Title
Novel Construct to Study the Functional Effects of SNPs on TrkA Protein Interactions and Localization

Permalink
https://escholarship.org/uc/item/30t9z470

Author
Chin, Brian Wai Wen

Publication Date
2015

Peer reviewed|Thesis/dissertation
A Novel Construct to Study the Functional Effects of SNPs on TrkA Protein Interactions and Localization

A thesis submitted in partial satisfaction of the requirements for the degree Master of Science in Biology by Brian Wai Wen Chin

Committee in charge:
Professor John Kelsoe, Chair
Professor Laurie Smith, Co-chair
Professor Deborah Yelon

2015
The Thesis of Brian Wai Wen Chin is approved, and it is acceptable in quality and form for publication on microfilm and electronically:

Co-Chair

Chair

University of California, San Diego

2015
# TABLE OF CONTENTS

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>Signature Page</td>
<td>iii</td>
</tr>
<tr>
<td>Table of Contents</td>
<td>iv</td>
</tr>
<tr>
<td>List of Figures</td>
<td>vi</td>
</tr>
<tr>
<td>List of Abbreviations</td>
<td>vii</td>
</tr>
<tr>
<td>Acknowledgements</td>
<td>ix</td>
</tr>
<tr>
<td>Abstract of the Thesis</td>
<td>x</td>
</tr>
<tr>
<td>Introduction</td>
<td>1</td>
</tr>
<tr>
<td>Overview of Bipolar Genetics and Importance of NTRK1</td>
<td>1</td>
</tr>
<tr>
<td>TrkA Structure and Function</td>
<td>3</td>
</tr>
<tr>
<td>TrkA Co-Factors and Signaling</td>
<td>5</td>
</tr>
<tr>
<td>Phosphatidylinositol Based Pathways</td>
<td>6</td>
</tr>
<tr>
<td>Grb2/Sos Adaptor Proteins</td>
<td>7</td>
</tr>
<tr>
<td>Signal Modifying Pathways</td>
<td>9</td>
</tr>
<tr>
<td>TrkA Interactions with p75</td>
<td>11</td>
</tr>
<tr>
<td>TrkA Post-translational Modification</td>
<td>12</td>
</tr>
<tr>
<td>Results</td>
<td>14</td>
</tr>
<tr>
<td>Cell Line Selection</td>
<td>14</td>
</tr>
<tr>
<td>Initial Studies with N-terminus HaloTag® TrkA Construct</td>
<td>16</td>
</tr>
<tr>
<td>Designing a Construct Capable of Producing Mature TrkA</td>
<td>18</td>
</tr>
<tr>
<td>Fluorescent Imaging of C-Terminus HaloTag® TrkA</td>
<td>19</td>
</tr>
<tr>
<td>Discussion</td>
<td>21</td>
</tr>
<tr>
<td>Development of the Construct</td>
<td>21</td>
</tr>
<tr>
<td>Section</td>
<td>Page</td>
</tr>
<tr>
<td>---------------------------------------------------------------</td>
<td>------</td>
</tr>
<tr>
<td>Future Uses/Follow-up Studies</td>
<td>22</td>
</tr>
<tr>
<td>Materials and Methods</td>
<td>25</td>
</tr>
<tr>
<td>Cloning</td>
<td>25</td>
</tr>
<tr>
<td>Cell Culture</td>
<td>26</td>
</tr>
<tr>
<td>HaloTag® Pulldown</td>
<td>26</td>
</tr>
<tr>
<td>Western Blots</td>
<td>27</td>
</tr>
<tr>
<td>Appendix</td>
<td>28</td>
</tr>
<tr>
<td>Wild-type and Mutant TrkA Sequence Alignment</td>
<td>28</td>
</tr>
<tr>
<td>References</td>
<td>33</td>
</tr>
</tbody>
</table>
LIST OF FIGURES

Figure 1: TrkA Structure and Phosphorylation Sites............................................. 4
Figure 2: TrkA Co-factors and Pathways................................................................. 5
Figure 3: PI Based Pathways.................................................................................. 6
Figure 4: Grb/Sos Adaptor Proteins........................................................................ 7
Figure 5: Signal Modifying Pathways..................................................................... 9
Figure 6: Transfection Efficiency Tests using GFP.................................................. 14
   Figure 6A: Transfection of SK-N-MC via Lipofectin®............................................ 14
   Figure 6B: Transfection of HEK-293T via Lipofectin®.......................................... 14
   Figure 6C: Transfection of HEK-293T via Lipofectamine®..................................... 14
Figure 7: HEK-293T Cell Lysate anti-SHC Western Blot.......................................... 15
Figure 8: N-Terminus HaloTag® TrkA anti-Total TrkA Western Blot.................... 17
   Figure 8A: NGF Treated....................................................................................... 17
   Figure 8B: NGF Untreated................................................................................. 17
   Figure 8C: Untransfected HEK-293T Cell Lysate.............................................. 17
Figure 9: C-Terminus HaloTag® TrkA anti-Total TrkA Western Blot............... 19
Figure 10: Live Cell Visualization of C-Terminus HaloTag® TrkA..................... 20
LIST OF ABBREVIATIONS

Abl: Abelson murine leukemia viral oncogene
Akt: protein kinase B
ANK3: ankyrin-3
Bcl-2: B-cell lymphoma 2
BD: Bipolar disorder
CACNG2: calcium channel, voltage-dependent, gamma subunit 2 (stargazin)
Crk: CT10 regulator of kinase
FKHRL1: forkhead transcription factor
FRS2: fibroblast growth factor receptor substrate 2
GAB2: GRB2 associated binding protein 2
GFP: green fluorescent protein
GSK: glycogen synthase kinase
HEK-293T: human embryonic kidney 293T cell line
MAPK: mitogen activated protein kinase
NFκB: nuclear factor kappa B
NGF: nerve growth factor
NTRK1: neurotrophic tyrosine receptor kinase 1
PDK1: phosphoinositol dependent kinase 1
PI: phosphatidylinositol
PI3K: phosphatidylinositol 3 kinase
PKC: protein kinase C
PLCγ1: phospholipase C gamma 1
p75: low-affinity nerve growth factor receptor
Rac: Ras-related C3 botulinum toxin substrate
Raf: rapidly accelerated fibrosarcoma
Rap1: Ras proximate 1
Ras: rat sarcoma
RTK: receptor tyrosine kinase
SHC: Src homology 2 domain containing transforming protein
SH2B1: Src homology 2-B adaptor protein 1
SH2B2: Src homology 2-B adaptor protein 2
SK-N-MC: human neuroblastoma cell line
Sos: son of sevenless
ACKNOWLEDGEMENTS

I would like to acknowledge Professor John Kelsoe for providing me the opportunity to work on this project. It has been a truly eye-opening experience, and I have developed a great deal both as a scientist and as a person while tackling the challenges encountered along the way.

I would also like to acknowledge our lab manager, Tanya Shekhtman, for helping me hone my technical skills and providing so much support throughout my endeavors. Her guidance helped orient me during the early stages of my project and helped me maintain my traction throughout.

Furthermore, I would like to thank our neighbors the Zhou lab for their assistance. In particular, I would like to acknowledge Xianjin Zhou and Baohu Ji whose combined expertise provided valuable insight for overcoming many of the obstacles I encountered.

Finally, I want to thank Dr. Laurie Smith and Dr. Deborah Yelon for their counsel and participation on my committee. It has been a pleasure and an honor to work with them.
ABSTRACT OF THE THESIS

A Novel Construct to Study the Functional Effects of SNPs on TrkA Protein Interactions and Localization

by

Brian Wai Wen Chin

Master of Science in Biology

University of California, San Diego, 2015

Professor John Kelsoe, Chair
Professor Laurie Smith, Co-Chair

A unique family has previously been reported where medullary cystic kidney disease and bipolar disorder segregated very closely together (Kimmel et al, 2005). This lead to the isolation of a novel SNP (rs144901788) in the NTRK1 gene. NTRK1 codes for a neurotrophic receptor tyrosine kinase known as TrkA
that signals down well-known survival and mitogenic pathways such as MAPK/ERK. Understanding how TrkA function is modified in these individuals may help shed light on a possible mechanism for bipolar disorder.

In order to examine any functional effects said SNP may have TrkA function, we procured a construct from Kazusa containing TrkA with Promega’s proprietary HaloTag® conjugated to the N-terminus. Based off the initial results with the construct, we determined that protein product would not provide an accurate reproduction of TrkA function. As a result, we produced an alternate construct capable of producing tagged, recombinant TrkA. Here, we characterize the products of said construct via western blot and live-cell fluorescent visualization and demonstrate its suitability as a model for studying SNPs in TrkA.
INTRODUCTION

Bipolar Disorder (BD) is a mood disorder that is characterized by severe fluctuations between manic and depressive phases. The intensity and duration of these phases can vary significantly between individuals affected by the disorder (NIH, n.d.). Given the broad spectrum of symptoms, it would be greatly beneficial to find a mechanism through which such a range could be caused. One of the more popular theories is that the bipolar spectrum arises from the amalgamation of many susceptibility alleles within the human genome (Kelsoe 2003). We can then associate specific genotypes and genes with varying severities of bipolar disorder. These genes and genotypes can then be further examined to determine the physical mechanisms through which they may play a role in bipolar disorder.

Overview of Bipolar Genetics and Importance of NTRK1

Much of the work done so far to find suspect genes behind BD has been conducted in the form of genome-wide association studies meant to link bipolar phenotypes with target genes. This sort of broad, shotgun approach to bipolar has helped shed light on some of the most prominent genes behind bipolar susceptibility such as calcium channel CACNG2 and sodium channel associated protein ANK3 (Koefoed et al, 2011). However, rarer or less obvious alleles tend to fly under the radar of large studies such as these. Studying these rare alleles can shed light on peripheral pathways that may play a role in BD in addition to
enhancing our understanding of the molecular mechanisms of BD. In order to pick-up on these more discrete mutations, linkage studies are often performed. Given the heritable nature of bipolar disorder, it is sometimes possible to locate susceptibility genes by tracing bipolar segregation with another phenotype (Kelsoe 2003, DePaulo 2004). In this case, we sequenced bipolar individuals within a family where BD was heavily linked with medullary cystic kidney disease (Kimmel et al, 2005). By examining the region around the MCKD-associated mucin 1 gene, we were able to isolate a single nucleotide polymorphism (SNP) in the neurotrophic tyrosine receptor kinase 1 (NTRK1) gene. This particular SNP, rs144901788, is a guanine to adenine substitution at position 1474 in the NTRK1 gene. Consequently, this causes amino acid 492 to change from a glutamate to a lysine. NTRK1 codes for the TrkA receptor tyrosine kinase, which helps promote neuronal proliferation and differentiation. Consistent with its function, it has also been shown to signal through large mitogenic pathways such as MAPK. Additionally, it is thought that TrkA mainly effects cholinergic neurons, which have been implicated to play a role in depression when either over or under-stimulated (Gibbons et al, 2009, Mineur et al, 2010). As a result, it may be possible that differences in TrkA function can affect the abundance or development of cholinergic neurons in a manner that causes the depressive phenotypes seen in BD. This would identify NTRK1 as a BD susceptibility gene, which would help further explain BD heritability.
TrkA Structure and Function

TrkA, NTRK1’s protein product, is a high-affinity receptor tyrosine kinase (RTK) for nerve growth factor (NGF). As is common for RTKs, TrkA undergoes dimerization followed by auto-phosphorylation at various tyrosine residues upon binding its ligand: NGF. This subsequently activates the protein and allows it to initiate its signaling cascade. Abnormalities within this cascade may be responsible for BD susceptibility. The overall structure of the protein is composed of an immunoglobulin-like extracellular domain, a transmembrane domain, a juxtamembrane region, the kinase region, and a final C-terminal domain (Weismann et al, 2001). The extracellular domain is formed of two IgG-like regions and a leucine-rich motif cassette that contains three linked leucine-rich motifs. It is the second of these motifs that participates primarily in binding NGF (Windisch et al, 1995). Upon activation, TrkA trans-phosphorylates tyrosine residues: Y490, Y670, Y674, Y675, Y751, and Y785, which span the juxtamembrane, kinase, and C-terminal domains of the protein (Jung et al, 2013, Segal et al, 1996).
In general, TrkA is believed to mainly activate the MAPK pathway to promote cell survival and differentiation although this varies between splice forms. To date, TrkA has three characterized isoforms (TrkA I, TrkA II, and TrkA III). TrkA I differs from TrkA II by six amino acids although the primary difference between the two is the tissues in which they are expressed. TrkA I, the isoform without the six amino acid insert, is primarily expressed in non-neuronal tissues while TrkA II is primarily found within neurons (Bothwell, 1996). Nonetheless, their structure and functions remain similar (Barker et al, 1993, Brodeur et al, 2009). TrkA III differs much more significantly in both structure and function. It is by far the smallest isoform, which is partially due to a truncated extracellular signaling region that does not respond to NGF. Additionally, TrkA III is
constitutively active and primarily works through the PI3K pathway to promote cellular survival and angiogenesis (Brodeur et al, 2009). For our purposes, we will primarily be focusing on the TrkA II isoform as it is responsive to NGF and is the main variant found within neurons.

**TrkA Co-Factors and Signaling**

TrkA has a plethora of co-factors found within neurons through which it enacts neuron cellular proliferation and survival. This cascade can be roughly divided into phosphatidylinositol (PI) based pathways, Grb2/Sos adaptor proteins, and signal modifying pathways. As for how these co-factors may play a role in BD, all of these pathways are capable of effecting TrkA mitogenic activity in some manner. The subsequent effect on cell proliferation could be the root of the observed BD susceptibility.

*Figure 2: TrkA Co-factors and Pathways. We can categorize these pathways into three main “types”*
Among TrkA’s many signaling pathways are two PI based co-factors. One is phosphatidylinositol 3 kinase (PI3K), which has been shown to bind TrkA when tyrosine 751 (Y751) is phosphorylated. This specific tyrosine residue is located at the very end of the protein’s kinase domain. Upon activation, PI3K begins to phosphorylate membrane phosphoinositides that then go on to bind with PDK1 (Obermeier et al, 1993). PDK1 then in turn phosphorylates Akt, which leads to the downstream activation of various pathways such as the transcription factor NFκB, GSK (glycogen synthase kinase), forkhead transcription factor (FKHRL1), and Bcl-2 (B-cell lymphoma 2 that assist in promoting cellular survival (Patapoutian et al, 2001, Soltoff et al, 1992). Alternatively, it has also been shown that PI3K is capable of activating PKC (protein kinase C) activity in certain cells, which promotes cell growth and differentiation (Plo et al, 2004).

The other major PI based co-factor that interacts with TrkA is phospholipase C gamma 1 (PLCγ1). The protein itself binds to phosphorylated tyrosine 785 (Y785) within the non-catalytic C-terminal end region. Since
PLCγ1’s and PI3K’s binding sites are at distinct tyrosine residues, both co-factors are capable of associating with TrkA at the same time (Obermeier et al, 1993). Upon binding, PLCγ1 catalyzes the production of inositol triphosphate (IP3), which in turn stimulates Ca^{2+} release and activates PKC (Althaus et al, 1997, Plo et al, 2004). The activated PKC then feeds into the MAPK pathway via MEK1/2. In this sense, PKC serves as a joining point for the signaling cascades induced by PI3K and PLCγ1. The main difference, in this case, is that PLCγ1 primarily signals through PKC while PI3K works through both PKC and Akt. Overall, changes to either of these pathways may influence neuronal proliferation and survival thereby increasing the chance of BD.

**Grb2/Sos Adaptor Proteins**

There are four main Grb2/Sos adaptor proteins that bind to TrkA, and two each bind to either the tyrosine 490 (Y490) or within the kinase domain. Of the two that bind to tyrosine 490, the most well-known is SHC. It is phosphorylated upon binding to activated TrkA, which enables it to then bind to the Grb2/Sos...
complex. The Grb2/Sos complex subsequently binds and activates the Ras G-protein, which phosphorylates Raf and feeds into the MAPK pathway (Rozakis-Adcock, 1992, McCormick, 1994). Similarly, FRS2 is another protein partner that is capable of binding TrkA’s phosphorylated Y490, and it has been speculated that it acts as a competitor with SHC. However, it has also been shown to be capable of binding the Grb2/Sos and thereby is capable of activating Ras and thereby activating MAPK as well (Sofroniew et al, 2001, Ong et al, 2000). In either case, binding of adaptor proteins to Y490 is one of the oldest and most well understood pathways through which TrkA activates MAPK. Additionally, our SNP of interest, rs144901788, is located very close to this binding site. As a result, modulation of Y490 phosphorylation and SHC binding are the two main ways through which we believe our SNP may be affecting TrkA function.

However, it is crucial to consider alternatives. The SH2B family of adaptor proteins, SH2B1 and SH2B2, primarily bind to TrkA via the kinase domain and have been shown to be capable of binding even in TrkA mutants that lacked Y490 and Y785 (Qian et al, 1998, Qian et al, 2001). They also assist with cell survival by binding the Grb2/Sos complex; thereby activating the MAPK pathway through Ras. It has also been thought that they may play a novel role in TrkA regulation through modulation of the TrkA’s auto-phosphorylation (Qian et al, 1998, Qian et al, 2001). Therefore, an alternative method through which SNP-induced changes may cause TrkA misregulation is through its interactions with the SH2B proteins. Overall, MAPK is a well-characterized mitogenic pathway,
and modifications to the manner through which TrkA signals through MAPK has the potential to cause the neuronal differences that we suspect may underlie BD.

Signal Modifying Pathways

As previously mentioned, the SH2B family is believed to be capable of modifying TrkA’s phosphorylation state. In this sense, SNP-induced changes in TrkA may also influence its activity by changing the way SH2B modifies its auto-phosphorylation. Similarly, Abl non-receptor tyrosine kinase is also believed to play a role in inhibiting TrkA auto-phosphorylation (Koch et al, 2008). Abl has been shown to directly bind to TrkA via its activation loop to TrkA juxtamembrane region even in the absence of phosphorylation, and it is believed to form a complex with Crk, which also interacts with FRS2 and various other co-factors of TrkA (Koch et al, 2000, Yano et al, 2000). Therefore, it is possible that BD
susceptibility may arise from changes to the TrkA protein that decrease Abl’s ability to inhibit TrkA phosphorylation thereby misregulating TrkA signaling.

General TrkA signaling can also be modified in a spatiotemporal manner via receptor endocytosis and activity duration. Additionally, these properties are more unique to TrkA. In fact, it has been postulated that this may be the reason why TrkA is capable of inducing neuronal proliferation and differentiation over other RTKs (Wu et al, 2001). One of TrkA’s co-factors, GAB2, has been shown to be capable of producing long-term MAPK activation when bound to TrkA via Rap1 GTPase activation. Rap1 is then capable of phosphorylating Raf, which feeds into the MAPK pathway as aforementioned (Wu et al, 2001). A notable difference between this pathway and the traditional Grb2/Sos to Ras pathway is the duration of MAPK activation and its uniqueness to TrkA versus other RTKs. Activation of MAPK by Ras is a common pathway among RTKs and mainly happens in a transient fashion. Activation of MAPK through GAB2/Rap1, however, has been shown to last much longer following the initial stimulation by NGF. In this sense, a mutation affecting the TrkA/GAB2 interaction could also feasibly lead to increased or decreased downstream signaling. Furthermore, GAB2/Rap1 activation of MAPK differs spatially compared to that of Grb2/Sos, due to the formation of signaling endosomes (Zeiller et al, 2012). Also, it is known that another TrkA co-factor, Rac, assists in the endocytosis of TrkA (Valdez et al, 2007). It is possible that SNPs may change the dynamics between TrkA and these co-factors changing TrkA localization; thereby changing its
signaling properties. These changes may then be responsible for the BD phenotypes observed.

**TrkA Interactions with p75**

NGF has two known receptors: TrkA and p75. Classically, TrkA acts as a high-affinity receptor that promotes cellular growth and differentiation while p75 is a separate, low-affinity receptor that causes cellular apoptosis. More recent views, however, have suggested that p75 may be tied to the regulation of TrkA. Studies have shown that TrkA activity changes depending on the ratio of p75 to TrkA within the cell (Twiss et al, 1998). In fact, further research into this area has revealed that a 29 amino acid region within the intracellular domain of p75 is capable of enhancing TrkA activity. It is also suggested that the juxtamembrane region of p75 causes conformational changes to the extracellular domain of TrkA thereby regulating its specificity (Matusica et al, 2013, Ho et al, 2011). This has led to speculation by some that TrkA may not naturally be a high-affinity receptor for NGF, but may instead form the high-affinity NGF receptor upon interacting with p75 (Wehrman et al, 2007). This is supported by data that demonstrates TrkA and p75 complexing prior to NGF stimulation (Iacaruso et al, 2011). In either case, p75 has been shown to modulate TrkA activation via the PI3K pathway, SHC pathway, and ubiquination. It assists in the activation of TrkA downstream signaling by either enhancing SHC phosphorylation or as a necessary TrkA co-factor for PI3K signaling (Negrini et al, 2013, Epa et al, 2004).
In the case of ubiquination, the presence of p75 is necessary for polyubiquination of TrkA, which regulates the internalization and activation of specific signaling pathways by the receptor (Geetha et al, 2005). Given the multitude of ways that p75 affects TrkA signaling, it is possible that our SNP may modify TrkA/p75 cross-talk.

**TrkA Post-translational Modification**

In addition to its plethora of co-factors, TrkA is also known to undergo both glycosylation and ubiquination, which adds a further layer of complexity to its signaling activity. It is believed that TrkA is N-glycosylated in its active form, and it has been shown that glycosylation plays a role in localizing the TrkA to the membrane. Glycosylation of the TrkA receptor has also been shown to be required for activation of the downstream pathways (Watson et al, 1998). Whether this is due to the glycosylation itself or the subsequent localization of TrkA has yet to be determined.

Ubiquination of TrkA is also known to play a role in receptor localization and motility. However, unlike glycosylation, ubiquination of the TrkA receptor causes internalization of the receptor. This internalization can lead to the degradation/recycling of the receptor (Makkerh et al, 2005). However, it is also known to aid in receptor complex stabilization (Song et al, 2011), which may lead to the formation of signaling endosomes within the cell (Harrington et al, 2012). This in turn, may be responsible for modifying TrkA’s temporal signaling
dynamics in a manner that allows it to promote neuronal division/survival (Chan et al, 2012). It should also be noted that TrkA is not inherently ubiquinated. It has been shown that TrkA undergoes polyubiquination upon activation by NGF and that the presence of p75 is required for this to occur (Geetha et al, 2005, Piper et al, 2011). In either case, both glycosylation and ubiquination have been shown to play essential roles in TrkA signaling and SNP-induced changes to modification sites may have an impact on TrkA function.

To date, there have been multiple studies on TrkA and its interacting protein partners that have been performed using co-immunoprecipitation (co-IP) techniques with stable transfectant cell lines (such as PC12) that over-express TrkA. While this has proven an effective system for understanding normal TrkA interactions, it would be difficult to adapt for use in studying the impact specific SNPs may have on TrkA function. While it would be theoretically possible to generate the desired SNPs using CRISPR/Cas9 nucleases, such an approach would come with its own set of obstacles such as off-site cleavage (Zhang et al, 2014) Furthermore, a significant amount of work and screening would be required for each mutant to be tested in order to ensure the integrity of the mutant gene and homogeneity of the cultured cells. As a result, we believe it would be valuable to develop a method by which specific TrkA SNPs, such as the one present within the aforementioned family, could be examined for possible functional ramifications. Study of these effects may help elucidate a pathway or at least part of a mechanism for bipolar disorder.
RESULTS

Cell Line Selection

The ideal cell line would be readily transfectable and express the protein at a relatively high level. Initial experiments were done in SK-N-MC cells, but transfection of the cell lines proved inefficient (Figure 6A). To remedy this, the HEK-293T cell line was chosen as a host for our construct due its ability to be easily transfected, and we were able to attain around 30-40% efficiency using Lipofectin® and up to 70-80% efficiency using Lipofectamine® 3000 (Figure 6B/6C).

Figure 6: A. Transfection efficiency test of SK-N-MC with GFP cDNA using Lipofectin® transfection reagent. Image captured at 20X magnification. B. Transfection of HEK-293T cells with GFP cDNA using Lipofectin® transfection reagent. Image captured at 40X magnification. C. Transfection of HEK-293T cells with GFP cDNA using Lipofectamine® 3000 transfection reagent. Image was captured at 20X magnification.
Furthermore, HEK-293T is a commonly used cell line for the purpose of protein expression (Thomas et al., 2005). Theoretically, this makes the HEK-293T cell line well suited for our purposes of expressing mutant TrkA. However, it is also equally important that HEK-293T express TrkA’s co-factors in order for us to examine any functional effects a mutation may have on protein binding. In our case, we chose to examine HEK-293T cells for the expression of SHC given rs144901788’s proximity to its binding site. It was found that the line does indeed express SHC, an important adaptor protein that is believed to play a role in connecting TrkA to the MAPK pathway (Figure 7).

Figure 7: Western blot of HEK-293T and SK-N-MC cell lysates probed with anti-SHC antibodies. All 3 isoforms of SHC are present in appreciable amounts in both cell lines.
As seen in the blot, HEK-293T cells express the 46kDa, 52kDa, and 66kDa forms of SHC each of which is known to be phosphorylated in response to receptor tyrosine kinase activation (Pelicci et al, 1992). Presence of SHC within these cells would potentially let us monitor the downstream effects of mutant TrkA on the MAPK pathway.

**Initial Studies with N-terminus HaloTag® TrkA Construct (pFN21AE2094)**

As one of our first candidates for studying the effects of SNPs, in particular rs144901788, we tested a commercially available construct from Kazusa (clone name: pFN21AE2094) that contained the TrkA gene preceded by a HaloTag® sequence on the N-terminus. Primer-based mutagenesis was used to generate the SNP. The construct was then transfected into both HEK-293T and SK-N-MC cells before being treated with 100ng/ml of NGF. TrkA complexes were isolated via affinity beads, and purified protein was then probed for total TrkA, pTrkA (Y490), phosphorylated SHC, and total SHC. Blots for phosphorylated SHC, total SHC, and TrkA phosphorylation at Y490 came up blank (not shown) and repeated attempts produced similar results. Probing for total TrkA produced a band with a molecular weight of 85kDa (Figure 8A/8B), and repeated attempts continued to only find this form of TrkA. Signal intensity for this particular band showed no correlation in regards to NGF treatment. Given the size of the band, we concluded that this was likely an immature form of TrkA consisting of only the core protein (Watson et al, 1999). Also interesting to note is the disappearance of many of the bands found when probing untransfected cell lysates directly for
TrkA (Figure 8C). This demonstrates the efficacy of using the HaloTag® to isolate the recombinant TrkA only.

Figure 8: A. Probing for total TrkA in transfected HEK-293T cells treated with 100ng/µl NGF for 5min before isolating TrkA. Cells were transfected with either mutant or wild-type pFN21AE2094 (TrkA with N-terminus HaloTag®). B. Probing for total TrkA in transfected HEK-293T cells left untreated before isolating TrkA. Similar to Figure 8A, cells were transfected with either wild-type or mutant pFN21AE2094. C. Untransfected HEK-293T cells were examined for any endogenous TrkA by probing the lysate directly.
Designing a Construct Capable of Producing Mature TrkA

Existing literature has often described the 110kDa and 140kDa forms of TrkA as more mature, glycosylated forms of the protein (Watson et al, 1999), so we sought a way to reproduce this in vitro. Towards this end, we sub-cloned the TrkA gene into a pHTC HaloTag® CMV-neo (pHTC) vector so the tag would be on the C-terminus of the protein. This would help us determine if placement of the tag affected protein processing/size. Interestingly, we had to switch polymerases from Taq, which was used for all work done with pFN21AE2094, to Phusion in order achieve amplification with the pHTC vector. We used restriction enzyme-based sub-cloning to insert the NTRK1 gene into the vector, and primer-based mutagenesis was once again employed to generate the mutant of interest (Sequence data can be found in Appendix). Transfection into HEK-293T cells and subsequent analysis of the isolated TrkA protein revealed three major bands that, given their sizes, were identified as 85kDa TrkA, 110kDa TrkA, and 140kDa TrkA (Figure 9).
Fluorescent Imaging of C-Terminus HaloTag® TrkA

It has also been previously observed that the 85kDa and 110kDa forms are located intracellularly while the 140kDa form is located on the cell surface (Watson et al, 1999). Now that we were capable of producing 140kDa TrkA, we decided to examine localization of the recombinant protein by fluorescently imaging the TrkA protein via fluorescent HaloTag® binding ligands. From the capture images, it is possible to determine that the TrkA receptor is located on the surface of the cell, which helps indicate the maturity of the protein (Figure 9).

Figure 9: HEK-293T cells were transfected with either wild-type or mutant pHTC TrkA where the HaloTag® is located on the C-terminus. Both conditions were then either treated with 100 ng/µl NGF for 5 min or left untreated. As can be seen, shifting the HaloTag® to the C-terminus causes the appearance of two additional bands (110 and 140) compared to the N-terminus HaloTag® pFN21AE2094 variant (Figure 8).
We detected no visible difference between the wild-type TrkA and the mutant at 10X, 20X, and 40X magnifications. Admittedly, greater magnifications would be necessary to really examine protein localization in detail, and the formation of TrkA signaling endosomes upon stimulation has been previously described in the literature (Grimes et al, 1996).

On a separate note, while our protein blots do show the presence of the 85kDa and 110kDa forms present within cells transfected with the pHTC construct, we did not notice a strong intracellular signal. It may be that these immature proteins do not generate a strong fluorescent signal due to their sequestration within the endoplasmic reticulum and golgi bodies as part of the secretory pathway and processing of transmembrane proteins.

Figure 10: Live cell visualization of C-terminus HaloTag® TrkA within cells using HaloTag® Oregon Green® Ligand. Ligand binds to the HaloTag® motif on the recombinant TrkA and can be used to visualize the protein localization within the cells. 20X and 40X magnifications included for both wild-type and mutant for additional clarity. An image of a GFP control is located on the far right. The differences seen between the control and the transfected samples helps emphasize the surface localization of the TrkA.
DISCUSSION

Development of the Construct

Here we developed and presented a model system that can be used for further studies into TrkA function. In particular this system allows us to study the impact specific SNPs have on a proteomic level. As we have shown, primer-based mutagenesis can be utilized to induce desired SNPs in the construct, which can then be transfected into a suitable cell line for production of the recombinant protein (Sequence data can be found in Appendix). This allows the construct to be easily adapted to study the proteomics of any SNPs of interest. Furthermore, the included C-terminus HaloTag® allows for cellular production of recombinant 85kDa, 110kDa, and 140kDa TrkA. Interestingly, we observed that differential placement of the HaloTag® on either the N-terminus or the C-terminus of the TrkA sequence causes a distinct difference in the size(s) of the final protein product (Figures 8 and 9). This is likely due to differences in protein processing where placement of the HaloTag® on the N-terminus of the protein ends up obscuring signals necessary for proper localization/translation. Furthermore, it has previously been described in the literature that TrkA comes in 85, 110, and 140kDa forms with the size difference being due to varying degrees of glycosylation. This glycosylation has also been linked with maturation of the protein with the 140kDa form being the mature receptor that binds NGF (Watson et al, 1999). On the other hand, the 85kDa and 110kDa are known to be immature forms that are localized intracellular and thus do not get exposed to
NGF. This is why we felt that the original N-terminus HaloTag® plasmid (pFN21AE2094) was not a good model for the system we wished to study, and it was paramount that our construct be able to produce the fully glycosylated, mature protein. Our results have shown that this is the case with our current construct in regards to both protein size and localization (Figures 9 and 10).

**Future Uses/Follow-up Studies**

This construct also offers several advantages in terms of isolating the mutant TrkA complexes. Compared to conventional immunoprecipitation techniques, the inclusion of a HaloTag® allows for separation of the mutant TrkA from the cell lysate and any endogenous TrkA that may be present via affinity beads. Thus, any differences detected in the isolated proteins when compared to the wild-type may be associated with the mutation in question. This approach would be useful in the case of examining SNPs such as the previously mentioned (rs144901788). Given its proximity to the SHC binding site on TrkA, rs144901788 may be affecting the interaction between TrkA and SHC in some manner.

Following affinity bead binding, the mutant TrkA complex can be released via the included TEV site and probed for protein interactions via western blots in a manner similar to Co-IP. It is also possible to dissociate and elute any bound proteins following isolation of the TrkA complex without releasing the TrkA itself by treating the complexes with SDS. If rs144901788 turns out not to have an effect on SHC, this technique may be used to isolate the mutant TrkA’s protein
partners in preparation for mass spectrometry experiments to examine possible differences between wild-type and mutant’s interactomes.

Further exploration of alternative mechanisms would also be possible because the HaloTag® motif affords flexibility in terms of manipulating and monitoring the recombinant protein beyond just purification. For example, a possible alternative mechanism through which rs144901788 might act is the modification of normal TrkA spatiotemporal localization. TrkA is known to be endocytosed and form signaling vesicles following activation, which is speculated to play a role in its unique ability to promote neuron mitogenesis (Grimes et al, 1996). It has been previously reported that TrkA recycling also involves a regulatory element within the juxtamembrane domain of the protein (Chen et al, 2005). Since rs144901788 is within relative proximity to the juxtamembrane domain, it might be possible that the SNP is influencing this signal in some fashion. To address this question, HaloTag® specific fluorescent ligands could be employed to perform fluorescent cellular imaging of the recombinant protein. This could provide valuable insight into any changes in cellular localization in response to NGF that may be attributed to the mutation. By fluorescently labeling the mutant protein, it would be possible to detect any changes in protein distribution that may be indicative of a functional effect. Other mechanisms may involve any of the other aforementioned co-factors such as FRS2 or p75.

In any case, being able to study the functional effects of SNPs in TrkA will help us gain a better understanding as to how the sequence affects its structure/function as well as its interactions with partner proteins. Going forward,
we hope to be able to use it to elucidate how SNPs linked to bipolar disorder such as rs144901788 may cause the phenotypes associated with the disease. Ideally, this will provide some insight for proposing a possible mechanism for bipolar disorder. This is particularly exciting because it has the potential to expand our understanding of bipolar susceptibility. Finally, studying the interplay between TrkA and its various co-factors may unveil even more susceptibility alleles in addition to providing more general insight into how the co-factors may affect TrkA and each other.
MATERIALS AND METHODS

Cloning

Original clone pFN21AE2094 containing TrkA with N-terminus HaloTag® was obtained from Kazusa, and stock pHTC HaloTag® CMV-neo vector was obtained from Promega. Subcloning of the TrkA gene from the original pFN21AE2094 vector into the pHTC vector was achieved by adding XbaI and XhoI restriction enzyme sites onto the N-terminus and C-terminus, respectively, of the TrkA gene. Restriction enzymes, ligase, and their respective buffers used during the process were obtained from New England Biolabs and custom primers were ordered from Integrated DNA Technologies. SNP-containing TrkA mutants were generated by primer-based mutagenesis using the QuikChange Lightning kit from Agilent Technologies. When working with the pHTC clones, we had to replace the included polymerase with Phusion polymerase from New England Biolabs. Agarose gels were run throughout the process to quality check PCR and monitor the cloning process. Agarose concentration used in gels was 0.8% with 2µl ethidium bromide added per 50ml gel. Qiagen PCR clean-up kits were used following each PCR to remove extraneous material. Following initial ligation, expansion of the plasmid was achieved via transformation into XL10 Gold cells (Promega). Qiagen mini-preps were then used to isolate the plasmid DNA. Sequencing to ensure quality of the clones was performed by Eton Biology.
**Cell Culture**

HEK-293T cells were grown in DMEM (Gibco) supplemented with 10% fetal bovine serum (Gibco) and were a gift from Dr. Xianjin Zhou. Cells were grown in T75 and T25 flasks. Transfections were carried out in 6-well plates pre-coated with poly-D-lysine from Millipore. Transfections carried out using Lipofectin® transfection reagent (Life Technologies) were performed at a ratio of 2μg of DNA to 10μl of Lipofectin® while transfections done with Lipofectamine® 3000 transfection reagent were carried out at a ratio of 2.5μg of DNA to 7.5μl of Lipofectamine® 3000. HEK-293T cells were plated 1 day prior to transfection so as to be approximately 90% confluent on the day of transfection. Following transfection, cells were incubated for 48hrs before being treated with NGF at a final concentration of 100ng/μl for 5min before having their growth medium replaced with ice-cold DPBS to stop the reaction. Cells were then harvested for protein. To help determine transfection efficiency, cells were transfected with a vector containing GFP gifted from Dr. Xianjin Zhou. To visualize recombinant TrkA within live cells, TrkA was labelled using HaloTag® Oregon Green® ligand from Promega according to provided protocol. Visualized cells were transfected 48hrs prior to examination.

**HaloTag® Pull-Down**

Isolation of the TrkA complexes was achieved with the HaloTag® Mammalian Pull-Down System from Promega (Cat #: G6504). HEK-293T cells being harvested for protein were pelleted by centrifugation at 1600 RPM for 5
minutes. Excess media was aspirated off, and cell pellet was then lysed with included Mammalian Lysis Buffer before addition of protease inhibitor cocktail (Promega) and Halt™ phosphatase inhibitor (Life Technologies). TrkA complexes were bound to affinity beads over 1hr at room temperature on a tube rotator followed by three washes (down from the recommended five). Bound complexes were then treated with ProTEV Plus for 1hr at room temperature on a tube rotator to release the TrkA complexes.

**Western Blots**

Isolated protein complexes were denatured by heating for 5mins at 95°C, and separation was then performed with 4-15% pre-cast polyacrylamide gels from Bio-Rad. Proteins were then transferred onto a PVDF membrane before blocked with 3% BSA for 1hr. Membranes then were incubated with primary antibody overnight (~16hr) before being washed twice with both 1% BSA and TBST (total 4 washes) for 10min each. Blots were then incubated with secondary antibody for one hour followed by another four washes (same conditions as before) with an additional 5min wash with dH2O. Membranes were then developed for 5min with Pierce ECL Plus solution before being imaged via X-ray film at various time exposures.
APPENDIX

Wild-type TrkA (Subject) and Mutant rs144901788 TrkA (Query) Alignment

<table>
<thead>
<tr>
<th>Query</th>
<th>Sbjct</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>ATGCTGGAGGCGGACGCGGGCGAGCTTGGCAGCTGGCCAGCTGAGGGCTGGGAGCCGGGCGA</td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>ATGCTGGAGGCGGACGCGGGCGAGCTTGGCAGCTGGCCAGCTGAGGGCTGGGAGCCGGGCGA</td>
<td></td>
</tr>
<tr>
<td>61</td>
<td>AGCCTGCTGGCTTGCTGATGACTGGGCTGACCCTGCGGCCGACCCCTGCGGCATGCTCCAC</td>
<td></td>
</tr>
<tr>
<td>61</td>
<td>AGCCTGCTGGCTTGCTGATGACTGGGCTGACCCTGCGGCCGACCCCTGCGGCATGCTCCAC</td>
<td></td>
</tr>
<tr>
<td>121</td>
<td>TGCCCCCAAGCGCTCTCGGGGAATCGGCGACCCCGGATGCGGCCCTCAGGTCATGCTCCAC</td>
<td></td>
</tr>
<tr>
<td>121</td>
<td>TGCCCCCAAGCGCTCTCGGGGAATCGGCGACCCCGGATGCGGCCCTCAGGTCATGCTCCAC</td>
<td></td>
</tr>
<tr>
<td>181</td>
<td>CACCTGGCGACGAGATGCAGTCTCATCGAGACGCACTGCTGAGCAGGAGTGGGGAGCTCAGCAGC</td>
<td></td>
</tr>
<tr>
<td>181</td>
<td>CACCTGGCGACGAGATGCAGTCTCATCGAGACGCACTGCTGAGCAGGAGTGGGGAGCTCAGCAGC</td>
<td></td>
</tr>
<tr>
<td>241</td>
<td>CATCTGCTGGCCACGAGACTGGTAGCTCATGCTATGGAGACGAGGAGTGGGGAGCTCAGCAGC</td>
<td></td>
</tr>
<tr>
<td>241</td>
<td>CATCTGCTGGCCACGAGACTGGTAGCTCATGCTATGGAGACGAGGAGTGGGGAGCTCAGCAGC</td>
<td></td>
</tr>
<tr>
<td>301</td>
<td>AGTGGTCTCCGTTGCTGAGGCGAGACGAGTCTCATGCTATGGAGACGAGGAGTGGGGAGCTCAGC</td>
<td></td>
</tr>
<tr>
<td>301</td>
<td>AGTGGTCTCCGTTGCTGAGGCGAGACGAGTCTCATGCTATGGAGACGAGGAGTGGGGAGCTCAGC</td>
<td></td>
</tr>
<tr>
<td>361</td>
<td>AATCTTCTTCCAATATGCTGGAGACGAGTCTCATGCTATGGAGACGAGGAGTGGGGAGCTCAGC</td>
<td></td>
</tr>
<tr>
<td>361</td>
<td>AATCTTCTTCCAATATGCTGGAGACGAGTCTCATGCTATGGAGACGAGGAGTGGGGAGCTCAGC</td>
<td></td>
</tr>
<tr>
<td>421</td>
<td>CAGGAATGGCTCTTGCGGGGACACCTGCGACTGTCTTTGTGGCCTGCGCTGCACAGGAGGAGTGGGGAGCTCAGC</td>
<td></td>
</tr>
<tr>
<td>421</td>
<td>CAGGAATGGCTCTTGCGGGGACACCTGCGACTGTCTTTGTGGCCTGCGCTGCACAGGAGGAGTGGGGAGCTCAGC</td>
<td></td>
</tr>
<tr>
<td>Query</td>
<td>Start</td>
<td>End</td>
</tr>
<tr>
<td>--------</td>
<td>-------</td>
<td>-------</td>
</tr>
<tr>
<td>2221</td>
<td>2221</td>
<td>2280</td>
</tr>
<tr>
<td>Sbjct</td>
<td>2221</td>
<td>2280</td>
</tr>
<tr>
<td>2281</td>
<td>2281</td>
<td>2340</td>
</tr>
<tr>
<td>Sbjct</td>
<td>2281</td>
<td>2340</td>
</tr>
<tr>
<td>2341</td>
<td>2341</td>
<td>2388</td>
</tr>
<tr>
<td>Sbjct</td>
<td>2341</td>
<td>2388</td>
</tr>
</tbody>
</table>
REFERENCES


