HRP at a dilution of 1:300 for 15 min at room temperature followed with Western Lighting Chemiluminescence (PerkinElmer Life Sciences, Boston). For supershift of nuclear NFκB, supernatants containing the nuclear extracts from LPS-treated HEK TLR4/MD2 cells in the presence or absence of Tamiflu were analyzed by EMSA to detect NF-κB DNA binding activity using a 3′ biotinylated double-stranded oligonucleotide (oligo) sequence 5’ AGT TGA GGGGAC TTT CCC AGGC 3′ against the NF-κB binding site. Non biotinylated oligo probe was used as a NF-κB binding competitor. Anti-NFκBp65 antibody at 0.1 μg/μL was used to block NF-κB binding to probe. The blot was probed with streptavidin-HRP followed with Western Lighting Chemiluminescence (PerkinElmer Life Sciences, Boston). Quantitative analysis was done by assessing the pixel density of band corrected for background in each lane using Corel Photo Paint 8.0 software. Each bar in the figure represents the mean corrected pixel density+SEM for n=5–10 repeated measurements. The data are a representation of one out of four independent experiments showing similar results.

**Co-immunoprecipitation and western blots**

HEK-TLR4/MD2 cells were left cultured in media or in media containing 5 μg/mL LPS for 5 min. Cells are pelleted and lysed. Cell lysates were immunoprecipitated with 2 μg of rat anti TLR4/MD2 (MTS510, Santa Cruz Biotech) antibodies for 18 h. Following immunoprecipitation, complexes were isolated using protein G magnetic beads and
resolved by 8\% gel electrophoresis (SDS-PAGE). Proteins are transferred to PVDF membrane blot. The blot was probed for either MyD88 (33 kDa) with rabbit anti-MyD88 (HFL-296, Santa Cruz Biotech), or biotinylated MAL2 lectin. The initial IP blot was stripped and further probed for described proteins. In addition, nuclear lysates were resolved by 8\% gel electrophoresis (SDS-PAGE) and the blot probed for NFKB p65 (Rel A) phospho specific pS529 with rabbit antipS529 (Rockland Immunoochemicals, Inc.), stripped and further probed with anti-\(\beta\)-actin. All blots were washed and followed with HRP conjugated secondary IgG antibodies and Western Lightning Chemiluminescence Reagent Plus. The chemiluminescence reaction was analyzed with either a Fluorchem HD2 Imaging System (Alpha Immunotech, San Leandro, CA, USA) or X-ray film. Sample concentration for gel loading was determined by Bradford reagent.
RESULTS

Neu1 sialidase forms a complex with naïve and stimulated TLRs (TLR2, TLR3 and TLR4 receptors)

Using a well established sialidase assay (Woronowicz et al., 2004, we were able to show that a sialidase is induced when ligand Lipopolysaccharide (LPS) binds to the Toll-like receptor 4 (TLR4) and functions to activate the TLR4. There are four known human sialidases and they are lysosomal Neu1 (Lukong et al., 2000; Nan et al., 2007; Starcher et al., 2008), cytosolic Neu2 and the plasma membrane bound Neu3 (Rodriguez et al., 2001; Sasaki et al., 2003; Papini et al., 2004). The fourth sialidase Neu4 is localized to either the mitochondrial (Yamaguchi et al., 2005) compartment or the lysosomal lumen (Seyrantepe et al., 2003). The first step was to identify the mammalian sialidase. Based on the neutralizing effect of specific antibodies against the four sialidases, Neu1 was found to be the candidate sialidase activated upon LPS binding to TLR4. Co-immunoprecipitation experiments using HEK-TLR4/MD2 cells further demonstrated that Neu1 (Figure 4.1) and not Neu2, -3 or -4 (Figure 4.2) forms a complex with naïve or LPS-stimulated TLR4 receptors. Although Neu2, -3 and -4 were not detected in the lysates from co-immunoprecipitation with TLR4 receptor, they were present in the whole cell lysates from LPS-treated HEK-TLR4 cells (Figure 4.2). Since HEK-TLR4 cells have a specific chimeric TLR4 with an in frame C-terminal YFP, we were able to show that Neu1 also co-immunoprecipitated with TLR4 using anti-GFP antibodies (Figure 4.1).
Conversely, TLR4 receptors co-immunoprecipitated with Neu1 in cell lysates from naïve or LPS-treated HEK-TLR4 cells but this was not seen in cell lysates from Tamiflu and LPS-treated cells. Perhaps, Tamiflu bound to Neu1 in cell lysates blocked anti-Neu1 antibodies in the immunoprecipitation assay. Surprisingly, Neu1 also co-immunoprecipitated with TLR2 and TLR3 receptors in naïve or ligand-treated HEK-TLR2 or HEK-TLR3 cells, respectively (Figure 4.3). Taken together, these results indicate that Neu1/TLR complexes are present on the cell membrane prior to ligand binding, which has not been previously observed. These findings suggest that Neu1 may be a common requisite intermediate in regulating pathogen-molecule induced TLR receptors.
Figure 4.1. Neu1 co-immunoprecipitates with TLR4. HEK-TLR4/MD2 cells with in-frame YFP were pretreated with 200 μM Tamiflu for 30 min followed with 5 μg/mL LPS for 5 min or left untreated as control (media). Cells were pelleted, lysed in lysis buffer and the protein lysates were immunoprecipitated with antibodies against the indicated proteins for 18 hrs. Immunocomplexes were isolated using protein G magnetic beads, resolved by SDS-PAGE and the blot probed with antibodies against the indicated proteins. The data are a representation of one out of five independent experiments showing similar results.
Figure 4.2. Neu2, 3 and 4 do not co-immunoprecipitate with TLR4. HEK-TLR4/MD2 cells with in-frame YFP were pretreated with 200 μM Tamiflu for 30 min followed with 5 μg/mL LPS for 5 min or left untreated as control (media). Cells were pelleted, lysed in lysis buffer and the protein lysates were immunoprecipitated with antibodies against the indicated proteins for 18 hrs. Immunocomplexes were isolated using protein G magnetic beads, resolved by SDS-PAGE and the blot probed with antibodies against the indicated proteins.
IP: anti-TLR4
Blot: anti-Neu2 47.5

Blot: anti-Neu3 47.5

Blot: anti-Neu4 47.5

Blot: anti-Neu2
→ Neu2 (42.0 kDa)

Blot: anti-Neu3
→ Neu3 (48.0 kDa)

Blot: anti-Neu4
→ Neu4 (53.0 kDa)
Figure 4.3. Neu1 co-immunoprecipitates with TLR2 and -3. HEK-TLR2 and HEK-TLR3 cells were stimulated with 200 μg/mL zymosan A or 20 μg/mL polyIC, respectively or left untreated as media control. Cells were pretreated with 200 μM Tamiflu for 30 min followed with 5 μg/mL LPS for 5 min or left untreated as control (media) and then were pelleted, lysed in lysis buffer and the protein lysates were immunoprecipitated with antibodies against the indicated proteins for 18 hrs. Immunocomplexes were isolated using protein G magnetic beads, resolved by SDS-PAGE and the blot probed with antibodies against the indicated proteins.
**Sialidase inhibitor Tamiflu inhibits LPS-induced NFκB activation in BMC-2 macrophage cells, DC-2.4 dendritic cells and primary bone marrow (BM) macrophages**

If LPS-induced NFκB activation is dependent on Neu1 sialidase activity, then neuraminidase inhibitor like Tamiflu should have an inhibitory effect on LPS-induced NFκB activation in TLR4-expressing cells. Immunocytochemistry analyses demonstrated that Tamiflu significantly inhibited NFκB activation and its translocation to the nucleus in HEK-TLR4/MD2 cells. Optimal activation of NF-kB requires phosphorylation in the transactivation domain of p65. This transactivation domain of p65 subunit is responsible for the interaction with the inhibitor IkB and contains the phosphorylation sites. A phospho-specific polyclonal antibody against the human NFκBp65 pS529 (pNFκB) was used here which has minimal reactivity with non-phosphorylated p65. This phospho-specific antibody reacts with a peptide sequence (PNGLLpSGDEDFC) corresponding a significant reduction compared to the LPS controls but not a complete block in NFκB-pS529 (Figure 4.4). Using EMSA analyses, the nuclear extracts of LPS-treated cells also contain the specific active form of NFκB DNA binding activity which is reduced to control levels in cells pretreated with Tamiflu (Figure 4.5). Anti-NFκBp65 antibodies block NFκB binding to the oligonucleotide during the reaction, reconfirming the specificity of the NFκB binding reaction (Figure 4.6).
Figure 4.4. LPS induces phosphorylated NFκBp65 pS529 (pNFκB) in TLR4-expressing cells Western blot analyses of phosphorylated NFκB (pS529) in nuclear lysates. HEK-TLR4/MD2 cells were pretreated with 400 μM Tamiflu for 30 min followed with 3 μg/mL LPS at the indicated times. Nuclear lysates from the cells were separated by SDS-PAGE and probed with antibodies against the indicated proteins. Quantitative analysis was done by assessing the density of band corrected for background in each lane using Corel Photo Paint 8.0 software. The data are a representation of one out of three independent experiments showing similar results.
Figure 4.5. LPS induces NFκBp65 in TLR4-expressing cell line. Electrophoretic mobility shift assay (EMSA) Supernatants containing the nuclear extracts from LPS-treated HEK-TLR4/MD2 cells in the presence or absence of Tamiflu were analyzed by EMSA to detect NF-κB DNA binding activity using a 3′ biotinylated double-stranded oligonucleotide (oligo) sequence 5′ AGT TGA GGG GAC TTT CCC AGG C 3′ against the NF-κB binding site. The blot was probed with streptavidin-HRP followed with Western Lighting Chemiluminescence (PerkinElmer Life Sciences, Boston). Quantitative analysis was done by assessing the pixel density of band corrected for background in each lane using Corel Photo Paint 8.0 software. Each bar in the figure represents the mean corrected pixel density±SEM for n=5 repeated measurements. The data are a representation of one out of four independent experiments showing similar results.
Supernatants containing the nuclear extracts from LPS-treated HEK-TLR4/MD2 cells in the presence or absence of Tamiflu were analyzed by EMSA to detect NF-κB DNA binding activity using a 3’ biotinylated double-stranded oligonucleotide (oligo) sequence 5’ AGT TGA GGG GAC TTT CCC AGG C 3’ against the NF-κB binding site. Non-biotinylated oligo probe was used as a NF-κB binding competitor. Anti-NFκBp65 antibody at 0.1 μg/μL was used to block NF-κB binding to probe. The blot was probed with streptavidin-HRP followed with Western Lighting Chemiluminescence (PerkinElmer Life Sciences, Boston, USA)
Identification of sialyl residue(s) involved in TLR ligand-induced NFκB activation

We showed evidence that Neu1 sialidase is already in complex with TLR-2, -3 and -4 receptors on the cell surface, and is induced upon ligand binding to either receptor. So we think that activation of Neu1 sialidase by TLR ligands binding to their receptors may potentially target one or more of N-glycan residues located within the ectodomain of TLR receptors. Mutational studies of TLR glycosylated residues have suggested that these N-glycan residues play a critical role in receptor activation and their modification may be a cellular mechanism of controlling TLR activation in promoting dimerization (da Silva et al., 2002; Weber et al., 2004; Sun et al., 2006). To determine whether or not a sialyl residue(s) is involved in TLR ligand induced NFκB activation, it should be possible to prevent NFκB activation by blocking these residues with specific lectins prior to ligand stimulation. It is noteworthy that neither MAL-2 nor SNA treatment of the BMC-2 cells blocked LPS binding to TLR4. MAL-2 lectin blot analyses revealed that immunoprecipitated LPS-stimulated TLR4 receptors lacked α-2,3-sialyl specific residues compared to non-stimulated cells (Figure 4.7). Together, these results signify the importance of sialyl α-2,3-linked β-galactosyl residues of TLR4 distant from LPS binding sites as targets for Neu1 sialidase activity associated with TLR ligand treated cells.
Figure 4.7. MAL-2 lectin blot. HEK-TLR4/MD2 cells were stimulated with 5 μg/mL LPS for 5 min or left untreated as control (media). Immunoprecipitation using anti-TLR4 antibodies was performed on whole-cell lysates from media control or LPS-stimulated cells. The blots were probed with biotinylated MAL-2 lectin followed with HRP conjugated streptavidin, and stripped and further probed with anti-TLR4 and HRP conjugated secondary antibody. Three independent experiments are shown with similar results.
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<th>Experiment</th>
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<td>Blot: Biotin-MAL-2</td>
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Removal of α-2,3-sialyl residues linked to β-galactosides by Neu1 sialidase associated with LPS treated TLR4-expressing cells is essential for TLR dimerization to facilitate TLR4/MyD88 complex recruitment

If the hydrolysis of α-2,3-sialyl residues on TLR4 receptors by Neu1 sialidase activity is essential for MyD88/TLR4 complex formation, we should be able to inhibit MyD88/TLR4 recruitment and formation with either α-2,3-sialyl specific MAL-2 lectin or neuraminidase inhibitor, Tamiflu. Using MyD88 co-immunoprecipitation with TLR4 in cell lysates from LPS stimulated HEK-TLR4/MD2 cells, both MAL-2 and Tamiflu pretreatment of LPS-stimulated cells caused a reduction of MyD88/TLR4 complex formation compared to LPS-stimulated cells alone (Figure 4.8).
Figure 4.8. Tamiflu and MALII inhibit LPS-induced TLR4/MyD88 complex formation. HEK-TLR4/MD2 cells were pretreated with either 200 μM Tamiflu or 100 μg/mL MAL-2 for 30 min followed with 5 μg/mL LPS for 5 min or left untreated as media control (media). Co-immunoprecipitation blots using the indicated antibodies were performed on whole-cell lysates from media control, LPS treated, LPS and Tamiflu, or LPS and MAL-2 treated cells. The blot was sequentially probed for the indicated proteins. The chemiluminescence reaction was analyzed with a Fluorchem HD2 Imaging system (San Leandro, CA, USA) or X-ray film. The data are a representation of one out of four independent experiments showing similar results.
IP: anti-TLR4
Blot: anti-TLR4
MW kDa
media LPS LPS + Tamiflu LPS + MAL-2
83 TLR4 (89 kDa)

IP: anti-TLR4
Blot: anti-MyD88
47 MyD88 (33 kDa)
33
DISCUSSION

The molecular mechanisms by which TLR receptors become activated or even inhibited are not well understood. The data presented in this report provide evidence that Neu1 sialidase may be an important intermediate link in the initial process of ligand induced TLR activation and subsequent cell function. They indicate an initial rapid activation of Neu1 activity which is only induced by TLR ligand binding. Central to this process is that Neu1 and not the other three mammalian sialidases forms a complex with TLR-2, -3 and -4 receptors in naïve and LPS-stimulated TLR4-expressing cells. This would actually make Neu1 complexed with TLRs readily available to be induced upon TLR ligand binding. The data presented in this report suggest that at least for TLR-2, -3 and -4 receptors the initial mechanism for TLR activation and subsequent cell function is dependent on Neu1 sialidase activity. Co-immunoprecipitation data indicate that Neu1/TLR complexes are already formed on the cell membrane in naïve TLR4-expressing HEK-TLR4 cell lines. TLR ligand binding to its receptor may induce allosteric conformational changes in the TLR receptor, which in turn might activate Neu1 sialidase. It is proposed that activated Neu1 would then hydrolyze sialic acids to facilitate TLR dimerization and activation. It is known that LPS signals through TLR4 in complex with MD-2 and CD14, and each of these proteins is glycosylated (Ohnishi et al., 2001; da Silva et al., 2002). MD-2 and the amino terminal ectodomain of human TLR4 potentially contain two and nine N-glycosylation sites, respectively, based on their sequence analysis. Studies with TLR4 and MD-2 mutants lacking N-linked glycosylation sites have
shown that these N-linked sites are essential to maintaining the functional integrity of this LPS receptor complex (da Silva et al., 2002). It is possible that potentially one or more of these N-linked glycosylation sites of TLR4/MD2 may be targets for Neu1 sialidase activity and thus they may play an important role in the activation of the TLR receptors and subsequent cellular bioactivity. The findings in this report suggest that Neu1 sialidase may be a key regulator of pathogen-molecule induced TLR activation to generate a functional receptor.

Neu1 sialidase activity associated with TLR ligand treated live cells rapidly hydrolyzes α-2,3-sialyl residues linked to β-galactosides of TLR receptors to facilitate TLR dimerization, MyD88/TLR4 complex formation and subsequent cellular signaling to activate NFκB. How the process of Neu1 sialidase associated with TLR is induced by TLR ligand binding to its receptor remains unknown. It is possible that conformational changes induced by ligand binding within the ectodomain of the receptor may facilitate this Neu1 activation process. Indeed, others have demonstrated that the binding of DNA containing CpG leads to substantial conformational changes in the TLR9 ectodomain (Latz et al., 2007; Ewald et al., 2008; Haas et al., 2008). Perhaps, a similar TLR ligand induced conformational change(s) in TLR ectodomain might in turn induce Neu1 sialidase activity.

The data presented in this report further signifies an important role of Neu1 sialidase as an intermediate link in the initial process of ligand induced TLR activation and
subsequent cellular signaling. The premise is that Neu1 forms a complex with glycosylated TLR receptors within the ectodomain. Secondly, Neu1 may be a requisite intermediate in regulating TLR activation following TLR ligand binding to the receptor. Thirdly, activated Neu1 by TLR ligand binding to the receptor rapidly removes α-2,3-sialyl residues linked to β-galactosides on TLR ectodomains to generate a functional TLR receptor. Although there are four identified mammalian sialidases classified according to their subcellular localization, we show that the sialidases classified as cytosolic (Neu2), plasma membrane bound (Neu3) and Neu4 are not involved in the sialidase activity associated with TLR ligand treated live primary macrophage cells and macrophage and dendritic cell lines.

As mentioned earlier, TLR signaling events classically involve the MyD88/interleukin-1 receptor-associated kinase (IRAK) signaling pathway and the interferon-regulatory factor (IRF) family in TLR signaling. These downstream signaling events are the MyD88-dependent branch which leads to NFκB activation. Since MyD88 is the central adaptor molecule interacting with all TLRs except TLR3, the findings in this report indicate that the loss of α-2,3-sialyl residues enables TLR dimerization by removing a steric hinderance to receptor association, and the subsequent recruitment of MyD88 to the receptor.
In conclusion, we identify a critical sialyl residue(s) involved in LPS-induced TLR4 activation. The premise is that Neu1 sialidase activity associated with TLR ligand treated cells rapidly targets one or more $\alpha$-2,3-sialyl residues of TLR4. The removal of these residues by Neu1 sialidase activity facilitates TLR dimerization, MyD88/TLR complex formation and subsequent NFkB activation. These findings uncover a novel desialylation process of TLR receptors by Neu1 sialidase in complex with the receptor, which initiates receptor activation following ligand binding for cellular signaling.
CHAPTER 5

SUMMARY AND PERSPECTIVES

THE ROLE OF NEU1 IN Trk TYROSINE KINASE RECEPTOR ACTIVATION

Although there is evidence that glycosylation of Trk receptors is important for the localization of the receptor to the cell surface, the role of glycosylation in receptor-ligand interaction is not fully understood. Insight for the role of glycosylation in Trk receptor activation came from our published findings on the model of TrkA family of tyrosine-protein kinase demonstrating the importance of the removal of sialyl α-2,3-linked β-galactosyl residues of the Trk receptor by sialidase for receptor dimerization and subsequent activation. Here, we report that ligand binding to Trk induces Neu1 sialidase activity which influences the desialylation and, consequently, the induction of Trk receptor activation. These observations suggest for the first time that Trk receptor activation is regulated by Neu1 sialidase induction, and thus we identify a critical parameter involved with ligand binding to the Trk receptor, which has not been previously observed.

Also, we show that ligand-induced Neu1 activation is blocked in the presence of the neuraminidase inhibitor Tamiflu and anti-Neu1 antibodies. Tamiflu and the neutralizing antibody blocked both ligand-induced sialidase activity on the cell surface and
phosphorylation of Trk and subsequent neurite outgrowth. We also show evidence that Neu1 is activated in TrkB-expressing primary neurons. Our cumulative results reveal for the first time that Trk receptor activation is dependent on Neu1 induction upon ligand binding to the receptor and that Neu1 plays a critical role in the regulation of Trk receptor activation.

**THE MECHANISM OF LIGAND-INDUCED NEU1 SIALIDASE ACTIVATION**

To date, the precise molecular process(es) that modulate the activation and signaling of Trk receptors is not clear. Apart from identifying the Neu1 sialidase involvement in ligand-induced Trk receptor activation, we also aimed at elucidating the mechanism of Neu1 activation during this process.

Based on existing evidence that Trk receptors can be activated by G-protein coupled receptor (GPCR) ligands, we hypothesized that GPCRs are involved in this sialidase activation. A link between intracellular matrix metalloproteinase (MMP) activity and GPCR-mediated signaling has been reported to explain the mechanism for transactivation of epidermal growth factor receptor (EGFR). It is proposed that GPCR activation results in the activation of EGFR via MMP-mediated release of EGFR ligands. This mechanism of transactivation provided us a clue that perhaps MMPs are also involved in the activation of Neu1 sialidase. Using specific inhibitors in our published sialidase assay, we observed the inhibition of Neu1 sialidase in the presence of Gαi protein sensitive
pertussis toxin and broad range matrix metalloproteinase (MMP) inhibitors, galardin and piperazine. These data suggest that Ga\textsubscript{i} proteins in alliance with MMP(s) are involved in Neu1 activation associated with ligand-induced Trk activation in live Trk-expressing cells.

The precise molecular mechanism(s) underlying GPCR-mediated MMP activation still remains unknown. It can be speculated that neurotrophin binding to the receptor on the cell surface initiates GPCR-signaling via GPCR Ga\textsubscript{i} subunit proteins to activate MMP. It is well known that agonist-bound GPCRs activate numerous MMPs (Fischer \textit{et al.}, 2006) as well as members of the ADAM family of metalloproteases (Prenzel \textit{et al.}, 1999; Gooz \textit{et al.}, 2006). This paradigm would predict a conformational change following neurotrophin binding. It has been reported that upon association of NGF with TrkA, a minor conformational change can occur to form a complex (Woo \textit{et al.}, 1998). Others have shown that p75\textsuperscript{NTR} binds along the homodimeric interface of NGF, which disables NGF's symmetry-related second p75 binding site through an allosteric conformational change (He and Garcia, 2004). Surprisingly, we have shown in this report that NGF or BDNF binding to p75\textsuperscript{NTR} in 3T3 cells stably expressing p75\textsuperscript{NTR} induces sialidase activity which is inhibited by Tamiflu and by anti-Neu1 antibodies. It appears that Neu1 is an intermediate link in the initial process of neurotrophin induced p75\textsuperscript{NTR} activation, which has not been previously observed.
Tamiflu (oseltamivir phosphate) which is the ethyl ester pro-drug of oseltamivir carboxylate was found to be highly potent (IC$_{50}$ 3.876 μM) in inhibiting Neu1 activity induced by NGF treatment of live TrkA-PC12 cells. The other neuraminidase inhibitors oseltamivir carboxylate and zanamivir had limited inhibitory effect on Neu1 sialidase activity associated with NGF treated live TrkA-PC12 cells. The reason for this inhibitory potency of Tamiflu on Neu1 sialidase activity is unknown. However, it may be due to a unique orientation of Neu1 with the molecular multi-enzymatic complex that contains β-galactosidase and cathepsin A (Lukong et al., 2000) and elastin-binding protein (EBP) (Hinek et al., 2006), the complex of which would be associated within the ectodomain of Trk receptors. Stomatos and colleagues have also shown that Neu1 on the cell surface is tightly associated with a subunit of cathepsin A and the resulting complex influences cell surface sialic acid in activated cells and the production of IFNγ (Nan et al., 2007). It has also been shown using Neu1-deficient mice that they produce markedly less IgE and IgG1 antibodies following immunization with protein antigens, which may be the result of their failure to produce IL-4 cytokine (Chen et al., 1997). Another possibility may involve Tamiflu’s direct effect on Neu1 sialidase with specificity for sialyl α-2,3-linked β-galactosyl residues of Trk receptors. On the cell surface, Seyrantepe et al. have recently shown that Neu1 can actually activate phagocytosis in macrophages and dendritic cells through the desialylation of surface receptors, including Fc receptors for immunoglobulin G (FcγR) (Seyrantepe et al., 2010).
The data presented in this thesis further signifies an important role of Neu1 sialidase as an intermediate link in the initial process of ligand-induced Trk tyrosine kinase receptor activation and subsequent cellular function. The premise is that Neu1 forms a complex with glycosylated Trk receptors within the ectodomain, which is consistent with our previous report with TLR receptors (Amith et al., 2009). Secondly, Neu1 may be a requisite intermediate in regulating Trk activation following neurotrophin binding to the receptor. Thirdly, activated Neu1 by neurotrophin binding to the receptor predicts a rapid removal of α-2,3-sialyl residues linked to β-galactosides on Trk ectodomain to generate a functional Trk receptor based on our previous report (Amith et al., 2010). Fourthly, the potentiation of GPCR-signaling via membrane targeting of Gai subunit proteins and matrix metalloproteinase-9 activation by ligand binding to the receptor is involved in the activation process of Neu1 sialidase on the cell surface. Using confocal microscopy on permeabilized naïve and neurotrophin-treated cells and co-immunoprecipitation experiments, the additional intracellular and cell surface co-localization of Neu1, MMP-9 and TrkA validated the predicted association of MMP-9 with Neu1 in alliance with Trk receptors.

Taken all together, the findings in this thesis suggest that Neu1 sialidase and MMP-9 cross-talk in alliance with Trk receptors may be the key regulators of neurotrophin-induced Trk activation to generate a functional receptor. Further studies on the interaction of Neu1 and MMP-9 with Trk receptors can further be corroborated by using the
bioluminescent resonance energy transfer (BRET) analysis, a technique used to measure protein-protein interactions in cells.

In our experimental model of Trk receptor activation (Figure 5.1), we propose that when NGF binds to TrkA involving Neu1/MMP-9 tripartite complex, a conformational change in the receptor activates an adjacent GPCR via a heterotrimeric Gαi protein. This GPCR-mediated activation results in the subsequent activation of MMP-9 which cleaves the EBP from the elastin receptor complex of Neu1/cathepsin A/EBP on the cell surface. It is likely that the removal of EBP results in the catalytic activation of Neu1 and allows for desialylation of Trk receptors enabling the removal of steric hinderance to receptor association for Trk receptor activation.
Figure 5.1. Proposed model for NGF-induced TrkA activation via Neu1 activation. We propose that when NGF binds to TrkA receptor, it results in a conformational change activating one of the GPCR Ga\textsubscript{i} proteins, thus activating the MMP-9 which is already complexed to the Trk receptor. Activated MMP-9 then removes the elastin binding protein EBP from the Neu1/cathepsin A/EBP complex and catalytically induces Neu1 sialidase. Neu1 sialidase that is found in complex with TrkA receptor on the cell surface then specifically targets and hydrolyzes the \(\alpha\)-2,3-sialic acids on TrkA receptor, thus priming the receptor for dimerization and phosphorylation of the TrkA receptors.
IS NEU1 SIALIDASE A MASTER ENZYME REGULATING OTHER MEMBERS OF THE TYROSINE KINASE RECEPTORS?

The data presented in this thesis provide evidence for an important role for Neu1 sialidase in the activation of TrkA and TrkB receptors. Published reports from our laboratory have also shown that Toll-like receptor activation is also dependent on Neu1 activation. They suggest that Neu1 sialidase might be involved in activating various other membrane-associated receptors.

Preliminary experiments using sialidase assays were conducted on live A431 cells, a human epithelial carcinoma cell line that express both epidermal growth factor receptors (EGFRs) and Insulin receptors. Tamiflu and anti-Neu1 antibodies blocked sialidase activity associated with ligand binding to these receptors in live A431 cells (Figures 5.2 and 5.3). These observations suggest that Neu1 sialidase maybe a common enzyme involved in the regulation of other glycosylated tyrosine kinase receptors like EGFRs and Insulin receptors. Further experiments are needed to substantiate our preliminary findings, thus expanding the potential roles of Neu1 in the regulation of various immune and growth factor receptors.
Figure 5.2. EGF induces Neu1 sialidase activity in EGFR-expressing A431 cells. A431 (human carcinoma) were grown in media containing 5% fetal bovine sera for 24h at 37°C. After removing media, 0.138 mM 4-MUNANA (4-MU) substrate [2’-(4-methlyumbelliferyl)-α-D-N-acetylneuraminic acid] (Sigma) in Tris buffered saline pH 7.4 was added to cells alone (Control) with EGF (250 ug/mL) or with EGF in combination either with 250 ug/mL Tamiflu (broad range Neuraminidase inhibitor), or 250 ug/mL Anti-Neu1 (Neutralizing antibody against Neu1 sialidase). Sialidase activity is inhibited by the neutralizing effect of Anti-Neu1 antibody indicating the activation of Neu1 sialidase upon ligand stimulation. The substrate is hydrolysed by sialidase to give free 4-methylumbelliferone which has a fluorescence emission at 450nm (blue color) following excitation at 365nm. Fluorescent images were taken at 1 min after adding substrate using epi-fluorescent microscopy (40x objective). The mean fluorescence surrounding the cells for n ≥ 50 replicates in each of the images was measured using Image J Version 1.43 Software (NIH). The data are a representation of one out of three independent experiments showing similar results.
Figure 5.3. Insulin induces Neu1 sialidase activity in Insulin-expressing A431 cells.
A431 (human carcinoma) cells were grown in media containing 5% fetal bovine sera for 24h at 37°C. After removing media, 0.138 mM 4-MUNANA (4-MU) substrate [2’-(4-methlyumbelliferyl)-α-D-N-acetylneuraminic acid] (Sigma) in Tris buffered saline pH 7.4 was added to cells alone (Control) with Insulin (15 µM) or with Insulin in combination either with 250 µg/mL Tamiflu (broad range Neuraminidase inhibitor), or 250 µg/mL Anti-Neu1 (Neutralizing antibody against Neu1 sialidase). Sialidase activity is inhibited by the neutralizing effect of Anti-Neu1 antibody indicating the activation of Neu1 sialidase upon ligand stimulation. The substrate is hydrolysed by sialidase to give free 4-methylumbelliferone which has a fluorescence emission at 450nm (blue color) following excitation at 365 nm. Fluorescent images were taken at 1 min after adding substrate using epi-fluorescent microscopy (40x objective). The mean fluorescence surrounding the cells for n ≥ 50 replicates in each of the images was measured using Image J Version 1.43 Software (NIH). The data are a representation of one out of three independent experiments showing similar results.
Trk receptors play vital roles in various neurodegenerative disorders and cancers. Understanding the components of Trk receptor signalling is pivotal in elucidating their mechanism of action. Our data provide key insights to the molecular mechanism of Neu1 sialidase regulation of Trk activation and thus aid in providing opportunities for manipulation of Trk receptor activation in restoring impaired neuronal function.

This novel role for Neu1 sialidase in the control of receptor signaling will open a whole new area of glycobiology research and will widen the scope for the development of novel therapeutic drugs to combat human diseases.
REFERENCES


204


