A ROLE FOR BDNF-TRKB SIGNALING IN THE MODULATION OF SUPEROXIDE DISMUTASE-1 EXPRESSION

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DEDICATION

To Phil: For your exceeding patience and taking a chance on an unorthodox student.

To Genny: Nothing is worth doing without you.

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by

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Superoxide dismutase-1 (SOD1) has been implicated in the pathogenesis of familial amyotrophic lateral sclerosis (fALS), a degenerative motor neuron disease more commonly known as Lou Gehrig's disease. The mechanism by which it causes degeneration and the extent of its involvement are currently unknown, although the present consensus is that a toxic gain-of-function mutation is involved. Our lab has previously demonstrated, via a cDNA expression screen, that the TrkB receptor and the guanine nucleotide exchange factor, RasGRF-1, modulate SOD1 expression at the protein level, suggesting that cell signaling pathways associated with TrkB signaling are involved in regulating the expression of SOD1. Overexpressing these proteins for long or 'chronic' time periods of 24-48 hours in a motor neuron-like cell line lead to a significant decrease in SOD1 protein levels. Subsequent experiments using TrkB mutants and pharmacological inhibitors of pathways known to be associated with TrkB revealed that the kinase activity of the receptor is necessary and that partial TrkB signaling is sufficient for suppression. Conversely, treatment with brain-derived

neurotrophic factor (BDNF), an activator of this pathway, over shorter or 'acute' time periods increased SOD1 protein levels. Further analyses using qPCR, a human SOD1 promoter Lucifersase assay, and inhibitors of the proteasome and translation machinery provide evidence that in both the acute and chronic phase, BDNF-TrkB signaling is modulating SOD1 expression at the level of translation. Taken as a whole, these data demonstrate that BDNF-TrkB signaling is involved in the regulation of SOD1 expression via translation and that the expression pattern of SOD1 is bi-phasic in response to the duration of the stimulus. These findings may have implications for therapeutic modification of mutant SOD1 levels in ALS patients.

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PRIOR PUBLICATIONS

1) Somalinga, BR, **Day CE**, Wei S, Roth MG, Thomas PJ (2012) *TDP-43 identified from a genome wide RNAi screen for SOD1 regulators.* PloS One 7(4):e35818
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1) Day CE, Somalinga BR, Thomas PJ (2014) *Regulation of Superoxide Dismutase-1 through TrkB-BDNF signaling*

2) Day CE, Thomas PJ (2014) *ALS: molecular pathology, SOD1, and therapeutic strategies* (review article)

3) Somalinga BR, **Day CE**, Thomas PJ (2014) *Cross-talk between TDP-43 and Antioxidant Response Signaling*

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LIST OF DEFINITIONS

Akt – mouse strain Ak, thymoma
ALS – amyotrophic lateral sclerosis
ALS2 – alsin 2
AP-1 – activator protein 1
ARE – antioxidant response element
β-gal – beta galactosidase
Bcl-2 – b-cell lymphoma 2
BDNF – brain derived neurotrophic factor
C9orf72 – chromosome 9, open reading frame 72
CaMK – $Ca2^{+}$/calmodulin dependent protein kinase
CCS – copper chaperone of SOD
C/EBP – CCAAT-enhancer-binding protein
CHX -- cycloheximide
CMV – cytomegalovirus
CREB – cyclic adenosine monophosphate response element binding protein
DMSO – dimethyl sulfoxide
Elk1 – ETS domain-containing protein
ERK – extracellular signal regulated kinase
FTLD – fronto-temporal lobar dementia
FUS – fused in liposarcoma
HA – hemagglutinin
HEK – human embryonic kidney
HeLa – Henrietta Lacks
JNK – c-jun N-terminal kinase
MAD – median absolute deviation
MAPK – mitogen activated protein kinase
MEK – mitogen activated protein kinase kinase
mTOR – mammalian target of rapamycin
NFκB – nuclear factor kappa-light-chain-enhancer of activated B cells
NGF – nerve growth factor
NMDA – n-methyl d-aspartate
Nrf2 – Nuclear factor (erythroid-derived 2)-like 2
NSC34 – neuroblastoma spinal cord 34
OPTN – optineurin
PLC – phospholipase C
qPCR – quantitative polymerase chain reaction
Ras – rat sarcoma
RasGRF-1 – ras guanine nucleotide releasing factor 1
RSK – ribosomal s6 kinase
ROS – reactive oxygen species
siRNA – short interfering ribonucleic acid
SOD1 – superoxide dismutase-1
TAF-15 – TATA-binding protein-associated factor 2N
TDP-43 – transactive response DNA binding protein 43kDa
TSS – transcription start site
TrkB – tropomyosin-related kinase B
UBQLN2 – ubiquilin 2
XRE – xenobiotic response element

Chapter 1

Literature Review

1.1 Introduction

Amyotrophic lateral sclerosis (ALS) is a neurodegenerative disorder that specifically affects upper and lower motor neurons in the cortex, brain stem, and spinal cord (Ferraiuolo *et al*. 2011). ALS has two broad classifications, familial and sporadic, which have similar clinical manifestations although their root causes have not been determined and may differ significantly (Bento-Abreu *et al*. 2010). To date there is no known cure for ALS and treatments are limited in both scope and efficacy (Bensimon *et al*. 1994). The disease is (usually) fatal and life expectancy from time of diagnosis typically does not exceed 5 years. The aggressive course of the disease and its relatively high prevalence highlight the need for effective clinical treatments.

Multiple genes have been identified that account for a significant fraction of fALS. Among these is superoxide dismustase-1 (SOD1) which was first associated with ALS in 1993 making it the most extensively studied ALS-associated protein (Rosen *et al*. 1993). SOD1 is a 154 amino acid protein that forms a dimer which functions as a scavenger of free radicals, specifically superoxide (Boukaftane *et al*. 1998). Mutations in SOD1 destabilize the protein which makes it prone to aggregation in the cytosol, a common feature among neurodegenerative disorders and fALS. The vast majority of these mutations leave the enzymatic activity of SOD1 intact and SOD1 knock-out animals do not develop ALS-like symptoms, which suggests that mutant SOD1 (mutSOD1) causes disease through a toxic gain-of-function (Borchelt *et al*. 1994, Shefner *et al.* 1999). Mouse models expressing different levels of mutant SOD1 protein have also demonstrated that the levels of mutant expression correlate with disease onset and severity (Dal Canto, 1995). This lends further support to the notion that mutant SOD1 is in fact causal and that the degree to which it is expressed dictates disease course. Therefore, identifying means of regulating SOD1 expression may lead to more effective treatments.

Previous work from our lab has identified several genes via a cDNA expression screen

and a whole genome RNAi screen that affect SOD1 expression (Somalinga *et al.* 2011, 2012). While the list of potential targets was varied in terms of function, two genes were notable for their involvement in similar signaling pathways: TrkB and RasGRF-1.

TrkB (tropomyosin-related kinase B) is a neurotrophin receptor and is a member of the tyrosine kinase super-family of receptors (Gupta *et al.* 2013). In general, these receptors are cell surface receptors that transduce intercellular signals from growth and trophic factors and are important for development and survival of cells. TrkB is known to bind three neurotrophins, brain-derived neurotrophic factor (BDNF), NT-3, and NT-4, although among these only BDNF and NT-4 are specific for TrkB (Reichardt 2006). Binding of these ligands to the extracellular domain is thought to induce dimerization of the receptors and autophosphorylation of the intracellular kinase domain which allow the receptors to bind signaling effector molecules (Massa *et al*. 2010). Stimulation of TrkB leads to activation of the MEK/ERK, Akt, and PLCγ pathways (Kaplan and Miller 2000). The MEK/ERK and Akt pathways are coupled to TrkB through Grb2 and Shc which in turn signal through SOS/Ras and PI3K for MEK/ERK and Akt, respectively (Minichiello 2009).

RasGRF-1 is a guanine nucleotide releasing factor that is integrated in these pathways at the level of SOS/Ras and activates Ras (Feig 2011). Ras was initially described as a principle effector in the MEK/ERK pathway but has also been shown to drive Akt signaling, hence expression of RasGRF-1 and its action on Ras can produce similar effects to activation or overexpression of TrkB.

Several studies over the past decade have investigated the potential role of TrkB and its associated signaling pathways in ALS models. However, some of the reported results are contradictory, alternately showing that both activation and inhibition of BDNF-TrkB signaling

are beneficial to cell survival. This is further complicated by the non-cell autonomous nature of SOD1-ALS in that multiple cell types in the CNS must express mutant SOD1 in order to observe the pathological symptoms and disease progression (Yamanaka, 2008) and TrkB has been shown to mediate different responses in neurons and astrocytes (Colombo *et al*. 2012). There is also limited data within these studies as to the effect these pathways have on the expression of SOD1. Taken together with more recent data in which SOD1 may actually be transmitted from cell to cell (Polymenidou, 2011), this highlights the need to understand the regulation of SOD1 in specific cell types.

Here, using a motor neuron-like cell line, we show that BDNF-TrkB signaling regulates SOD1 expression in a bi-phasic manner. Short term stimulation with BDNF increases SOD1 protein levels approximately 2 to 4-fold while more chronic BDNF treatment, or overexpression of TrkB, significantly decreases SOD1 protein. Our data indicate that the kinase activity of TrkB is required for these effects and that partial TrkB signaling through either MEK/ERK-Akt or PLCγ is sufficient for suppression. Finally, we show that the modulation of SOD1 protein levels is translationally mediated.

1.2 Amyotrophic lateral sclerosis

Amyotrophic lateral sclerosis (ALS) is a neurodegenerative disease that affects motor neurons within the central nervous system. It is a progressively fatal adult-onset disorder with most patients having a life expectancy of no more than 5 years after onset of clinical symptoms. It is the most common disorder among the major motor neuron diseases with approximately 5,000 new cases diagnosed per year in the United States alone. Given its global incidence rate of 1 per 100,000 individuals and the aggressive course of the disease, finding suitable treatments and potential cures is a high priority (Redler and Dokholyan 2012).

ALS was first described over 140 years ago by French neurologist Jean-Martin Charcot, but did not gain wide attention until the mid-1900s when noted baseball player Lou Gehrig retired in 1939 due to the debilitating condition, hence the more common name, Lou Gehrig's Disease. This was followed in the early 1950s by an outbreak on the island of Guam among the Chamorro people caused by a naturally occurring neurotoxin in their diet, β-methylamino-L-alanine, or BMAA. Despite the increased public awareness and the newly focused scientific effort, it was another forty years before ALS was definitively linked to a genetic factor.

Clinically, ALS presents as weakness, twitching, and spasticity in the muscles of patients. These symptoms can be categorized as bulbar or spinal onset depending on their location, e.g. face vs. limbs, which typically correlate with upper or lower motor neuron dysfunction (Burrell *et al*. 2011). The disease normally manifests in one focal area and spreads contiguously, ultimately leading to the loss of diaphragmatic enervation which causes respiratory failure and death (Ravits *et al*. 2009). Additionally, only a small percent of patients display any overt dementia or loss of cognitive function, which is in line with ALS being classified as a motor neuron disease (Eisen *et al*. 1992). However, certain types of ALS present with frontotemporal

lobar dementia (FTLD or FTD), a type of dementia caused by atrophy of the frontal and temporal lobes of the brain. Therefore, it is possible that a higher number of patients do have some cognitive alterations but the rapid course of the motor symptoms obfuscates or precludes visible cognitive decline (Byrne *et al*. 2012).

The origin of ALS as a central or peripheral neuropathy is still a matter of debate. The clinical presentation of ALS is sometimes first seen as a disruption in the peripheral motor system which leads to subsequent disruption in central nerve fibers, thus leading to a 'dying-back' hypothesis of disease progression (Dadon-Nachum *et al*. 2010). Conversely, centrally located motor fibers that lack inputs from cortical motor units are apparently resistant to degeneration (Eisen 2009). This suggests that lower-motor neurons controlling peripheral responses require corticomotor input and thus supports a top-down or 'dying-forward' model of degeneration. It may be the case that both of these processes are at work and that the clinical presentation of an individual is greatly affected by their unique genetic composition and environment. The matter of neuropathic origin combined with the emerging genetic framework has led some researchers to suggest that ALS be redefined as a spectrum of highly similar disorders rather than a single disease (Turner *et al.* 2013).

1.3 Sporadic vs. Familial ALS

The etiology of ALS is fairly complex in that the vast majority of cases are sporadic (sALS) and have no known heritable components (Ferraiuolo *et al*. 2011). Taken at face value this suggests that there is a large environmental component to the development of ALS, but to date there is still a lack of understanding as to what environmental factors may be contributing or if they are in fact causal. The distinction between sporadic and familial is also somewhat

arbitrary as cases are classified based on patterns of inheritance and known genetic factors (Byrne *et al*. 2012). This however assumes that all disease-associated genes are sufficiently penetrant to present in successive generations. In fact, all of the mutations currently associated with familial ALS (fALS) have been identified in small subsets of patients with apparently sporadic disease (Chesi *et al*. 2013). Furthermore, recent research has uncovered previously unknown mutations in genes that are observed in both familial and sporadic cases (Chio *et al*. 2011). This suggests that a portion of the so-called sporadic cases may instead be familial cases with uncharacterized genetic abnormalities, mutations with low penetrance, or incomplete family history. Furthermore, it may simply be that these genetic factors act to predispose individuals to disease as opposed to being causal factors. The known ALS-associated genes are summarized below in the table.

Year	Gene	Function	Disease Phenotype	Ref.
1993	SOD1	Antioxidant	ALS	Rosen *et al.* 1993
2001	ALS2	Guanine nucleotide exchange factor	ALS	Hadano *et al.* 2001
2008	TDP-43	DNA/RNA binding, mRNA splicing	ALS-FTD	Kabashi *et al*. 2008
2009	FUS	DNA/RNA binding, transcription	ALS, some FTD	Kwiatkowski *et al.* 2009
2009	OPTN	Membrane traffiking, exocytosis	ALS-FTD	Maruyama *et al.* 2010
2011	C9orf72	Unknown	ALS-FTD	DeJesus-Hernandez
		(proposed guanine nucleotide exchange factor)		*et al.* 2011
2011	TAF15	Transcription, subunit of TFIID	ALS	Ticozzi *et al.* 2011
2011	UBQLN2	Ubiquitin-proteasomal degradation	ALS, some FTD	Deng *et al*. 2011

Table List of known ALS-associated mutations and their relative concurrence with frontotemporal dementia (FTD). Importantly, TDP-43 and C9orf72 proteins have been found in large percentages of sALS cases and mutations in these genes are known to occur in fALS, thus providing a link between sALS and fALS. However, it is important to note that SOD1-ALS and ALS cases associated with TDP-43 are considered mutually exclusive (Al-Chalabi *et al.* 2012).

The division between sporadic and familial ALS is further complicated by the cellular and molecular pathology observed in patients and animal models. The most common and prominent feature of ALS is proteinaceous aggregates in the cell bodies of neurons (Ince *et al*. 1998). The first gene to be associated with inherited ALS, SOD1, produces a protein that is prone to misfolding and aggregation when mutated (Rakhit *et al*. 2007). Similarly, the gene product of TARDBP, TDP-43, has been shown to be the major component of cellular inclusions in sporadic cases, however it is generally accepted that SOD1 and TDP-43 ALS are independent (Neuman *et al*. 2006). This suggests that there is a common mechanism of cellular degeneration between sALS and fALS. However, it should be noted that these proteins have drastically different functions. SOD1 is an antioxidant enzyme while TDP-43 is a DNA-RNA binding protein that regulates transcription and splicing. Other genes and their products associated with ALS have similarly disparate functions. So, while protein aggregation is a common feature in different types of ALS, it may not be the sole, definitive pathogenic feature.

1.4 Cellular and Molecular Pathology of ALS

The cellular and molecular pathology of ALS is extremely complicated. Numerous cellular processes, organelles, and proteins have been implicated in the disease (Martin *et al.* 2013). These include but are not limited to: increased oxidative stress, glutamate toxicity, mitochondrial dysfunction, disruption of axonal transport, and, as mentioned previously, proteinaceous aggregates (Redler and Dokholyan 2012). There is also evidence of a non-cell autonomous component to the disease as both microglia and astrocytes have been implicated via release of inflammatory factors or reduced glutamate buffering, respectively (Beers *et al*. 2008). These proposed mechanisms are summarized in Figure 1.1

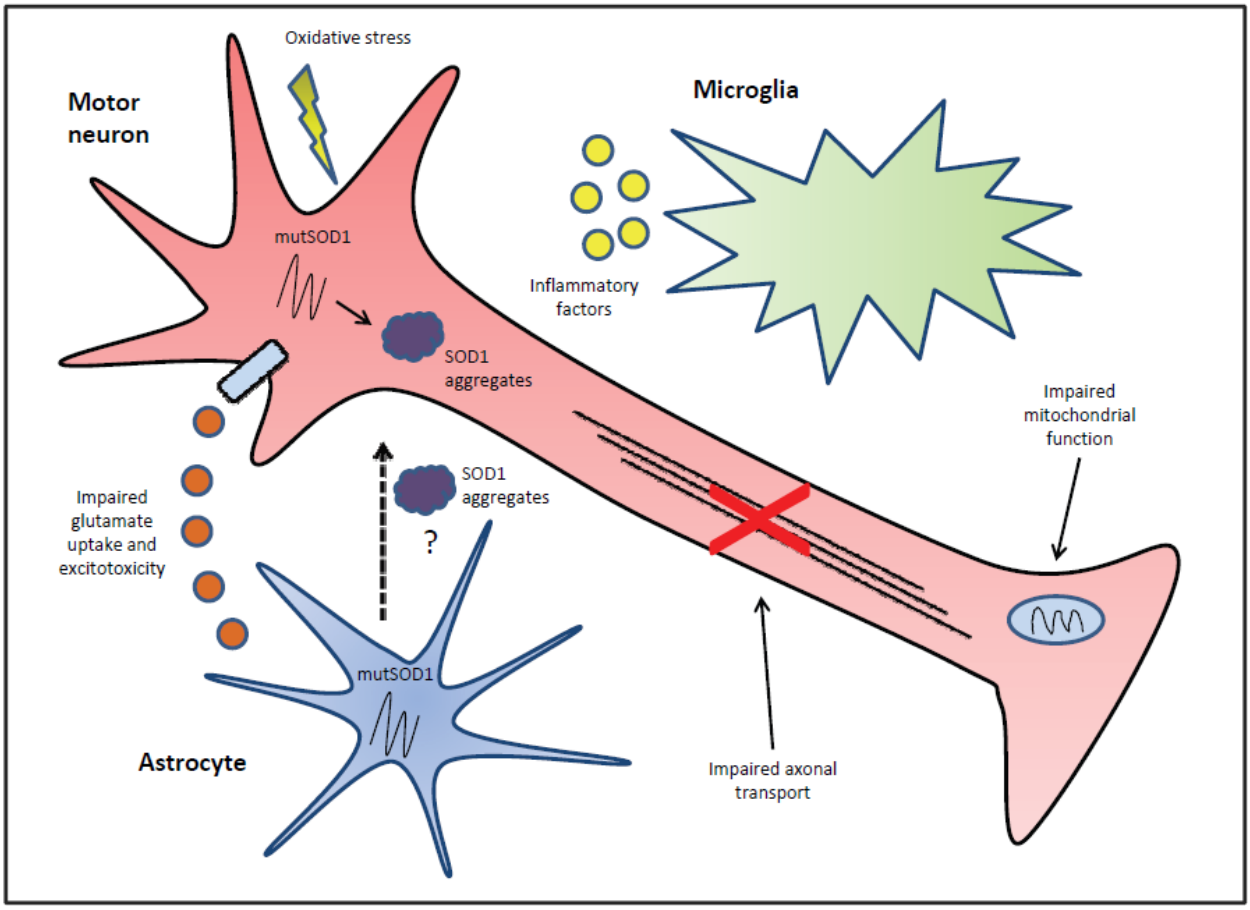


Figure 1.1 Summary of cellular processes that are linked to ALS pathology. The list of possible causal factors in ALS is extensive, ranging from cytoskeletal disturbances to excitotoxicity. However, only a few of these factors have been shown to be causal while most are probably downstream consequences of an initial toxic insult (Martin *et al.* 2013).

Oxidative stress is a common feature in neurodegeneration. This is most likely due to the unique environment of the brain which has a high oxidative metabolism and limited regenerative capabilities, thus perhaps leaving it prone to oxidative insult (Andersen 2004). Increased oxidative stress is also a common feature of aging, suggesting that its presence in neurodegenerative disorders is a result of a reduced ability to handle reactive oxygen species produced by a toxic insult such as protein aggregates. Peroxidated lipids and proteins damaged by oxidation have been observed in tissue of SOD1-ALS patients (Ferraiuolo *et al.* 2011). mRNA oxidation has also been observed both in patients and in mouse models of SOD1-ALS and these alterations were shown to occur before onset of symptoms in the animal models, suggesting that oxidative stress is an early event in disease pathogenesis (Chang *et al.* 2008). However, as mentioned previously, many SOD1 mutants retain at least some of their catalytic dismutase activity and SOD1 knockout animals do not display ALS-like pathology. This underscores the toxic-gain-of-function aspect of SOD1-ALS and suggests that oxidative damage, while apparently a pre-symptomatic event, occurs downstream from some other initial toxic insult.

Mitochondrial dysfunction is also commonly seen in SOD1 associated pathology. Vacuolization of mitochondria was first observed in mutSOD1 animal models almost twenty years ago (Wong *et al.* 1995). Subsequent research showed that aberrant SOD1 acquired the ability to associate with Bcl-2, an anti-apoptotic protein, or the mitochondrial membrane itself (Pasinelli *et al.* 2004, Vande Velde *et al.* 2008). In regards to Bcl-2, the authors provide evidence that both WT and mutSOD1 are capable of binding Bcl-2, and that Bcl-2 associates with aggregates of SOD1 in humans and mice. They propose a model in which abnormal or increased association of Bcl-2 to SOD1 due to aggregation depletes the cell of free Bcl-2, thus

preventing its anti-apoptotic activity. Combined with its proposed ability to associate with the mitochondrial membrane, this creates a scenario where SOD1 can cause direct damage to the mitochondria and then subsequently impairs mitochondrial regulation of apoptosis, leading to cell death. SOD1 mutants have also been shown to associate with and disrupt components of the axonal compartment (DeVos *et al*. 2008). This is relevant to mitochondrial function because of neurons are typically highly polarized cells that are dependent on kinesin and dynein transport from the soma to their distal compartments, which includes transport of mitochondria for local energy production. Disruption of the axonal compartment could create a lack of mitochondria in the axon and axon terminal leading to atrophy of these subcellular areas.

Glutamate toxicity is another hallmark of neurodegeneration, and is observed in SOD1-ALS as hyperactivation of motor neurons (Ilieva *et al.* 2009). Interestingly, this toxicity appears to be caused by a non-cell autonomous mechanism wherein there is a failure of glutamate buffering by neighboring astrocytes. Another key aspect of excitotoxicity is the involvement of mitochondrial disruption and perturbations in enzyme systems that generate ROS (Arundine *et al.* 2003), which makes them downstream actors in the cellular pathology of SOD1-ALS. However, the sole approved drug on the market for ALS, Riluzole, functions partially by inhibiting glutamate release and provides limited extension of survival (Auclair *et al*. 2010). This raises the possibility that glutamate toxicity is not a principal player in the disease process and simply exacerbates other mechanisms of cell toxicity. Alternatively, because neuronal hyper-excitability has been observed pre-symptomatically, it may be the case that the available treatment for ALS is simply applied too late in the disease process after, i.e. after symptomatic onset.

Similar to excitotoxicity, neuroinflammation is a non-cell autonomous component of

ALS. CNS immune cells such as microglia are known to be active in animal models of mutSOD1 and pro-inflammatory factors such as IL-8 have been observed in the cerebrospinal fluid of patients (Kuhle *et al.* 2009). Astrocytes expressing mutSOD1 have also been shown to have a heightened inflammatory response and blocking the release of inflammatory factors or transplantation of glial cells expressing WT-SOD1 have been shown to be beneficial in mouse models (Hensley *et al.* 2006 and Beers *et al* 2008, Lepore *et al.* 2008).

Of note in the pathology of ALS is that much of what has been described has emerged from SOD1 animal models. This is partially a historical artifact as most of the fALS associated proteins have been identified more recently. Despite this, the SOD1 models have served as a reliable basis for studying ALS as there is a great deal of overlap between the symptoms and cellular pathology observed in other fALS cases as well as sALS.

1.5 SOD1

Among the gene products that have been described in ALS, the best characterized to date is superoxide dismutase-1. SOD1 was first identified as being genetically linked to a subset of familial ALS patients in 1993 (Rosen *et al.*). It is a copper-zinc metalloenzyme that exists as a homodimer and catalyzes superoxide radicals to hydrogen peroxide and water. Mutations in SOD1 are autosomal dominant and cause the dimeric form to become unstable, leading to monomers that have a high propensity to form intracellular aggregates (Furukawa *et al*. 2005). To date, more than 160 mutations have been identified in SOD1 that are associated with ALS. While it appears that all of these mutations are destabilizing and lead to misfolding events, the degree to which they do so varies greatly.

Because SOD1 is an antioxidant enzyme, it was initially thought that mutations in SOD1

caused a loss-of-function and SOD1-ALS resulted from higher levels of reactive oxygen species (ROS). While oxidative stress has been observed in murine models of ALS, there is sufficient evidence to support this pathology being independent of SOD1 enzymatic activity (Bruijn *et al*. 1997). In fact, most mutSOD1 variants maintain some if not all of their dismutase activity, which would mean that increased oxidation is a downstream effect of accumulated mutSOD1 rather than a disruption of its enzymatic function. This notion is supported by other key findings from mutSOD1 transgenic mice that were generated in the wake of SOD1 being linked to ALS: first, the severity of the disease (age of onset and progression) correlates with specific mutations and their effect on the stability of the protein and its propensity to aggregate (Prudencio *et al*. 2009). For example, A4V, a common mutation in North American populations, is highly aggregation prone and has an extremely rapid disease course of 2 years or less, compared to H46R which is significantly less prone to aggregation and has an average course of 5+ years (Khare *et al*. 2006). Second, disease severity is dependent on gene copy number. When the initial animal models of SOD1-ALS were generated, different lines had different numbers of mutSOD1 genes. Animals with the same mutation displayed different onset and survival times depending on mutSOD1 gene dosage, with higher copy-number animals having shorter survival times (Gurney *et al*. 1994). This suggests that the relative expression of mutSOD1 is a determining factor. This is further supported by animals with conditional deletions of the mutSOD1 transgene. Animals with mutSOD1 that has been excised via Cre-mediated excision do not develop disease or have a significantly delayed onset and/or death (Wang *et al*. 2010). Finally, full knockouts of SOD1 do not develop ALS-like symptoms or pathology (Reaume *et al*. 1996). Taken together, these findings support the conclusion that mutSOD1 is, in fact, causal and that intracellular aggregates containing SOD1 are toxic to the cellular environment and lead

to loss of motor neurons in the CNS, via a toxic gain-of-function mechanism.

SOD1-ALS has also been shown to have a non-cell autonomous component. Importantly, expression of mutSOD1 in cell types outside of motor neurons appears to be required for the observed time course of the disease. Selectively decreasing mutSOD1 in motor neurons delays the onset of the disease but does not affect progression (Boillee *et al.* 2006). On the other hand, decreasing mutSOD1 expression in microglia or astrocytes significantly delays progression, but does not affect onset. (Boillee *et al.* 2006, Yamanaka *et al.* 2008). This suggests a model wherein mutSOD1 in motor neurons is the initial source of cellular damage which is then exacerbated by impairments in glial cells.

Due to the inherent instability of mutSOD1 compared to WT, it is prone to misfolding events. Misfolding presumably leads to aggregation of the mutant protein which is readily observed in diseased tissues from animal models and humans. What has been unclear until recently is whether WT-SOD1 participates in the aggregation process. This is important for human patients because the disease is autosomal-dominant, so there is typically one normal and one mutant copy of SOD1 present. Recent studies have provided some evidence for the involvement of WT-SOD1 in the disease process leading some to speculate that SOD1 has prion-like properties. Prions are infectious agents that are composed of amino acids as opposed to nucleic acids. They have been shown to be responsible for some spongiform encephalopathies with a mechanism of action that appears to be dependent on 'templating' an unfolded or non-native conformation onto natively folded proteins.

One study using animal models of SOD1 demonstrated accelerated onset of disease by overexpressing WT-hSOD1 with hSOD1-G85R. The double Tg animals had a significantly earlier age of onset than either non-Tg animals or G85R alone. Pathologically, young double

transgenic animals were more similar to single mutSOD1 animals that were at the end stages of disease (Wang et al. 2009) suggesting that WT-SOD1 does contribute to the disease process. A separate study using antibodies specific for WT or mutSOD1 in a cell culture model demonstrated that WT-SOD1 was converted to a misfolded state in the presence of mutSOD1, thus providing evidence for intermolecular transmission of misfolding (Grad et al. 2011). Around that same time, a separate group showed that exogenous aggregates of SOD1 could be taken up by cells and subsequently induce misfolding and aggregation. Furthermore, by fluorsecently tagging the aggregates of different SOD1 mutants, they showed that these aggregates could be transmitted from cell to cell in a co-culture setting (Munch et al. 2011).

The picture that emerges from this research is that SOD1-ALS has many singularly interesting components that contribute to the disease process. Given the non-cell autonomous nature of the disease, the evidence that ties disease onset and severity to SOD1 expression levels and aggregation, and the apparent prion-like properties of SOD1, there appear to be multiple points for possible therapy. Therefore, despite the complex, multicellular character of the disorder, targeting SOD1 aggregates or modulating SOD1 expression remain the most obvious avenues for treating SOD1-ALS.

1.6 Regulation of SOD1 expression

Much is known about the regulation of the human SOD1 gene. Perhaps most obviously, the SOD1 promoter has a binding site for Nrf2, an activator of antioxidant response genes (Dreger *et al*. 2009). Nrf2 is normally targeted for degradation by a complex of Keap1 and Cul3, an E3 ubiquitin ligase. When there is a buildup of reactive oxygen species in the cell, it is thought that Keap1 acts as a redox sensor, changing its conformation to free bound Nrf2 for

targeting to the nucleus where Nrf2 can then bind the promoter regions of antioxidant genes such as SOD1. Additionally, the proximal promoter region of SOD1 has been shown to contain putative binding sites for xenobiotic response elements, NFkB, C/EBP, AP-1, Egr-1, and Sp1 (Milani *et al.* 2011) (Fig 1.2). These elements suggest that production of SOD1 mRNA, and presumably protein, is regulated by an array of different cellular stimuli.

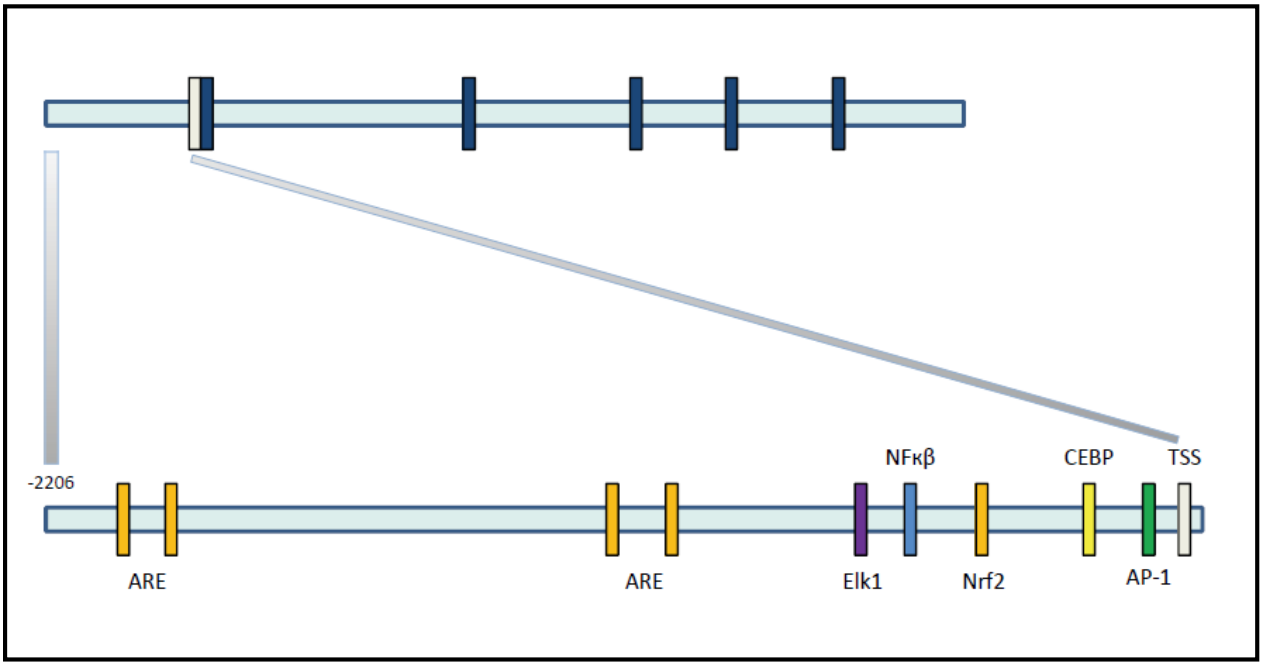


Figure 1.2 Proximal promoter region of the human SOD1 gene and organization of exons and introns. The SOD1 promoter contains putative binding sites for NfκB, Nrf2, Elk-1, the AP-1 complex, C/EBP (a CREB associated protein), in addition to several other antioxidant and xenobiotic response elements (Milani *et al.* 2011).

NFkB is principally involved in cellular immune responses but has also been shown to respond to cytokines and free radical damage. In neurons, NFkB can be activated by stimulation with growth factors such as BDNF or NGF, and it has been shown to play a part in synaptic plasticity due to its response to glutamate signaling (Gavalda *et al*. 2009). C/EBP is a bZIP transcription factor that is also involved in regulating immune and inflammatory responses. Similar to NFkB, C/EBP also responds to various growth stimuli (Calella *et al*. 2007). AP-1 is a transcription factor that is actually a complex of c-Fos and c-Jun proteins (Afonso *et al*. 2006). Both of these proteins are known to respond to stimuli that activate MAPK signaling, but c-Jun is primarily regulated through the JNK pathway, a pathway known to be involved in the cellular stress response. Egr-1, also known as Zif268, is a zinc-finger protein that has been shown to regulate transcription (Minc *et al*. 1999). While its precise function is still unclear it has been shown to be widely expressed in the brain and its expression is correlated with neural activity. Sp1 is another zinc-finger transcription factor that is known to respond to growth and immune factors. The assortment of factors that are predicted to induce SOD1 transcription suggest that in addition to its canonical response to Nrf2, SOD1 is activated as a general responder to cellular stress.

Interestingly, not much is known about the regulation of SOD1 post-transcriptionally. A recent study provided evidence of FMRP binding to mouse SOD1 mRNA through a unique triple-stem loop structure in the mRNA (Bechara *et al.* 2009). They propose that FMRP acted to stabilize SOD1 mRNA through this structure and thus promote SOD1 translation. However, this proposed mechanism is counter to the currently accepted function of FMRP as a repressor of synaptic translation and their results have not been independently verified. Despite this, there is evidence that SOD1 is actively translated in the axonal compartment so it is possible that SOD1

mRNA is transported and translated for compartment specific activity outside of the neural cell soma (Willis *et al.* 2005). Translation of synaptic proteins in response to neural activity is a well established phenomenon and there is a growing body of evidence that some of the same molecular players are involved in the axonal compartment (Deglincerti and Jaffrey 2012). Therefore it is a possibility that SOD1 mRNA is subject to similar activity dependent translation in neurons.

Post-translational regulation of SOD1 is better characterized. The enzymatic activity of SOD1 is known to be dependent on its metallo cofactors Zn and Cu and the delivery of Cu cofactor is dependent on the copper chaperone for SOD1 (CCS) protein (Culotta *et al* 1997). It is important to note however, that the folding of SOD1 is dependent on the presence of Zn and some disease causing mutations in SOD1 have been shown to perturb the protein's ability to bind Zn, thus leaving it more prone to misfolding events (Kayatekin *et al*. 2010). This suggests a model where impaired post-translational folding of mutSOD1 leads to an increased population of toxic non-native states that contribute to disease pathogenesis (Bruns and Kopito 2007). Moreover, the apo form of the enzyme is more readily targeted for proteasomal degradation via its association with CHIP, a binding partner of HSP70, and mutSOD1 variants have been shown to have shorter half-lives than WT-SOD1 (Urushitani *et al.* 2004, Borchelt *et al.* 1994). This suggests that there are additional alterations in post-translational regulation of mutSOD1 that lead to its aggregation.

1.7 Therapeutic Strategies for ALS

ALS is currently an untreatable disease with Riluzole being the only drug on the market that has shown any clinical efficacy. However, its effects are very limited and most patients gain

only a few months of survival time. Numerous other clinical trials are either underway or have been completed with limited or no success. This is most likely due to the complex nature of the disease along with our currently limited understanding of how the various cellular disruptions that have been identified may cooperate to produce a disease state.

Therapeutic strategies for ALS have generally targeted individual symptomatic aspects of the disease, such as microglial activation and inflammation, relieving mitochondrial damage, or reducing calcium-glutamate excitotoxicity. Chronic inflammatory responses are common in neurodegeneration and have been demonstrated to accelerate ALS progression suggesting that reducing inflammation in the CNS may be beneficial (Henkel *et al*. 2013). Dexpramipexole is a recent example of this type hypothesis. This compound was shown to stabilize mitochondrial function and confer cellular protection, presumably by slowing mitochondrial damage and preventing release of apoptotic agents. However it failed to show significant efficacy in Phase 3 trails in a study group of both sporadic and familial ALS patients. Other approaches have centered on providing trophic support for diseased cells or inhibiting processes that lead to cell death. Accordingly, several clinical trials of neurotrophic factors have been carried out with the hope that the maintenance and survival functions of these growth factors would slow or halt disease progression (Henriques *et al*. 2010). With a few minor exceptions, none of the known neurotrophic factors have demonstrated any significant clinical efficacy under the dosing regimens that were tested.

The majority of clinical trials for ALS mentioned above have centered on altering cell survival or affecting disease processes that may not be causal. However, what seems clear from animal models of SOD1-ALS and analyses of protein stability is that the amount of expressed mutSOD1 present correlates with disease severity. This leads to two obvious, broad categories

for therapy: dissolving or breaking up SOD1 aggregates and decreasing expression of SOD1.

Since these aggregates are presumed to be toxic to the cell, breaking them up or dissolving them could be beneficial. One way to accomplish this would be with small molecule compounds that bind the proteins that comprise the aggregates, thereby either preventing them from aggregating by stabilizing their mature form, or by dissociating them from existing aggregates. Cisplatin is an example of such a compound. It was recently shown that Cisplatin can bind dimeric apo-SOD1 to impart additional stability to the dimer interface. It was also demonstrated that applying Cisplatin to neuronal cells expressing mutSOD1 reduced the level of SOD1 oligomers (Banci *et al.* 2012). However, this approach has not been tested in human patients. A more recent study employed a chemical cross-linking strategy with maleimide compounds to stabilize SOD1 dimers (Auclair *et al*. 2010). They show that in the presence of their cross-linkers, both G93A and G85R variants of SOD1 are stable as dimers. Furthermore, they provide evidence that this stabilization restores the catalytic activity of the G85R variant, which is catalytically dead when aggregated. While these results are intruiging, they still need to be translated from the *in vitro* setting employed here. Another compound, Arimoclomal, is currently in clinical trials. It is thought to act by up-regulating molecular chaperone activity in the cell, thereby preventing partially folded proteins from aggregating and driving them towards their native states (Kim *et al*. 2013). Similarly, targeting the ubiquitin-proteasome system may be of some value as it has been shown in mouse models that aggregation may be reversible by enhancing proteasome function (Puttaparthi *et al.* 2003). However, these latter approaches ultimately may not be practical because broad, non-specific activation of chaperones or inhibition of proteasome activity may increase the concentration of other non-disease associated proteins that need to be degraded by the cell. Alternatively, manipulating endogenous signaling

pathways or transcription factors that affect expression of SOD1 may provide a more straightforward approach. The thought is that if SOD1 aggregates are indeed the toxic, pathogenic species, then reducing the amount of available SOD1 should reduce aggregate formation and in turn, ameliorate disease symptoms.

The variety of transcription factors that can bind the SOD1 promoter and the range of stimuli that they can respond to also suggest that controlling SOD1 expression at the level of transcription may be a viable therapeutic route. Murakami *et al.* conducted a high-throughput chemical screen to identify chemical compounds that affected SOD1 transcription. Using a Luciferase-SOD1 fusion protein driven by the genomic promoter, they identified a compound that reduced Luciferase signal in a dose-dependent manner. This result was confirmed biochemically by examining SOD1 protein levels and they report no cellular toxicity. Similarly, a recent study performed a screen for chemical inhibitors of SOD1 transcription (Wright *et al.* 2010). Using PC12 cells, a rat neural cell line, they identified a compound that reduced SOD1 expression. This was then confirmed in a human cell line, wherein they showed that treatment with the compound reduced endogenous SOD1 levels. However, the effect was more modest when tested in mouse spinal cord tissue. While the modest effect of these compounds may be of small benefit clinically, these studies support the idea of using high-throughput formats to identify modulators of SOD1 expression.

In further pursuit of reducing SOD1 using existing cellular mechanisms, other studies have attempted to use the RNAi machinery or antisense methods to target SOD1 mRNA in order to reduce protein levels. One recent study used such a strategy in a transgenic mouse model. Using two variants of the G93A mice, with high and low copy numbers of mutSOD1, they globally expressed a transgene for an allele-specific shRNA against mutSOD1 (Xia *et al.* 2006).

Despite only reducing mutSOD1 protein expression by 50% as detected by western blot from spinal cord extracts, mice that expressed the shRNA transgene showed significant delays in age of onset and had significant extensions in survival time. Similarly, another group used an shRNA to target the human SOD1 transgenes in G93A mice (Ralph *et a.l* 2005). An important difference in their strategy was that the delivery of the shRNA construct was targeted intramuscularly. The viral construct employed here had been shown previously to be retrogradedly transported to motor neurons and this was confirmed by IHC. Additionally, the construct was delivered by injection postnatally. Their analysis showed a significant reduction in human SOD1 which correlated with enhanced motor performance and delays in disease onset. Notably, there was no improvement in survival time after disease onset in animals receiving SOD1-shRNA compared to untreated controls. Both groups had a window of disease progression and survival of approximately forty days after onset. This highlights the importance of decreasing SOD1 levels in cell types beyond skeletal muscle and motor neurons and supports the notion of non-cell autonomy in SOD1-ALS. More recent work has attempted to replicate these findings using an AAV9 viral vector for delivery of SOD1 antisense RNA (Foust *et al*. 2013). AAV9 is a vector that has been modified from an adeno-associated virus, which is infectious to some primate species including humans but does not cause a severe immune response, making it a candidate for therapeutic use in human patients. This approach achieved similar levels of mutSOD1 knockdown of roughly 50% and the age of vector delivery correlated with the degree of improvement in age of disease onset and time of survival. Taken together these studies support the idea of reducing SOD1 protein as a therapeutic avenue.

Summary

There has been significant progress in our understanding of the underlying cellular mechanisms of ALS. The vast majority of the data on hand points to mutSOD1 as causal for a large subset of fALS, with misfolded and/or aggregated SOD1 having toxic effects in multiple cell types in the CNS. Pursuant to this, multiple studies, employing varied approaches, have shown that reducing mutSOD1 levels positively affects disease severity. Furthermore, the effect on disease onset and progression in animal models appears to be cell-type dependent as SOD1 reduction in motor neurons or glial cells differentially affects these aspects of disease. This produces a scenario where identifying endogenous gene products that affect SOD1 protein levels and how they may differ in multiple cell types may be of therapeutic benefit. This strategy could also provide new insight into the cellular pathology of the disease or how currently established mechanisms combine to produce cellular toxicity.

Chapter 2

Screening for Modulators of SOD1 Expression

2.1 Introduction

As mentioned above, a potential line of treatment for SOD1-ALS is modulating or perhaps controlling expression of SOD1. A straightforward way of pursuing this strategy would be to interrogate chemical or genomic libraries for products that alter SOD1 protein levels. However, this also requires a reliable, quantitative measure of SOD1 in a high-throughput setting. Previous research had used GFP or β-galactosidase fusion assays to monitor protein-protein interactions or aggregation in certain conditions. Additionally, similar approaches had been used to screen for compounds that affected SOD1 transcription or increased its degradation (Murakami *et al*. 2011, Broom *et al.* 2006). However, none of these approaches have examined endogenous proteins or signaling pathways that directly affect SOD1 expression. Recent work from our lab employed an expression screen of mouse CNS cDNA in combination with an assay to monitor protein folding and solubility (Somalinga *et al.* 2011). The study examined endogenous elements that may regulate SOD1 expression and solubility when upregulated, in line with the aforementioned therapeutic strategy of decreasing SOD1 expression and in turn, aggregates. A subsequent screen used a whole genome RNAi library in tandem with the same β-gal reporter system to examine the complementary aspect of genes that affect SOD1 when suppressed. Therefore, the rationale was to use an assay similar to those previously described to identify gene products, rather than compounds, that either modulate SOD1 expression or affect its solubility, in the hope that these would provide targets for therapy.

2.2 cDNA Expression Screen for Modulators of SOD1 Expression

To screen for cellular modulators of SOD1, a complementation assay developed in our labby was employed (Wigley *et al.* 2001). This assay makes use of the β-galactosidase (β-gal) enzyme as a reporter by fusing a small portion of the β-gal protein (α-fragment) to the C-terminus of a target protein, in this case SOD1. When co-expressed in cells with the main β-gal portion (ω-fragment) the complementary pieces form a functional enzyme that can produce a fluorescent signal when exposed to a substrate, FDG (Figure 2-1, left). Complementation and fluorescence are dependent on the solubility and expression level, respectively, of the target protein. This provides a means of quantitatively measuring protein solubility and expression.

Initial testing of a CMV driven human SOD1 (hSOD1) with this system in HEK293 cells revealed good correlation between the signal obtained in the fluorescence assay and detection of protein levels via western blot (Figure 2-1, right). Several different variants of hSOD1 were tested including the common mutations A4V and G93A. The preliminary data also demonstrated that the biochemical readouts correlated well with the respective stability of the SOD1 variants, i.e. mutant SOD1 is less stable which results in lower total protein and therefore less fluorescence in this assay.

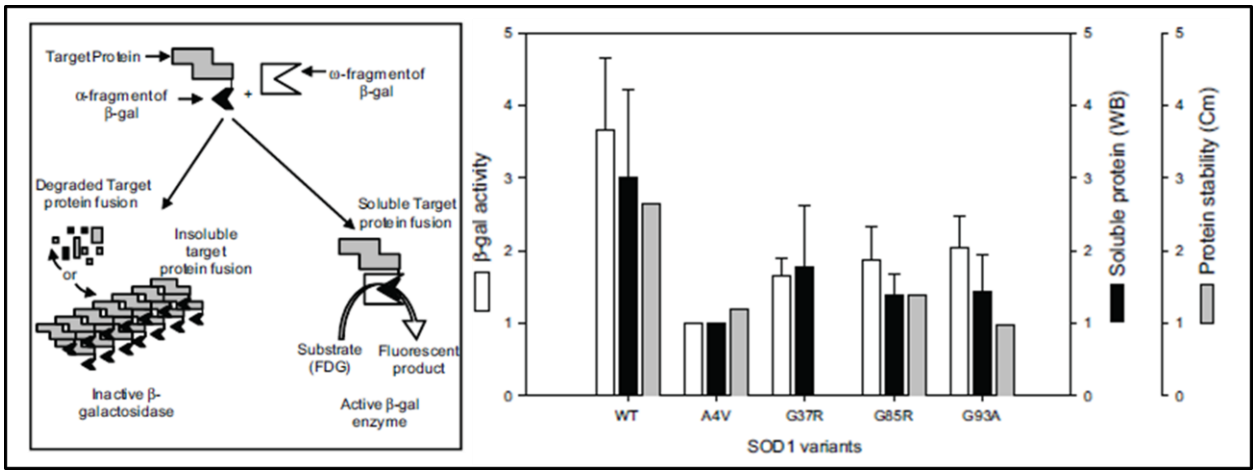


Figure 2-1 Summary of β-galactosidase complementation assay. Left – Aggregated, insoluble, or poorly expressed protein will result in low fluorescent signal due to a lack of functional enzyme. Right – β-gal activity/fluorescence (white bars) correlates very well with soluble protein levels as detected by western blot (black bars). For hSOD1, these measurements are also closely related to the relative stability of various mutants (reprinted from Somalinga *et al.* 2011).

Following optimization of the assay, the system was used to screen a library of approximately 350,000 cDNA clones from mouse central nervous system tissue. Primary enrichment was done using a fluorescence activated cell sorting approach. Enriched cDNAs from FACS were then divided into pools of 10 for secondary screening in a 96 well plate format. Secondary pools containing genes that enhanced fluorescent signal in the assay were selected and regrouped into pools of four cDNAs for tertiary screening, again in a 96 well format. Tertiary pools containing cDNAs that enhanced SOD1 signal were selected and single cDNA clones were isolated using LB-Ampicillin selection. The cut-off for selection in the tertiary screen was three standard deviations above the control. Single cDNAs were then screened and those that increased fluorescent signal in the β-gal assay were sequenced. The sequences obtained for the single cDNA clones were then analyzed and identified using BLAST. This resulted in a list of 15 targets that modulated SOD1 expession. The results for these targets obtained in the β-gal assay were then verified biochemically by western blot. Because the screening process was carried out in HEK293 cells with a CMV promoter driving SOD1-HAα expression, the identified SOD1 modulators were retested with a human-SOD1 promoter driving SOD1 expression to separate modulators of expression and solubility. The human-SOD1 promoter used here was composed of the 2.2kba immediately upstream of the SOD1 coding region. This promoter was cloned from human tissue and is highly similar to a SOD1 promoter used in separate study screening for chemical modulators of transcription (Broom *et al.* 2006). However, because the relative expression of SOD1 from this promoter is low in HEK293 cells, the experiments with this promoter were done in HeLa cells. From this set of experiments the most prominent hit was RasGRF-1 (Figure 2-2). This result also suggests that there may be promoter and/or cell type dependence for some of the targets identified in the screen.

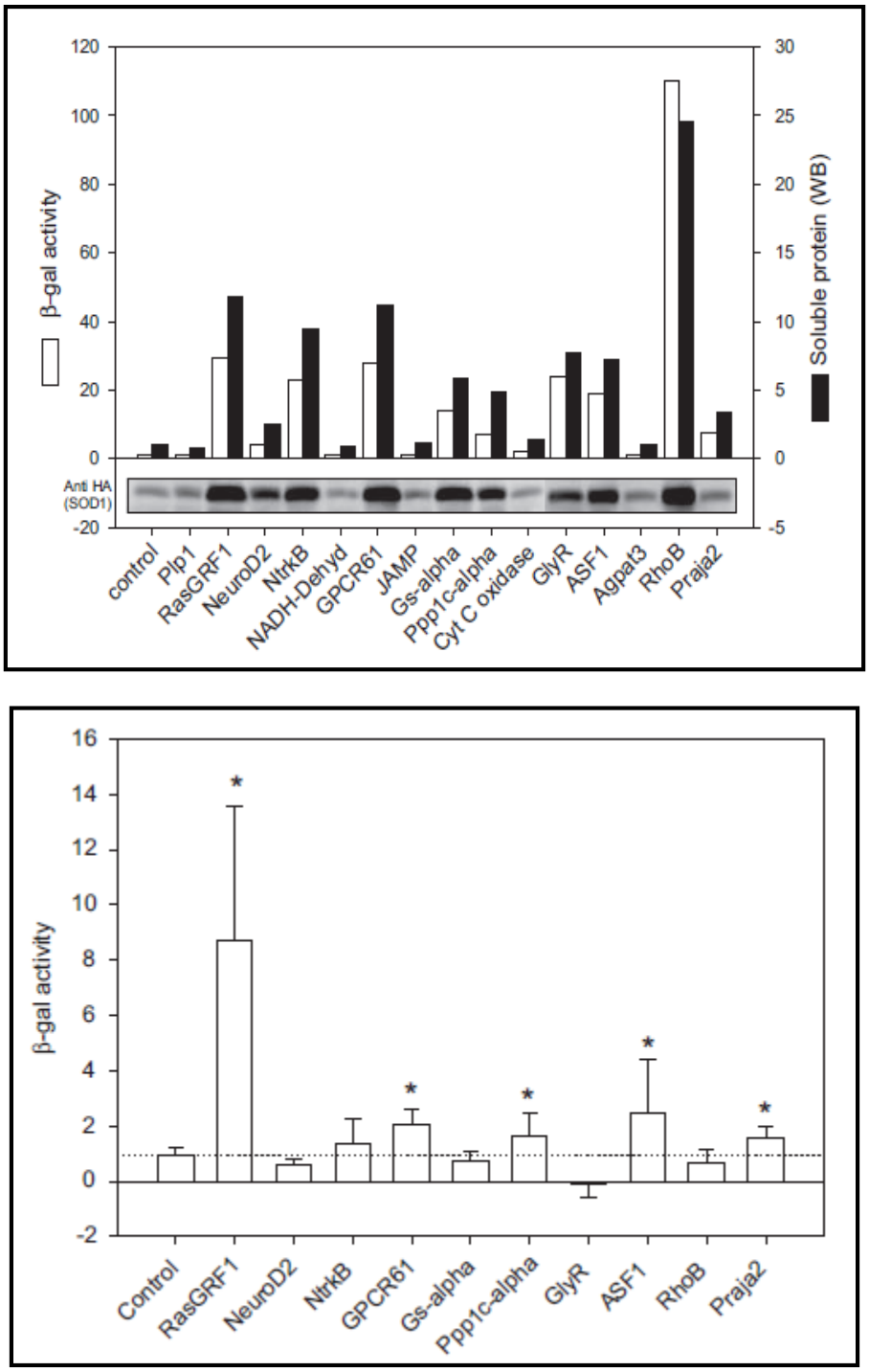


Figure 2-2 cDNA clones identified in the initial HEK293 screen (top) were re-examined in HeLa cells using a human SOD1 promoter (bottom). The most prominent hit was RasGRF-1 (reprinted from Somalinga *et al.* 2011).

2.3 Whole Genome RNAi Screen for Modulators of SOD1 Expression

While conducting the biochemical validation for the cDNA expression screen, we conducted a second screen using RNAi. Conceptually, the cDNA screen relied on high-expression, and presumably exaggerated function, of gene products to identify effectors of SOD1 expression. Therefore, the primary goal of the RNAi screen was to see if employing an inverse technique could also reveal modifiers of SOD1.

For the RNAi screen we employed the same β-gal assay system described for the cDNA expression screen. As seen during validation of the cDNA hits, HeLa cells displayed better relative expression of hSOD1 driven by the human SOD1 promoter when compared to other mammalian cells such as HEK293. The expression characteristics of HeLa cells combined with their viability in a high-throughput setting led us to select them for this screen. The β-gal assay system was then optimized for a high-throughput format in this cell line. Optimization was performed using an siRNA against the S4 subunit of the proteasome in concert with our SOD1 reporter. Knockdown of this subunit has been shown to return a similar result as inhibition of the proteasome, a simple and reliable method for increasing protein levels, and the results support this expectation (Fig. 2-3). Further optimization was done by varying the amount of transfected SOD1-HAα construct in order to achieve optimal stochiometry of our reporter elements as the ω-fragment is being expressed via a CMV promoter and thus at much higher levels. Our results show that a 4:1 ratio of α:ω was optimal under these conditions, therefore we proceeded the screen (Fig 2-3).

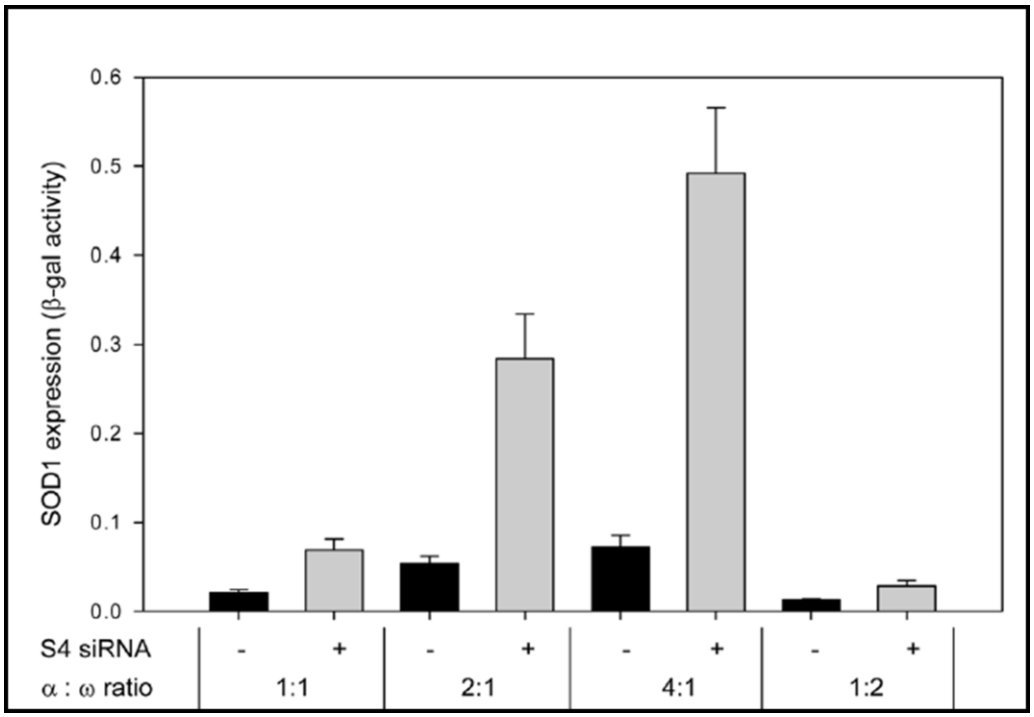


Figure 2-3 Optimization of β-gal assay. Knockdown of the S4 subunit of the proteasome increases SOD1 signal. Analysis of α:ω revealed that a 4:1 ratio gave the greatest change in signal to noise ration for SOD1 (reprinted from Somalinga *et al.* 2012).

Results from the screen were analyzed by two different statistical methods, median absolute deviation (MAD) and z-score. MAD is a median-based method that measures statistical dispersion and therefore can help rule out variability that may be inherent within a large data set. $MAD = 1.4826 \times median(|x_{ij} - median(x)|)$, where median(x) is the value of all the samples in one experimental plate and x_{ij} is the value of an individual well. The constant 1.4826 is used to normalize MAD to standard deviation where a normal distribution occurs. Z-score is a more conventional method that uses the mean and standard deviation of a set or population. $Z = (x - \mu)/\sigma$, where x is the value of an individual sample, μ is the mean of all samples (population), and σ is the standard deviation of the population. Comparing the hits from these different analyses yielded significant overlap in the datasets with approximately 90 percent of the z-score hits appearing amongst the MAD hits (Figure 2-4). Because the MAD method is more resistant to statistical outliers we chose to analyze the hits from this data set.

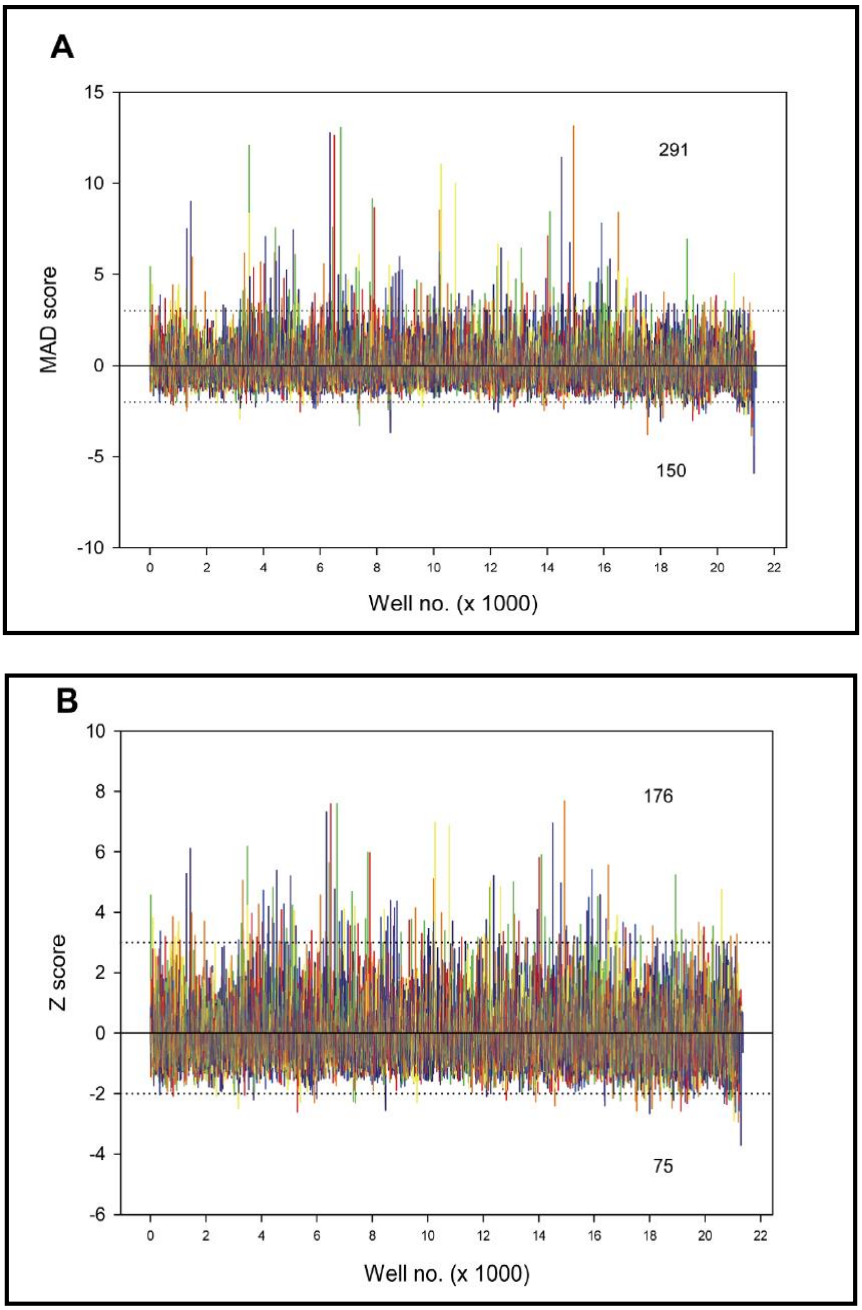


Figure 2-4 Analysis of RNAi screen. Two different statistical methods were employed for statistical analysis. a) MAD score –median absolute deviation b) z-score, raw value compared to population standard deviation and mean. Cutoffs for hits were set at +3SD and -2SD. The lower cutoff was adjusted to 2SD due to assay floor effects. Inset numbers indicate number of hits above or below the cutoffs for each method (reprinted from Somalinga *et al.* 2012).

We organized the hits from the screen into functional networks using pathway analysis software (Fig. 2-5, top). This allowed us to characterize specific sets of genes that may act in concert or respond within the same signaling network(s). Cross-referencing our pathway rankings with established pathway maps from the KEGG (Kyoto Encyclopedia for Genes and Genomes) database revealed several additional components of the MAP kinase signaling pathways. These included but were not limited to: ERK1/2, NFκB, P38, and Ras. Combined with the previous results from the cDNA expression screen, these data strongly implicate the MAP kinase pathways in the regulation of SOD1 protein levels (Fig. 2-5, bottom).

Rank	No. of molecules	Highly ranked protein interaction networks
1	50	Skeletal and Muscular System Development and Function, Tissue Morphology, Inflammatory Response
2	41	Carbohydrate Metabolism, Lipid Metabolism, Small Molecule Biochemistry
3	28	Cell Cycle, Cellular Growth and Proliferation, Embryonic Development
4	28	Cardiovascular Disease, Metabolic Disease, Lipid Metabolism
5	27	Lipid Metabolism, Molecular Transport, Small Molecule Biochemistry
6	27	Cell Cycle, Cell Death, Cardiovascular System Development and Function
7	26	Organismal Injury and Abnormalities, Cancer, Dermatological Diseases and Conditions
8	25	Protein Degradation, Protein Synthesis, Lipid Metabolism
9	23	Cell Death, Cellular Growth and Proliferation, Hematological System Development and Function

Network 2:
ADRA2A, ADRBK1, Angiotensin II receptor type 1, ANXA6, Ap1, BMP2, Calcineurin protein(s), CD3, CD9, CYCS (includes EG:54205), Cytochrome c, DBNL, DMBT1, EDNRA, **ERK**, **ERK1/2**, F3, Focal adhesion kinase, G alpha, G-protein beta, **GAB2**, Gpcr, GPR3, HSD17B10, **Insulin**, LDL, Lh, **Mapk**, Mmp, NEXN, **NFkB** (complex), NXF1, NXT1, **P38 MAPK**, Pdgf, PDGF BB, **PI3K**, PIK3CD, Pkc(s), PLA2, PLB1, **PLC**, **PLC gamma**, PLCD1, **PLCG1**, PSMA3, **Ras**, REL, RICS, RPL13, SERPINE1, SFRS3, SFTPA1, Shc, SLC2A6, SLC7A7, SOD1, SRC, TACR1, TBXA2R, TRIB3, TXNRD1, U2AF1, UGDH, **Vegf**, VIP, VIPR1, VSNL1, WTAP, XPO1

Figure 2-5 Organization of screen hits into functional networks. Top panel - networks were ranked according to the number of gene products in each set. Network names were assigned by the pathway analysis software. Bottom panel - several additional members of the MAP kinase pathways (in bold) were identified and grouped in the second highest interaction network.

2.4 Discussion

RasGRF-1

RasGRF-1 is a guanine nucleotide exchange factor for Ras (rat sarcoma) proteins, which are small GTPases that function in cellular signaling cascades. The identification of this protein from the cDNA screen is interesting on two fronts. First, RasGRF-1 is known to anchor/associate with microtubules which are known to be disrupted in some ALS patients (Forlani *et al*. 2006). Other proteins with similar structure and function to RasGRF-1 have been suggested to contribute to formation of aggregates in ALS cases and Alsin2, another fALS associated protein is also a Ras exchange factor (Lai *et al*. 2009). Second, Ras, the target of RasGRF-1, is known to be an integral component of the mitogen activated protein kinase (MAPK) signaling cascade. This cascade is fairly well characterized and some of the transcription factors that act downstream of MAPK activation have been shown to bind the promoter region of SOD1. Taken together these suggest the possibility that RasGRF-1 may play a role in SOD1-ALS either by contributing to the formation of SOD1 aggregates or by affecting the signaling machinery upstream of SOD1 transcription. As previously noted, disruption of SOD1 aggregates and reducing SOD1 protein levels have both been pursued as therapeutic strategies making RasGRF-1 an intriguing target.

RasGRF-1 produced similar effects in both cell types with different promoters (CMV vs. hSOD1). However, other targets such as G_s-alpha, a G-protein subunit, and NtrkB, a neurotrophin receptor, completely lost their effect on SOD1 when tested in HeLa cells with the hSOD1 promoter. This could be a result of different expression patterns within the two cell lines as they are derived from different human tissues (HEK cells are from kidney, HeLa from cervix) or from copy number variance as HEK293 cells have 64 chromosomes and HeLa 82.

Alternatively, it could be that some of these targets require components of the CMV promoter and their effect on SOD1 was non-specific or due to effects on solubility or post-translational modifications.

Interestingly, expression of RasGRF-1 is largely confined to the CNS (Shou *et al*. 1992). Three isoforms of RasGRF-1 are known to exist, all of which are expressed in the human brain (Feig 2011). The cDNA identified in the screen is that of the second isoform. This isoform contains the same three C-terminal domains from the full length protein: the Ras-activating domain (CDC25), a CDC25 stabilizer domain (REM), and a domain that mediates binding to NMDA receptors, a type of glutamate receptor in the CNS (ND) (Fig. 2-6). While little is known about the specific functions of isoform 2, studies on recombinant forms of the full-length isoform suggest that the N-terminal domains negatively regulate the GTP-exchange activity of the C-terminal domains (Baouz *et al.* 1997) This suggests that isoform 2 may exhibit increased or perhaps constitutive activity towards Ras. However, this remains to be described in a neuronal cell context.

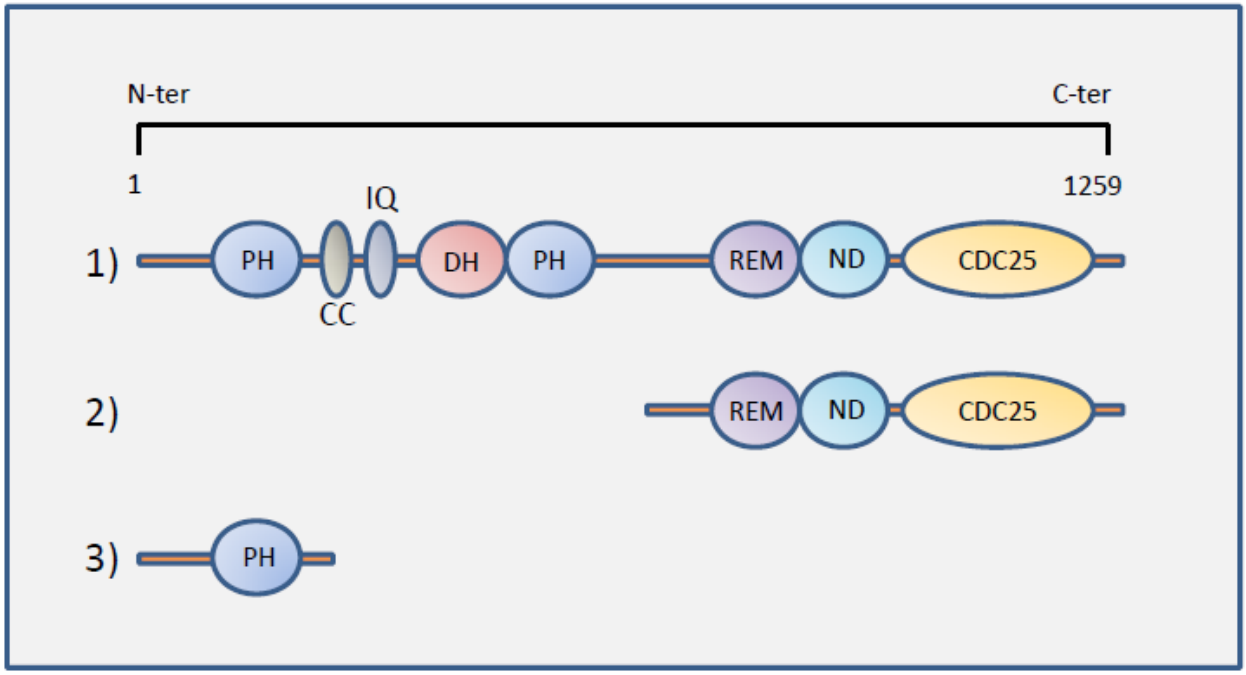


Figure 2-6 The isoform identified from the cDNA screen was isoform 2 (middle). This isoform maintains the domains necessary for GTPase activity and association to NMDA receptors but lacks the domains required for Ca^{2+}-induced activation **(**Feig 2011). PH = plextrin homology, CC = coiled coil, IQ = Calcium/calmodulin binding motif, DH = Dbl homology, REM = ras exchange motif, ND = NMDAR binding domain, CDC25 = ras activating domain.

MAP Kinase Pathways

Beyond RasGRF-1 we can look at the MAP kinase pathways themselves. The results from our RNAi screen revealed several additional components of the MAPK pathways as possible modulators of SOD1 protein. This suggests that these pathways may be targeted as a whole instead of focusing on individual components such as RasGRF-1.

MAP (mitogen activated protein) kinases are specific for serine and threonine amino acid sites and are similar to cyclin dependent kinases (Pearson *et al*. 2001). They constitute a vast and complex signaling network that coordinately regulates an array of cellular functions in response to specific external stimuli. These functions include but are not limited to: cell division, differentiation, cell survival, and apoptosis (Pearson *et al*. 2001). There are six distinguishable, known groups of MAPK proteins: extracellular regulated kinases (ERK1/2), ERK3/4, ERK5, ERK7/8, Jun NH2 terminal kinase (JNK), and p38 (Krishna and Narang 2008). Notably, two of these groups were identified in the RNAi screen, ERK1/2 and p38, and as previously stated, RasGRF-1 acts on Ras proteins which are upstream activators of ERK1/2 and p38.

Schematically, these pathways are laid out in a typical signaling cascade (Lewis *et al*. 1998). Cell surface receptors, such as receptor tyrosine kinases, are activated by various stimuli and can then be transduced by binding partners such as G-proteins (Pawson and Scott 1997). These transduction elements then pass on the signal through the first tier of MAPKs (MAP3Ks) at the membrane (Yuasa *et al*. 1998). The MAP3Ks bind and phosphorylate MAP2Ks which then bind and activate MAPKs. As an example, upon receptor activation, Raf proteins (MAP3K) would be recruited to the membrane and subsequently activate MEK1/2 (MAP2K) via phosphorylation. MEK1/2 would in turn phosphorylate and activate ERK1/2 (MAPK), which

can then be transported to the nucleus to exert its regulatory processes. This type of organization is important on multiple fronts. Primarily it allows for output specificity and signal integration. Specific phosphorylation events at the plasma membrane dictate which transduction elements will be activated and thus which MAP3Ks will be recruited and each tier of MAPK elements has specificity for the next tier. Furthermore, some of the primary transduction elements are known to respond to different stimuli and activate different sets of MAP3Ks. This allows for signal integration to occur between individual pathways which dictates how the output is carried forward, i.e. dialed up or down. In this manner the output through these pathways can be finely tuned according to the type, magnitude, duration, and coincidence of cellular stimuli. Assuming that SOD1 is a downstream target of these pathways, this suggests that there may be multiple points at which their signaling output may be effected to regulate SOD1 expression.

RasGRF-1 and MAPK Pathways in Neurons

As mentioned above, RasGRF-1 has been shown to associate with NMDA receptors. These glutamate receptors are coincidence detectors of pre and post-synaptic activity and thus serve as the initial basis for long-term potentiation and depression of neurons, the cellular correlates of memory (Sturani *et al*. 1997). However, the isoform we have identified here lacks the IQ domain of the full-length protein and therefore cannot respond to Ca2+ influx through these receptors. This suggests that GRF-1 isoform 2's response in neurons is dependent on another stimulus or that it regulates or amplifies the response of full-length RasGRF-1. However, there is evidence from mouse knockout models that isoform 2 has a function independent of full length RasGRF-1 (Giese *et al*. 2001).

In terms of neuronal function, the pathway that primarily mediates the activity of

RasGRF-1 is MEK/ERK. Upon phosphorylation, ERK can translocate to the nucleus where it can activate several transcription factors including CREB and Elk-1 which have been shown to be important for neuronal survival and memory formation. ERK activation is also known to participate in translation activation at the synapse in response to high frequency stimulation, an inducer of LTP through mTOR (Tsokas *et al*. 2007). ERK is also able to affect cell wide translation via phosphorylation of eIF4E and S6 kinase, two well established regulators of the translation machinery (Kelleher III *et al.* 2004). Thus it appears that RasGRF-1 activation may be able to affect gene expression both transcriptionally and translationally via MEK/ERK signaling.

Possible Connections to SOD1

As mentioned above, there are multiple levels at which the MAPK pathways can regulate gene expression, and possibly SOD1. Elk-1 is known to bind the SOD1 promoter and has been shown to upregulate SOD1 expression via ERK1/2 activation in mice (Chang *et al.* 1999 and Wang *et al*. 2011). As previously stated, SOD1 is actively translated in other neuronal compartments outside of the cell soma and given ERK's involvement in translational regulation it may be possible that ERK activity modulates SOD1 translation. Interestingly, mouse models of SOD1-ALS have shown that ERK may be upregulated in astrocytes of symptomatic mice (Chung *et al*. 2005). This upregulation may be part of a stress response to the toxicity induced by SOD1, but assuming that SOD1 is downstream of ERK, increased ERK phosphorylation and activity may actually increase SOD1 production and exacerbate the disease state.

Other recent work has examined the possible role of p38 in ALS. p38 was found to be activated pre-symptomatically in the cortical motor neurons of G93A mice and is associated with

TNFα receptors in spinal motor neurons (Holasek *et al*. 2005 and Veglianese *et al.* 2006). Canonically, p38 responds to cellular stress, so activation of p38 in these models may have been in response to early cellular toxicity or inflammatory factors released by neighboring microglia prior to motor neuron death. p38 activation has also been observed in motor neurons at end-stage time points and there is evidence of a motor neuron-specific p38-Fas cell death pathway (Dewil *et al.* 2007). Despite the links to ALS pathology demonstrated in these studies, none of them examined SOD1 expression. To date there is no direct link between p38 activation and upregulation of SOD1 expression. However, one study demonstrated that NfkB, which is a downstream target of p38, can bind to the SOD1 promoter and induce transcription, although it should be noted that this induction was done via PI3K/Akt signaling and not p38 (Rojo *et al.* 2004).

Summary

These results show that we have identified several targets within the MAP kinase pathways that may provide a means of modulating SOD1 expression. Going forward we can test this by overexpressing these components or by using pharmalogical inhibitors that show a high degree of specificity for MEK/ERK and p38 such as U0126 or SB220025 (SB22) (English and Cobb 2002). Additionally, early validation experiments suggest that there are cell type specific requirements for these gene products to act on SOD1. Therefore, we should perform additional validation in a cell line with neuronal characteristics that is more relevant to the disease setting. Also of note is that these pathways are known to respond to a number of growth factors and neurotrophins that act through tyrosine kinase receptors (Doanes *et al.* 1999 and Xing *et al.* 1998). This provides another group of targets that can help elucidate the possible connections

between the MAP kinase pathways and SOD1.

Acknowledgements:

Dr. Balajee Somalinga conducted the cDNA expression screen described above and referenced in Somalinga *et al*. 2011.

Dr. Balajee Somalinga and I collaborated on the RNAi whole-genome screen described above and referenced in Somalinga *et al.* 2012. I participated in the screen and subsequent data analysis.

Chapter 3

Signaling Pathways in the Regulation of SOD1 Expression

3.1 Introduction

The preliminary data from the screens provided sufficient evidence for pursuing specific elements of the MAP kinase pathways as targets for manipulating SOD1 expression. The data also suggested there may be differences in how these elements affect SOD1 depending on the cellular environment and/or the regulatory elements in the 5' region of SOD1. Furthermore, while these screens were done in mammalian cell lines, they were not of a neuronal lineage. Therefore our primary goals in following up the results from the screen were to test whether the observed effects occur in a neuronal cell line and to characterize any effects that might be promoter dependent.

We chose to move our cell culture experiments into the NSC34 cell line based on its well characterized neuronal characteristics. This cell line was established by Dr. Neil Cashman approximately twenty years ago and is a hybrid fusion of a mouse neuroblastoma (N18TG2) and primary motor neurons from the mouse spinal cord (Cashman *et al*. 1992). These cells synthesize, release, and take up acetylcholine, fire action potentials, express neuronal intermediate filaments, and form functional synapses when co-cultured with myotubes, all of which are features of functional motor neurons. (Cashman *et al*. 1992). Furthermore, all of these characteristics are absent in the parental neuroblastoma cell line, suggesting that the predominant character of the resulting fusion is neuronal. This cell line has also been previously used as a culture model for fALS as NSC34 cells that have been transiently or stably transfected with mutSOD1 develop SOD1 aggregates and cell toxicity, which recapitulate major features of the disease (Gomes *et al.* 2007, Magrane *et al*. 2009, and Nishitoh *et al.* 2008). In total, these features outline the utility of this cell line as a system to study SOD1-ALS.

3.2 Expression of RasGRF-1 in NSC34 cells decreases SOD1 protein

The first set of experiments involved examining the effects of a subset of the cDNA screen targets in our motor neuron cell line due to possible cell type differences observed during the screen. Preliminary experiments were performed using a Myc-tagged SOD1-A4V construct driven by the human SOD1 promoter (Fig 3-1). Cells were seeded to 6-well plates and transfected 24hrs later at 50-60% confluency with Myc-SOD1-A4Vand either RasGRF-1 or an empty pcDNA3.1 vector to control for total DNA content. Strikingly, overexpression of RasGRF-1 significantly reduced SOD1 protein levels (Fig 3-2a), which is in marked contrast to the response seen in HeLa cells (Fig 3-2b). To rule out any off-target effects of the Myc-tag or the SOD1 construct we repeated this experiment using the SOD1-A4V-HAa construct used in the screens. Similar results were obtained with this construct demonstrating that there is no effect due to the different tags (Fig 3-2b).

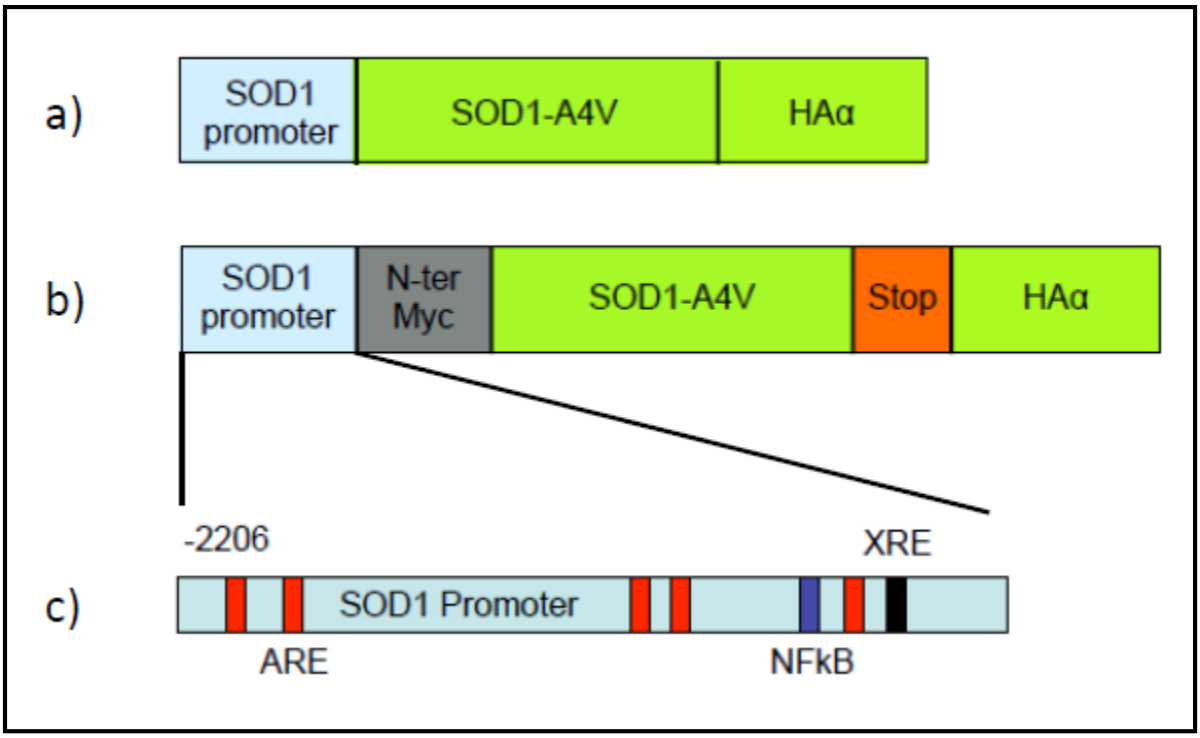


Figure 3-1 Human SOD1 constructs. a) SOD1 construct used in the screens; 2.2kb SOD1 promoter cloned from human tissue is drivinig expression of a SOD1 cDNA (A4V variant shown here) fused to an HA tag and the β-galtactosidase alpha fragment. b) A similar construct was prepared by cloning in an N-terminal Myc tag between the promoter and the beginning of the SOD1 cDNA. A stop codon sequence was inserted on the 3' end of the SOD1 cDNA upstream of the HA tag. c) A basic schematic of the cloned human SOD1 promoter. The promoter is 2.2kb and is similar to that described by Broom *et al*. 2006. Example regulatory sites are shown for antioxidant response elements (ARE), xenobiotic response elements (XRE) and NFκB.

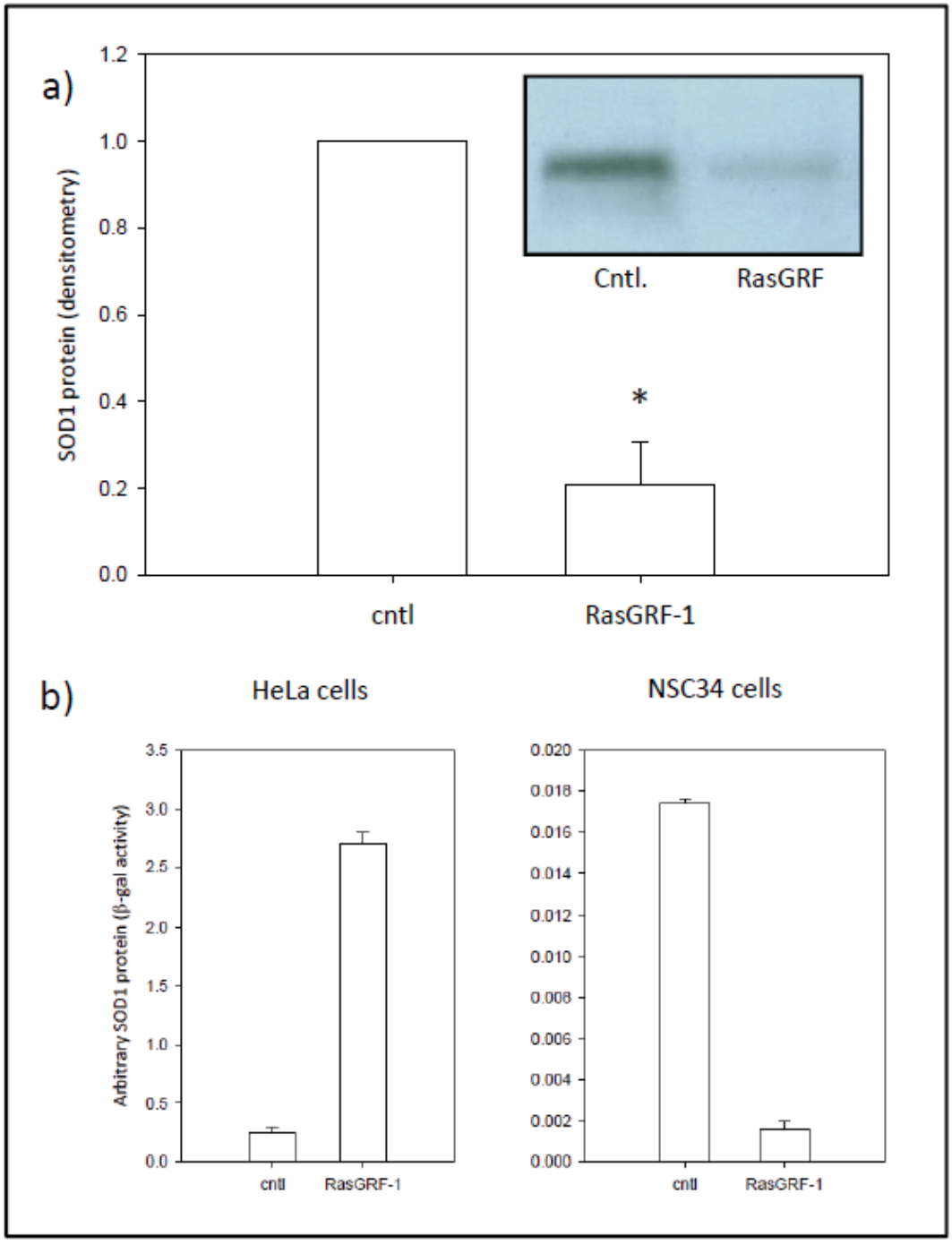


Figure 3-2 Expression of RasGRF-1 in NSC34 cells decreases hSOD1 protein levels. a) Quantification of Myc-tagged hSOD1 expression -/+ RasGRF-1 from western blots, inset: representative western blot for α-Myc SOD1, (n=4). b) Test of alternative hSOD1 construct with HAα fusion in NSC34 cells. Results are similar for both constructs. Samples are prepared from the soluble fractions of cell lysates. * = p-value > 0.05

Since RasGRF-1 is an activator of Ras and Ras is an upstream effector of MEK/ERK we hypothesized that overexpression of RasGRF-1 may mediate suppression of SOD1 protein through this signaling module. In support of this hypothesis we observed an increase in phospho-ERK levels in some of our experiments when RasGRF-1 is overexpressed. To test this we repeated our overexpression experiment with an inhibitor of MEK activity, U0126 **(**Favata *et al.* 1998**)**. U0126 has been shown to be highly selective for MEK1/2, which specifically activates ERK1/2; therefore, by treating with U0126 we should be able to block the effects of RasGRF-1 downstream. We tested U0126 at several different concentrations to establish effectiveness in our cell line. ERK phosphorylation was completely abolished at all of the concentrations that were examined and there were no visible signs of cell toxicity. Surprisingly, when RasGRF-1 was overexpressed with U0126, we see no rescue of SOD1 protein levels (Fig 3-3a). This suggests that RasGRF-1 exerts its effects on SOD1 through another signaling pathway and that ERK1/2 activity may actually be positively regulating SOD1 levels (Fig 3-3b**)**.

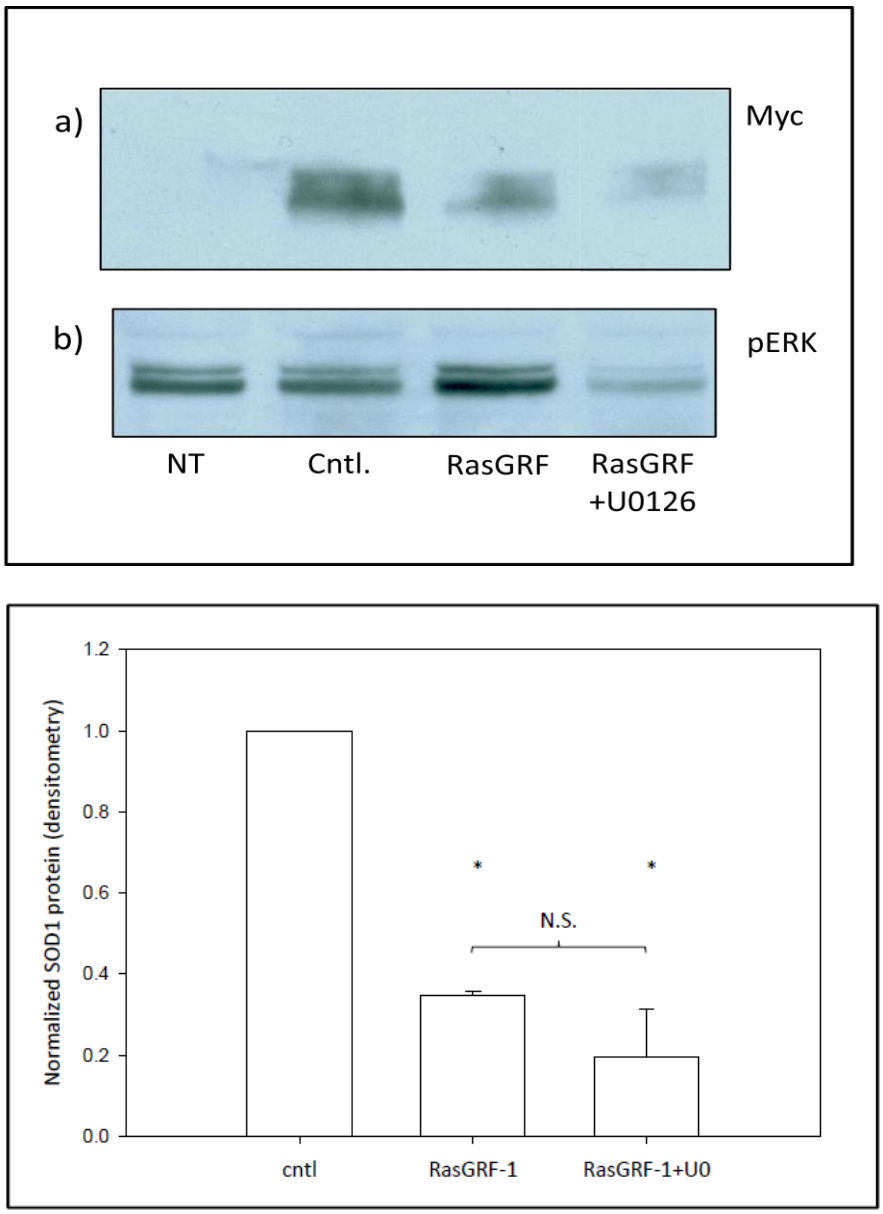
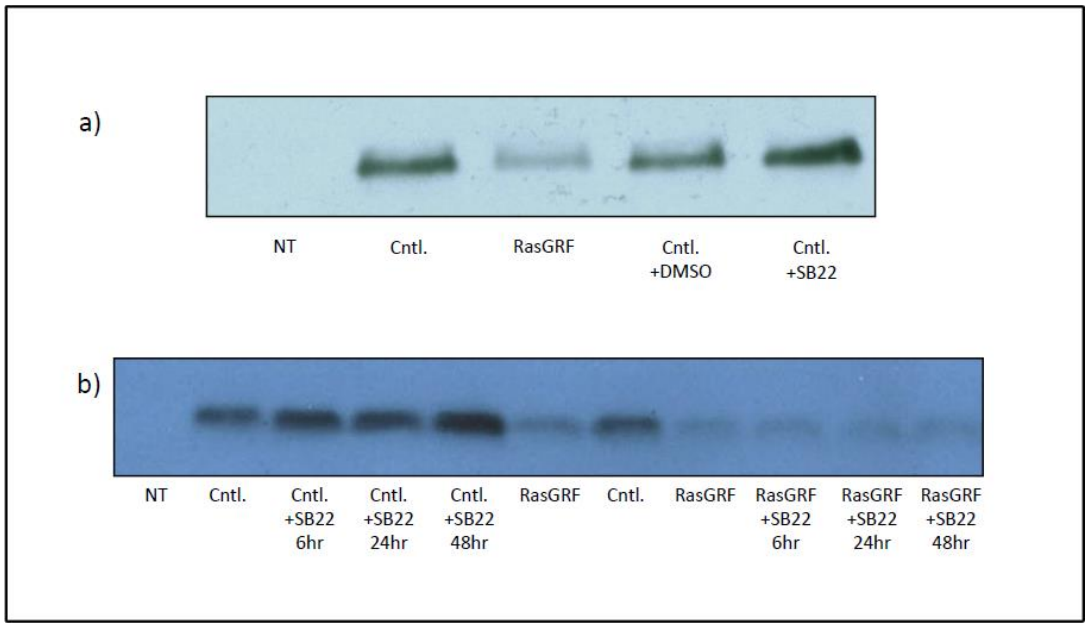


Figure 3-3 Application of U0126 does not rescue the effects of RasGRF-1 on SOD1 expression. a) western blot for anti-Myc for hSOD1; Cells were transfected with Myc-SOD1-A4V with either an empty vector (cntl.) or RasGRF-1. NT= non-transfected for SOD1-A4V, empty vector mock transfection. Expression of Myc-SOD1-A4V is again decreased by expression of RasGRF-1 and application of U0126, Top panel: a) MEK/ERK inhibitor does not block this response. b) western blot for anti-phospho ERK. pERK levels are increased with expression of RasGRF-1 and are significantly decreased with application of U0126 (5μM). Bottom panel: quantification of hSOD1 protein (n=3). * = p-value > 0.05 between experimental conditions and controls. Results bwtween RasGRF-1 +/- U0126 were not statistically significant.

3.3 Inhibition of p38 increases SOD1 protein

Given the preliminary results with RasGRF-1 expression and the hits from the screen, we decided to look at an alternate pathway. In addition to MEK/ERK, Ras has been shown to activate p38 in some neuronal cell types (Mullen *et al.* 2012). p38 was also one of the targets that was identified in the RNAi screen. Therefore it is possible that RasGRF-1 is acting via a Ras-p38 axis to modulate SOD1 expression. To test this we employed an inhibitor of p38 activity, SB22. Similar to U0126, SB22 has been shown to have a high degree of specificity for its target kinase (Jackson *et al.* 1998).

After performing a dose-response experiment to establish that NSCs would tolerate SB22 treatment, we performed pilot experiments to establish the effect of p38 inhibition on SOD1 without RasGRF-1 expression. Samples were treated either with SB22 or an equivalent volume of vehicle (DMSO) to rule out off-target effects. Surprisingly, basal inhibition of p38 led to a increase in SOD1 levels **(**Fig 3-4a**).** This suggests two possibilities: 1) RasGRF-1 is acting through an effector other than ERK1/2 or p38 or 2) the response we see from basal inhibition of p38 is different than that in stimulated cells, i.e. with active RasGRF-1. To test this we re-examined inhibition of p38 in the presence of RasGRF-1. Additional conditions were added to examine the duration of inhibition of p38, because our initial experimental samples were treated with the inhibitor at the time of transfection. SB22 did not block the effects of RasGRF-1 at any of the tested time points. We again saw an increase in SOD1 levels with SB22 in the absence of RasGRF-1 but only for longer durations of treatment at 24 and 48hrs **(**Fig 3-4b**)** This suggests that the effects of RasGRF-1 and SB22 are independent and acting though separate signaling mechanisms. It also seems likely that the increase we see in SOD1 with SB22 application is non-specific owing to the duration of the treatment necessary for this effect.



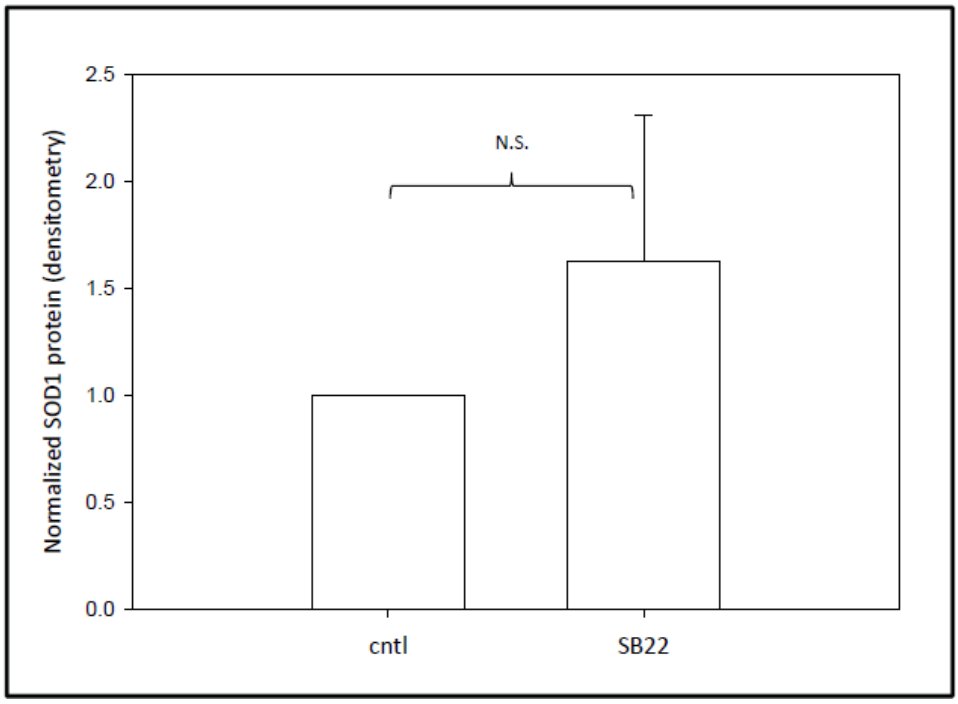


Figure 3-4 – Basal inhibition of p38 via SB22 produces an increase in hSOD1 protein levels. a) αMyc western blot for Myc-SOD1-A4V, SB22 was applied for the duration of the transfection at a concentration of 1.5μM. b) To test if the RasGRF-1 induced suppression of hSOD1 was due to p38 activity we repeated our RasGRF-1 transfections +/- SB22 for varying amounts of time. Times listed are from the end of the experiment, i.e. 6hrs = treatment for final 6hrs of the experiment prior to harvesting. Total transfection time = 48hrs. (n=4)

3.4 Chronic BDNF treatment decreases SOD1 protein levels

As previously mentioned, there is an array of cell-surface receptors that respond to growth factors and neurotrophins that are known to activate the MAP kinase pathways. These include receptors for platelet-derived growth factor, vascular endothelial growth factor, and receptors for the major neurotrophins NGF, BDNF, NT-4 and NT-3. While none of these ligands were identified as hits in our screens, it is possible that this is a result of cell-specific regulation and expression and that testing the effects of these receptors and their ligands on SOD1 in a neuronal context will provide a different result.

From an initial literature review of NSC34 cells we knew that they expressed some of the aforementioned receptors, specifically Trk receptors B and C, which are the principal receptors for BDNF and NT-3, respectively (Matusica *et al*. 2008). Furthermore it is known that Ras and ERK1/2 are downstream effectors of Trk receptor activity, as are PI3K and PLCγ, which were also identified in the RNAi screen (Fig 3-10).

If we assume that expression of RasGRF-1 is driving activity of Ras and its associated pathways, then it seems reasonable that stimulation of these pathways with a Trk ligand would produce a similar result. To test this hypothesis, we chose to use BDNF, a ligand for TrkB, which has a reported ED_{50} of 10 ng/ml in cell culture. Application of a single dose of BDNF for 24hrs at 50ng/ml produced a similar effect to expression of RasGRF-1 in the same experiment which supports the notion that suppression of SOD1 protein levels is due to sustained activation of the pathways downstream of BDNF and RasGRF-1 (Fig 3-5).

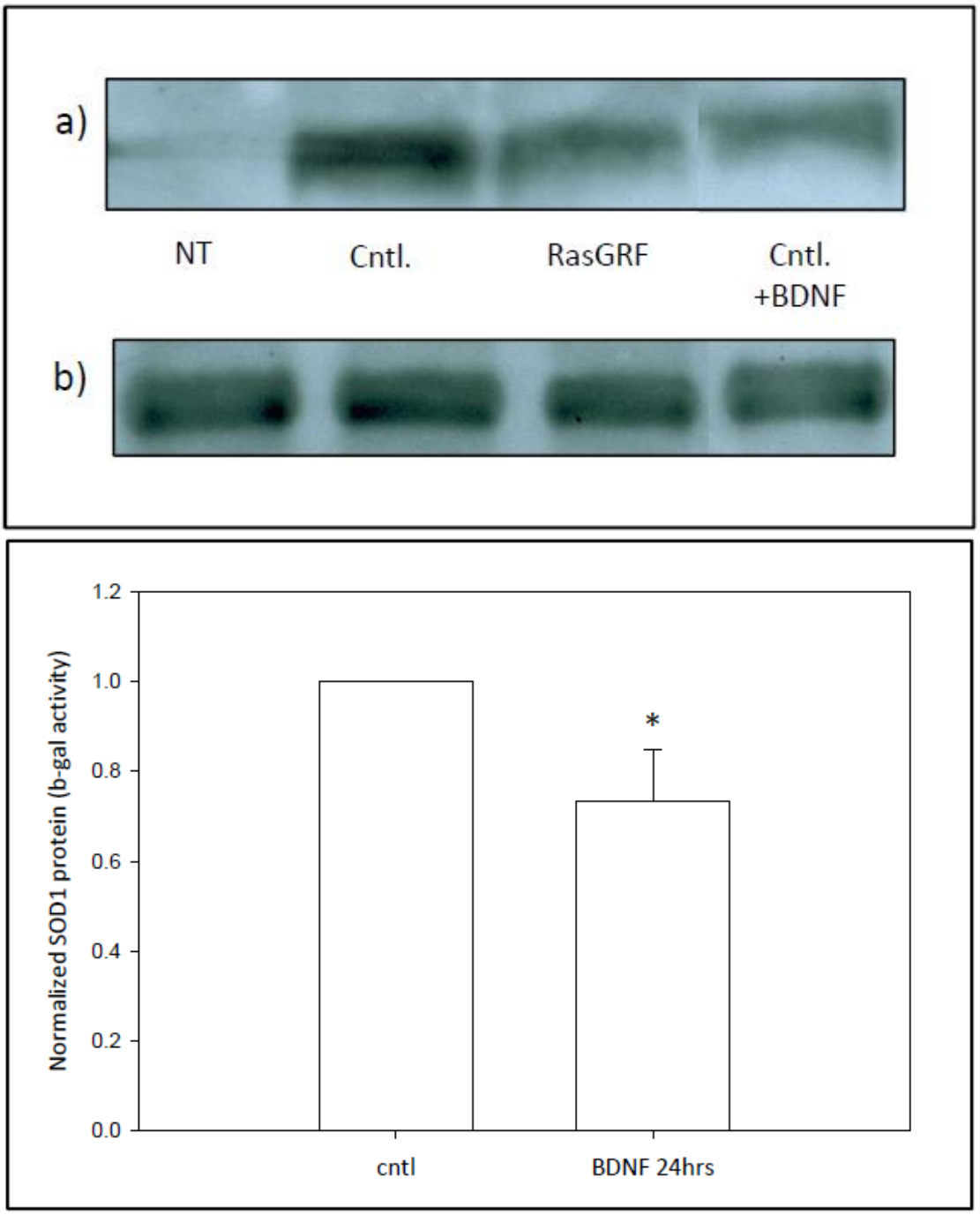


Figure 3-5 – Chronic application of BDNF reduces hSOD1 protein levels. Cells were transfected with Myc-SOD1-A4V and either co-transfected with RasGRF-1 or an empty vector. 24hrs after transfection, BDNF was applied at 50ng/ml. Cells were incubated an additional 24hrs and then harvested. a) western blot for Myc-tagged hSOD1. b) western blot for actin, loading control. NT = mock transfected. Bottom panel – β-gal assay for chronic BDNF treatment (n=6). * = p-value > 0.05

In order to provide additional validation of our preliminary results with RasGRF-1 and BDNF, we repeated the chronic application of BDNF in an organotypic culture. We prepared spinal cord sections from non-transgenic p7-10 mice in 6 well plates containing a mixture of MEM and high glucose HBSS. The sections were incubated in conditions identical to our cell culture and allowed to recover for 24hrs following tissue harvesting. Sections were then treated for 24hrs with a single application of 100ug/ml BDNF or vehicle. Following BDNF application the sections were pooled across conditions in each plate and whole cell lysates were prepared. Analysis by western blot showed that SOD1 protein levels were decreased in BDNF treated sections (Figure 3-6). This result was comparable to results obtained in recent reports using shRNA and other methods to knock down SOD1.

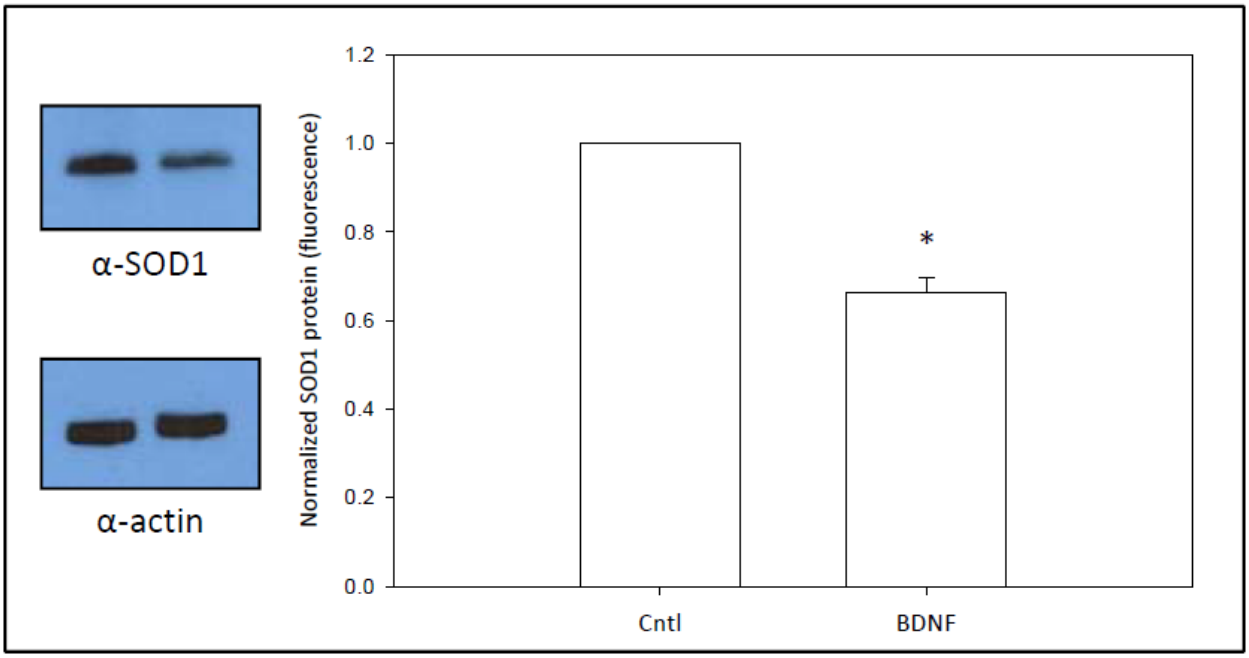


Figure 3-6 BDNF was applied to organotypic mouse spinal cord slices for 24 hours. Western blots were performed for SOD1and actin and insets representative of the results obtained. The graph to the right is the quantification of SOD1 protein levels -/+ BDNF made from a Typhoon Imager and Image J (n=3), * = p-value > 0.05

3.5 Trk Receptors

The tropomyosin kinase receptor B (TrkB) is part of the family of receptor tyrosine kinases and is part of a subfamily that includes TrkA and TrkC (Klein *et al*. 1989). These receptors were first described from the isolation of a novel oncogene that contained several exons found in non-muscular tropomyosin (Coulier *et al*. 1990). Further analysis showed that the tropomyosin exons were fused to domains encoding a previously unidentified tyrosine kinase, hence the name tropomyosin kinase. Subsequent studies demonstrated that these receptors bound the previously identified neurotrophins nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF), and neurotrophins 3 and 4 (NT-3, NT-4) (Squinto *et al*. 1991). NGF, BDNF, and NT-4 display specificity in their binding. NGF only binds to TrkA and BDNF while NT-4 only binds to TrkB, albeit with differing affinity than BDNF. NT-3 binds all three of the Trk receptors but binds TrkC with a much higher affinity.

Structurally, the Trk receptors are highly similar. The N-terminal region is comprised of leucine rich repeat domains sandwiched between two cysteine clusters (Haniu *et al.* 1995). These are followed by two immunoglobulin-like domains which at least partially confer ligand specificity and are the major site of ligand binding (Huang and Reichardt, 2003). The C-terminal kinase domain is the most conserved among the receptors, with only slight variations in the number and placement of tyrosine-phosphorylation sites which dictates downstream signaling capabilities. Additionally, TrkB has an alternatively spliced variant that has a unique truncated intracellular domain (Fig 3-7).

The neurotrophins function as dimers and binding to their target receptor induces and/or stabilizes receptor dimerization. This in turn leads to autophosphorylation within the kinase

domain and recruitment of adaptor proteins that allow activation of downstream signaling components. Following ligand binding and receptor activation, the receptors are internalized via endosomes, presumably to be degraded by the lysosome or to be recycled to the cell surface. Interestingly though, it has been shown that following internalization, Trk receptors remain capable of attracting adaptor proteins and mediating signaling (Howe *et al*. 2001). However, it is still unclear how this affects the dynamics of the Trk signaling process.

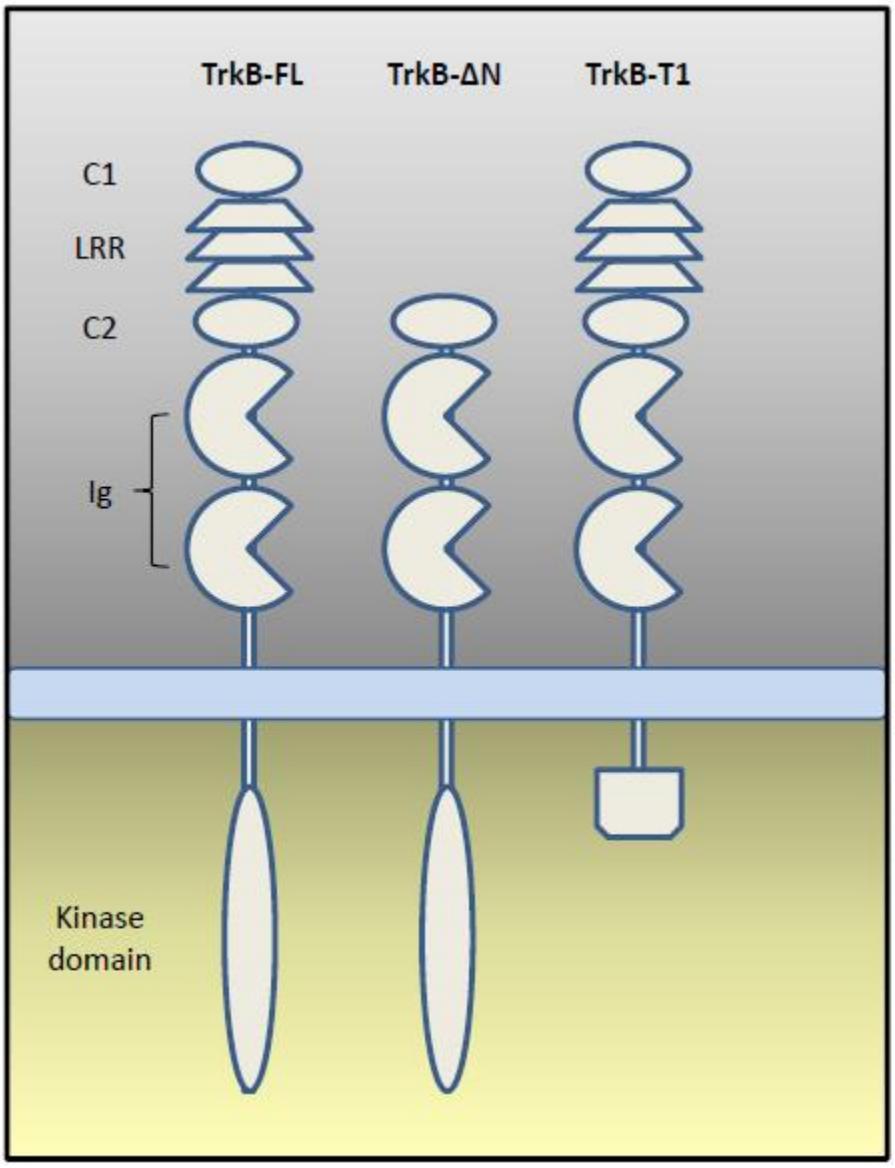


Figure 3-7 Organization of Trk receptors. The cysteine clusters and LRRs have been shown to stabilize receptor dimers upon neurotrophin binding. The cysteine clusters also contain multiple sites for N-glycosylation and thus trafficking to the membrane (Haniu *et al.* 1995). Specificity for binding is conferred by the Ig-like regions and extracellular juxtamembrane regions. The intracellular kinase domain is highly conserved but carries unique signaling capabilities for each receptor. The suggested organization for truncated TrkB is shown to the right (Huang and Reichardt, 2003).

3.6 Overexpression of TrkB produces similar effects as RasGRF-1 and chronic BDNF

In light of our results with BDNF it seems curious that its receptor, TrkB, was not one of the validated hits from the cDNA expression screen. However, it should be noted that TrkB actually was a candidate in the initial tertiary pool from the cDNA screen in HEK293 cells but its effects on SOD1 could not be reproduced in HeLa cells. This again suggests the possibility of cell-type dependence as we have observed with expression RasGRF-1. To examine the effects of TrkB on SOD1, we re-visited our overexpression experiments and transfected cells with our myc-tagged SOD1 and either RasGRF-1 or TrkB. Our results show that expression of TrkB significantly reduces SOD1 protein expression, similar to RasGRF-1 (Fig 3-8).

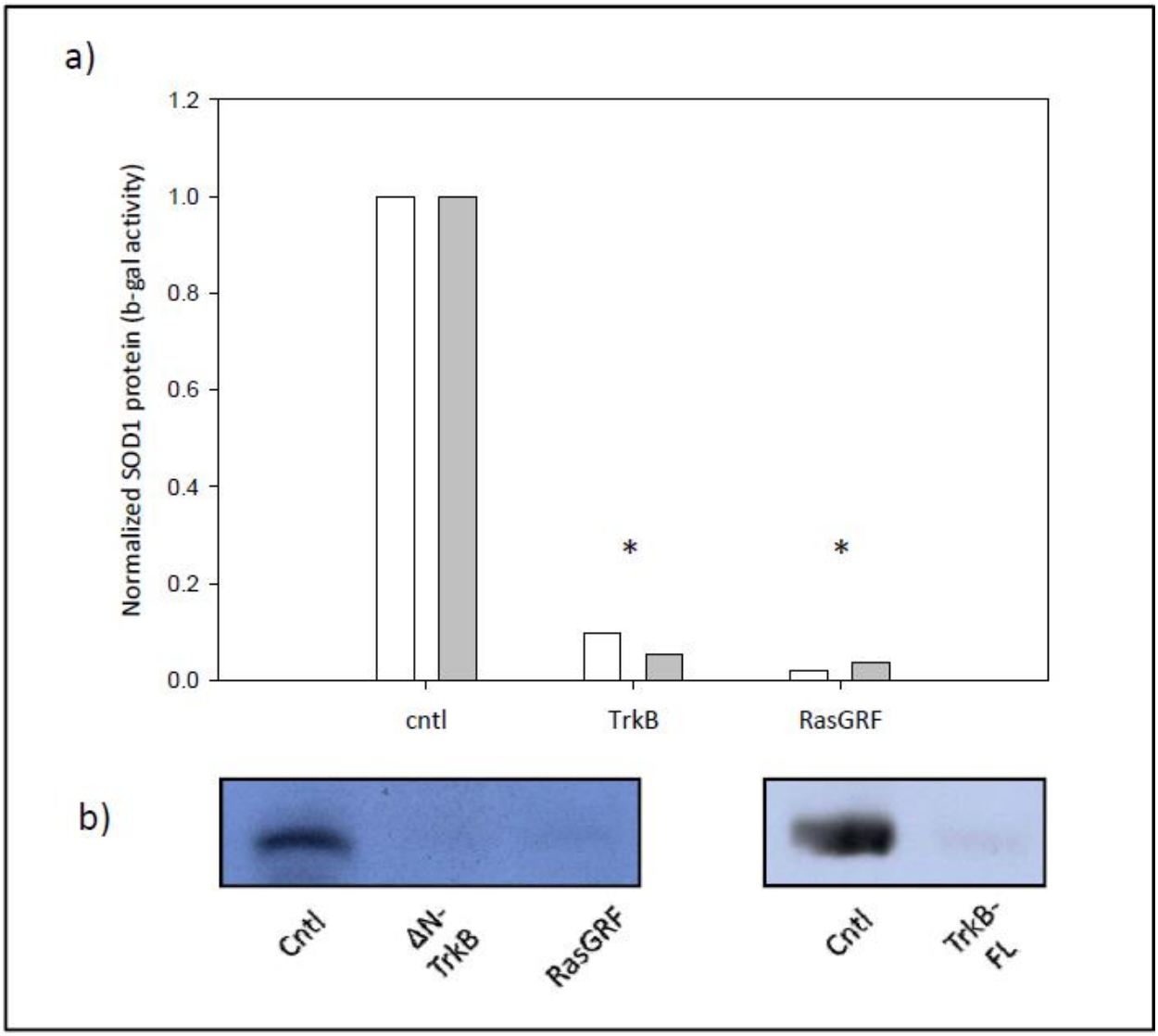


Figure 3-8 – Overexpression of TrkB in NSC34 cells. a) TrkB expression significantly reduces SOD1 signal as assessed in our β-gal assay, similar to our previous observations with RasGRF-1 in this cell line. b) representative western blots for HA-tagged SOD1; β-gal assay signal correlates with hSOD1.HA protein levels. Cntl = empty vector transfection, ΔN-TrkB = N-terminally truncated TrkB cDNA from the initial screen which is N-terminally truncatred, TrkB-FL = full length TrkB. RasGRF-1 is the same expression construct used in previous experiments, (n=4).

At this point it should be noted that the TrkB clone we isolated is N-terminally (ΔN-TrkB) truncated and corresponds to a recently identified splice variant (Fig 3-9). While this truncated variant contains the full intracellular kinase domain, the transmembrane span, and the extracellular binding region for BDNF, it lacks the amino acids for the signal sequence due to an alternative translation start site and thus it is predicted to be inefficiently inserted into the plasma membrane (Luberg *et al*. 2010, Fig 3-9). However, this has not been examined directly.

In order to rule out that this effect was due to some non-specific characteristics of ΔN-TrkB, we repeated the experiment with a full length TrkB (FL-TrkB) construct expressed in a similar vector. We saw a similar effect with FL-TrkB (Fig 3-8c). This suggests that the deleted residues in ΔN-TrkB are not required for the observed effects on SOD1 and that as long as the ligand binding and kinase domains are intact, TrkB is capable of reducing SOD1 expression.

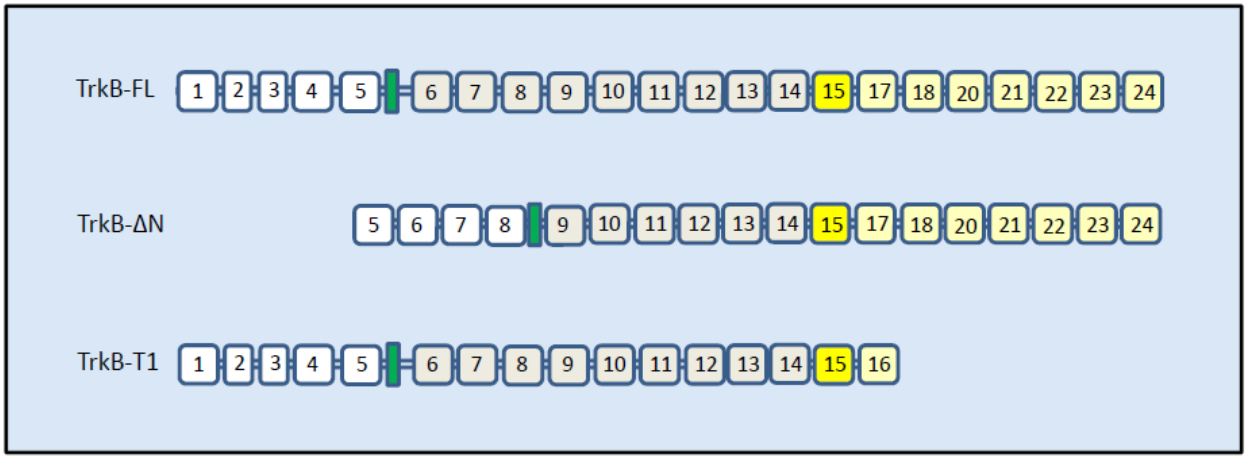


Figure 3-9 A recently identified splice variant of TrkB contains an alternate translation start site at the beginning of exon 9 and therefore lacks the first 75 amino acids of the extracellular portion of the full length protein. This truncation also lacks a signal sequence and therefore is most likely not targeted to the cell membrane (Luberg *et al.* 2010).

3.7 TrkB Signaling

TrkB was first identified as a novel tyrosine kinase that was expressed during development in a murine model and, as previously mentioned, showed a high degree of similarity to the previously identified TrkA (Klein *et al*. 1989). Shortly thereafter it was shown that TrkB was in fact the receptor for BDNF and also bound NT-3 (Soppet *et al.* 1991).

Full length TrkB is 822 amino acids in length and has multiple phosphorylation sites in its intracellular kinase domain (ICD). Ligand binding to receptors triggers autophosphorylation of the kinase domains. The phosphorylation events are apparently sequential in that the initial activation depends on the presence and binding of ATP with subsequent phosphorylation being dependent on the concentration of the ICD (Iwasaki *et al.* 1997). It has also been shown that mutating the ATP binding site of TrkB eliminates phosphorylation of other tyrosines within the kinase domain and inhibits signaling (Luikart *et al*. 2008). Taken together this suggests a model wherein ATP binding stabilizes the kinase domains which then allows for further phosphorylation.

Binding of BDNF to TrkB is known to activate three distinct signaling pathways. Phosphorylation of Y515 activates the Ras-MEK-ERK and PI3K-Akt pathways and Y816 activates PLCγ signaling (Fig 3-10). These pathways are known to participate in a variety of cellular processes, but in neurons, they are involved in growth and differentiation, survival, and synaptic plasticity and neurotransmission, respectively (Boulle *et al*. 2012). Additionally, TrkB has been shown to be active in the absence of neurotrophin binding, either through local clustering of receptors in lipid rafts or through transactivation by G-protein coupled receptors (GPCR) (Swift *et al.* 2011, Lee and Chao 2001).

Phospho-Y515 leads to activated Ras through recruitment of Grb2 bound to SOS, a

guanine nucleotide exchange factor for Ras. Activated Ras is then capable of stimulating MEK-ERK through Raf1, but also p38MAPK and PI3 kinases, the latter of which is also activated by Grb2-Gab1 complexes in the same phosphorylation event, suggesting a point of crosstalk between the MEK-ERK and Akt pathways. The downstream effectors from Ras-MEK-ERK include the ribosomal s6 kinase (RSK) family and cyclic-AMP response element binding protein (CREB). Activation of RSK proteins may stimulate transcription through several effectors, including, but not limited to c-Fos, CREB, ATF-1, and MEF2c (Romeo *et al*. 2012). RSK proteins have also been shown to regulate translation by inhibiting the kinase activity of eEF2K, thus promoting EEF2 function. CREB is also known to regulate transcription of c-Fos in addition to many other neuronal targets including BDNF. Furthermore, RSK activity suppresses both Bad and DAPK-mediated apoptosis thereby contributing to cellular survival.

Activation of PI3K-Akt has been shown to promote neuronal survival through a well characterized signaling cascade. Phosphorylation at Y515 recruits Grb2-Gab1 complexes which activate PI3 kinases. These kinases stimulate the production of phosphotidylinositides (PI) which in turn activate PDK-1, a PI dependent kinase. PDK-1 activates Akt, also known as protein kinase-B, which inhibits apoptosis by inhibiting several downstream elements including GSK3β, FKHRL1, and Bad. Akt substrates also include a subset of RSK proteins that act on the translation machinery, thereby promoting protein synthesis. Activation of RSKs also acts on Raptor, a component of the mTORC1 complex, which is known to regulate protein synthesis in response to the energy status of the cell.

Phospho-Y816 stimulates production of IP3 and DAG via hydrolysis of PIP_2. IP3 induces the release of internal Ca^{2+} stores which trigger isoforms of protein kinase C (PKC) and calmodulin kinases (CaMK) while DAG also regulates a subset of PKCs. Some of the PKC

isoforms are also known to act on Raf-MEK activity, thus providing another point of crosstalk between the pathways downstream of TrkB.

The most prominent function of TrkB-PLCγ signaling as described in mouse models appears to be transcriptional programming in the early phase of hippocampal-LTP via CREB and CaMKII. Homozygous Y816F mice display severely reduced induction of LTP in the CA1 region of the hippocampus, which is similar to the effect seen in forebrain knockouts of TrkB (Minichiello *et al.* 2002).

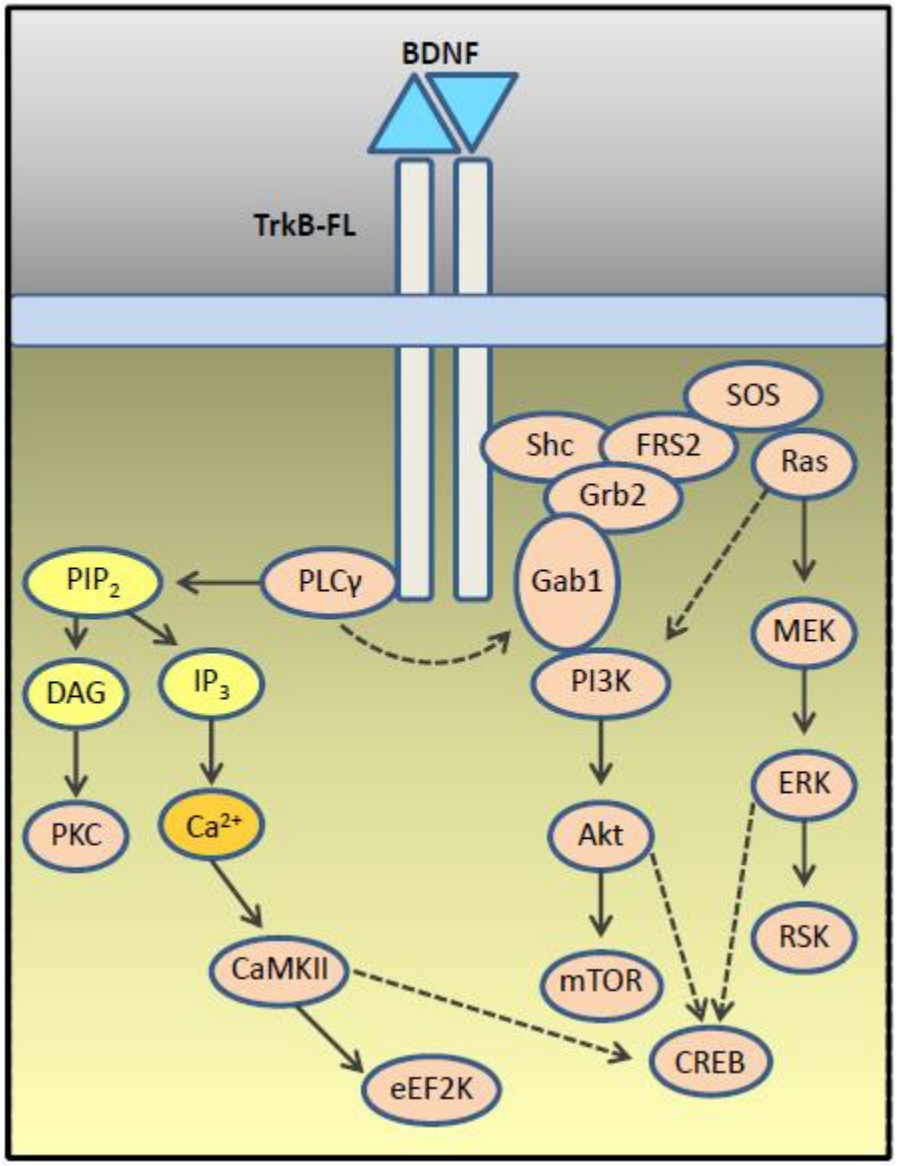


Figure 3-10 Schematic of TrkB signaling components. BDNF homodimer binds and induces receptor dimerization. Phosphorylation at Y515 leads to recruitment of SHC/Grb2 adapter complex which activates MEK-ERK through the SOS-Ras-Raf cascade or PI3K-Akt via Gab1. Phosphorylation atY816 leads to binding of PLCγ which hydrolyzes PIP_2 to IP_3 and diacylglycerol which stimulates release of internal calcium stores and the protein kinase C pathway (Nagahara and Tuszynski, 2011).

3.8 The kinase activity of TrkB is required for the reduction in SOD1 protein

Having established that expression of RasGRF-1 and TrkB and long-term application of BDNF have similar effects on expression of hSOD1, we decided to investigate the signaling processes of TrkB that were mediating our observed effects. As previously stated there are several well defined phosphorylation sites in the intracellular domain of TrkB. When phosphorylated these sites recruit specific adaptor proteins that link to TrkB activation to MEK/ERK, PI3K, and PLCγ signaling. Therefore, by mutating or disrupting these sites in a way in which they cannot be phosphorylated, one can interrupt specific aspects of TrkB signaling.

To test the involvement of these pathways downstream of TrkB we employed different TrkB variants with mutations to sites that mediate the receptor's signaling processes. These included Y515F, which is a mutant for the Grb2 binding site and blocks activation of ERK and PI3K, and Y816F which mutates the docking site for PLCγ signaling. We also used a K571N mutant, which results in an inactive TrkB. This amino acid site is within the activation loop of TrkB and substitutions at specific sites within this loop cause the receptor to lose its kinase activity, i.e. it is 'kinase dead' (KD-TrkB). Similar mutations have been described for F616 (Chen *et al.* 2005).

As previously noted, there are several transcription factors downstream of ERK and PI3K that have been shown to bind the SOD1 promoter. Additionally, activation of these signaling components in response to TrkB activity is known to regulate the translation machinery. This led us to predict that mutating the Grb2 binding site in TrkB, Y515, would abrogate the suppression of SOD1 signal we observe with WT-TrkB. However, when we expressed the Y515F mutant there was no difference in SOD1 expression when compared to expression of WT-TrkB. This suggested that the other major pathway, PLCγ, may be responsible for the

reduction in SOD1. Surprisingly, the Y816F mutant also failed to significantly rescue SOD1 expression. This result suggested a few possibilities: 1) the decrease in SOD1 is independent of TrkB activity; 2) there is sufficient redundancy (crosstalk) within the pathways that activation of either ERK/PI3K or PLCγ is capable of modulating SOD1; 3) the decrease in SOD1 requires signaling machinery outside of ERK, PI3K, and PLCγ; 4) the exogenous TrkB mutants are activating endogenous TrkB. To test this first possibility we utilized a kinase dead-TrkB, K571N. If the effects are dependent on TrkB activation then expression of this mutant should have no effect on SOD1 levels. In line with this hypothesis, K571N caused no significant changes in SOD1 signal, therefore the effect on SOD1 expression is not TrkB independent. The latter two possibilities can be tested by mutating both Y515 and Y816. If this double mutant fully rescues SOD1 levels in a manner similar to (KD-TrkB), it would suggest that the reduction in SOD1 is due to crosstalk between the two signaling arms and that this crosstalk can be initiated from either site of activation, Y515 or Y816. Alternatively, if Y515/816F does not completely restore SOD1 expression to that seen in the control condition, this suggest that there are either additional signaling components of TrkB outside of the major pathways that are acting on SOD1 or perhaps more simply, that endogenous TrkB is activated by the expressed mutants. With expression of TrkB Y515/816F, only a partial reduction of SOD1 levels was observed, which is consistent with the latter two possibilities mentioned above (Fig 3-11).

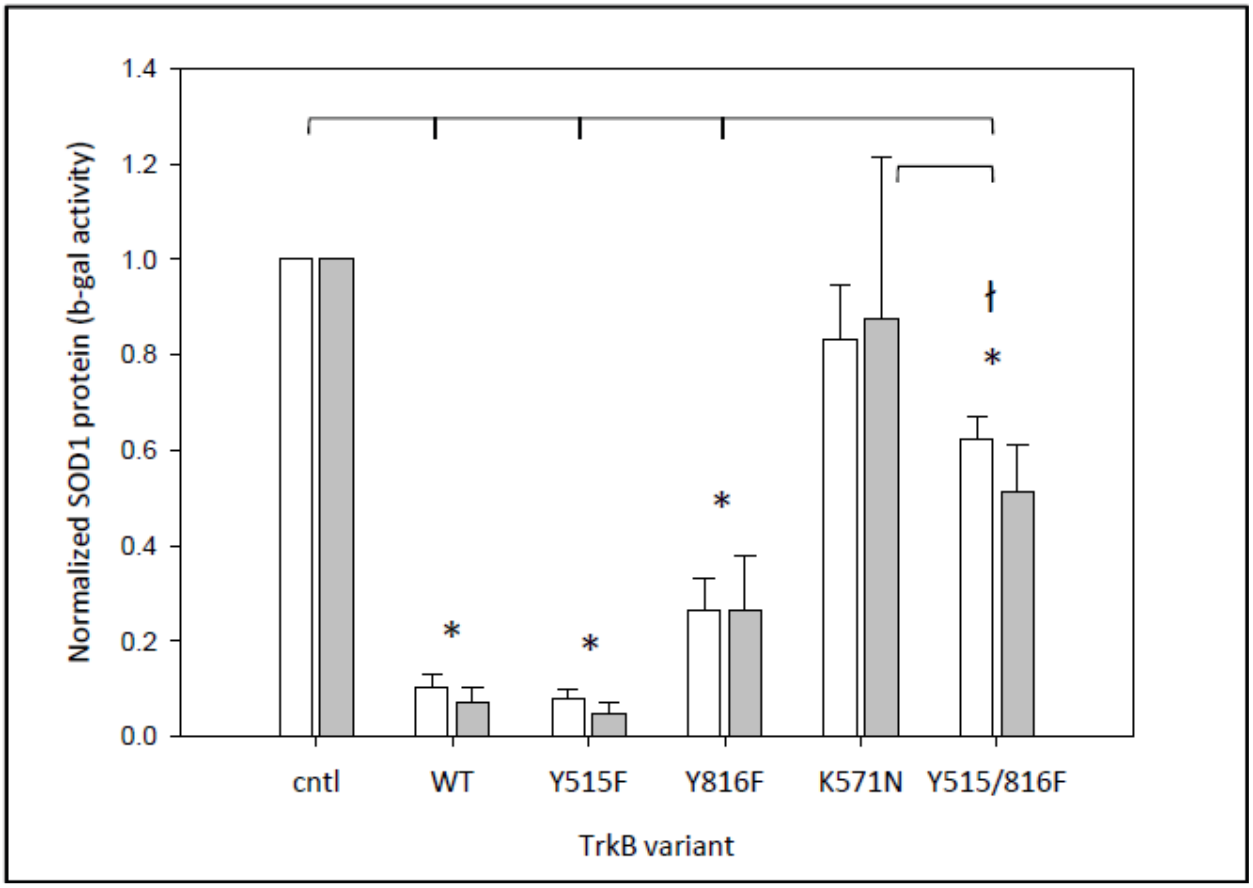


Figure 3-11 Overexpression of TrkB and TrkB mutants on SOD1 protein levels. a) β-gal assay Co-transfections of WT-SOD1 (white bars) or SOD1-A4V (gray bars), here with variants of TrkB. WT= WT TrkB, Y515F= ERK/Akt signaling TrkB mutant, Y816F= PLCγ signaling TrkB mutant, K571N= kinase dead TrkB, Y515/816F= ERK/Akt- PLCγ TrkB double mutant. White bars = WT hSOD1, grey bars = A4V hSOD1. * = p-value > 0.05 between control and mutant(s); ł = p-value > 0.05 between K571N and Y515/816F.

In order to further clarify the possible components involved in suppressing SOD1 protein levels we turned to a dual approach with MAP kinase inhibitors and our TrkB mutants. The strategy was to use the Y816F mutant, which is inactive for PLCγ, in tandem with inhibitors for MEK/ERK (U0126) or PI3K/Akt (Triciribine, a.k.a. API-2). In this manner, we should be able to parse out the individual components of TrkB signaling that emerge from phosphorylation of Y515. We repeated our transfections of SOD1 WT or A4V with and without TrkB-Y816F as previously described. 24 hours post-transfection, we added either U0126, API-2, or both, with control conditions receiving an equal volume of DMSO (vehicle). While the combination of inhibitors on top of TrkB-Y816F partially interfered with suppression of SOD1, neither inhibitor provided any significant change when used independently with TrkB-Y816F (Fig 3-12). Additionally, these inhibitors actually decreased SOD1 levels in the absence of TrkB which is reminiscent of the data with U0126. When we expanded our experiments to include WT-TrkB and Y515F with the compound treatments, we obtained similar results wherein the compound treatments on top of the expressed receptors have little effect on SOD1 levels (Fig 3-13). In total, these data support the latter two possibilities listed above in which the exogenously expressed TrkB mutants are interacting with endogenous TrkB receptors to affect SOD1 levels or there are additional active signaling components that are unaffected by the mutations or compounds tested here.

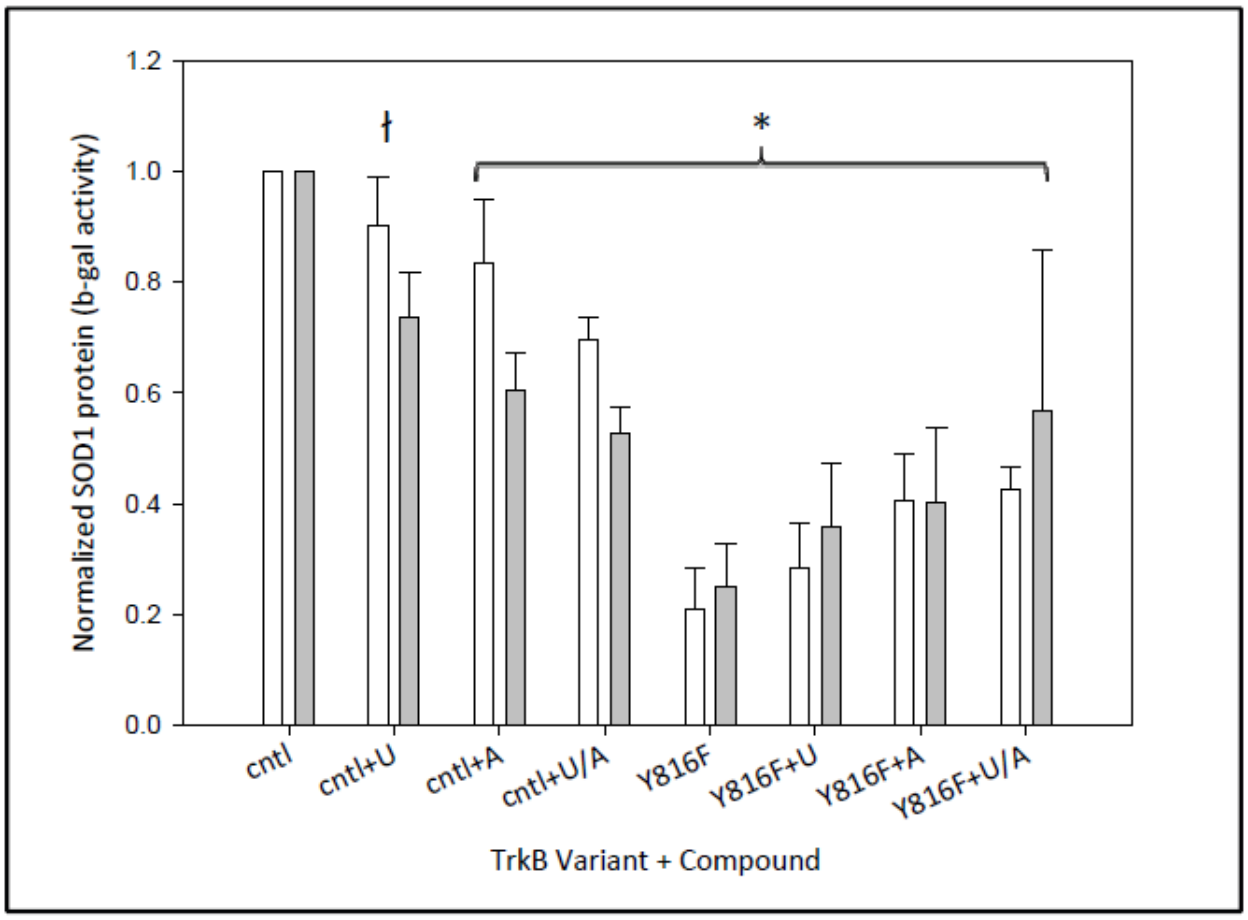


Figure 3-12 Overexpression of TrkB variants +/- compounds. Compound treatments to assess specific components of signaling pathways downstream of TrkB. U= U0126, MEK/ERK inhibitor; A= API-2, Akt inhibitor. U0126 was applied at 5uM, API-2 at 1.5uM for final 24hrs of experiment. 48hrs total time post transfection. White bars = WT hSOD1, gray bars = A4V hSOD1. * = p-value > 0.05, ł = p-value > 0.05 for A4V only.

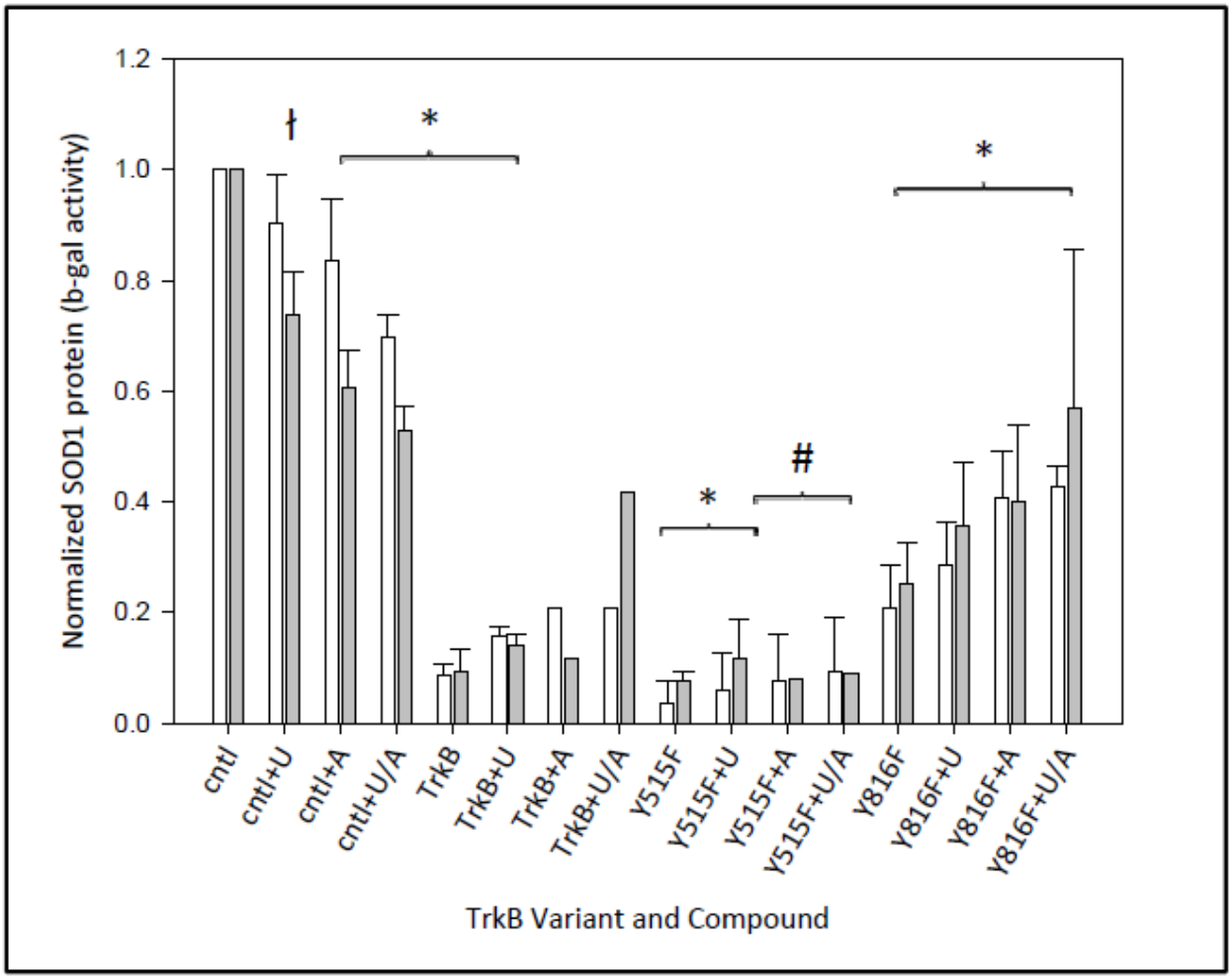


Figure 3-13 Expression of TrkB variants +/- compounds expanded to include WT-TrkB and Y515F. U= U0126, MEK/ERK inhibitor; A= API-2, Akt inhibitor. White bars = WT hSOD1, gray bars = A4V hSOD1. * = p-value > 0.05, ł = p-value > 0.05 for A4V only, # = p-value > 0.05 for WT only.

3.9 Acute application of BDNF increases SOD1 protein

To this point the data support the idea that TrkB-BDNF signaling is involved in regulating SOD1 protein levels. However, BDNF is most often released in an acute, transient manner in response to synaptic activity as opposed to the more chronic applications we have employed thus far, both with BDNF and TrkB. More specifically, BDNF is known to initate transltional and transcriptional response over minutes to hours. Additionally, a recent report demonstrated that TrkB and its associated pathways have different modes of activation that are dependent on acute or gradual increases in concentration (Ji *et al.* 2010). More specifically, the investigators show that when BDNF is delivered as a single dose, TrkB and ERK1/2 are activated very rapidly and then slowly return to baseline states of phosphorylation. Conversely, gradually increasing BDNF over several minutes up to the same concentration produces a more gradual increase in TrkB and ERK activation that is then sustained for several hours. This suggests that the kinetics by which TrkB is activated also dictate the kinetics of its downstream pathways. These findings have implications for how target genes of BDNF-TrkB signaling are regulated. This led us to test acute application of BDNF in the cell culture model.

We repeated our transfections for hSOD1 in NSC34 cells. 24hrs post-transfection we again applied BDNF at 50ng/ml. However, we analyzed SOD1 levels after 30 or 60 minutes of treatment with control conditions receiving vehicle. Surprisingly, hSOD1 protein increased in this time frame (Fig 3-14a). We re-tested acute BDNF treatment without transfection of hSOD1 to examine any possible effects on endogenous SOD1. Again, we see a similar fold increase in endogenous SOD1 levels and in both cases these changes correlated with increases in phosphorylation of ERK, a readout of BDNF activity (Fig 3-14b). These results suggest either that there are separate responses in SOD1 expression depending on the mode of TrkB activity or

that the response is bi-phasic, with an acute increase in SOD1 protein followed by a subsequent decrease relative to control levels.

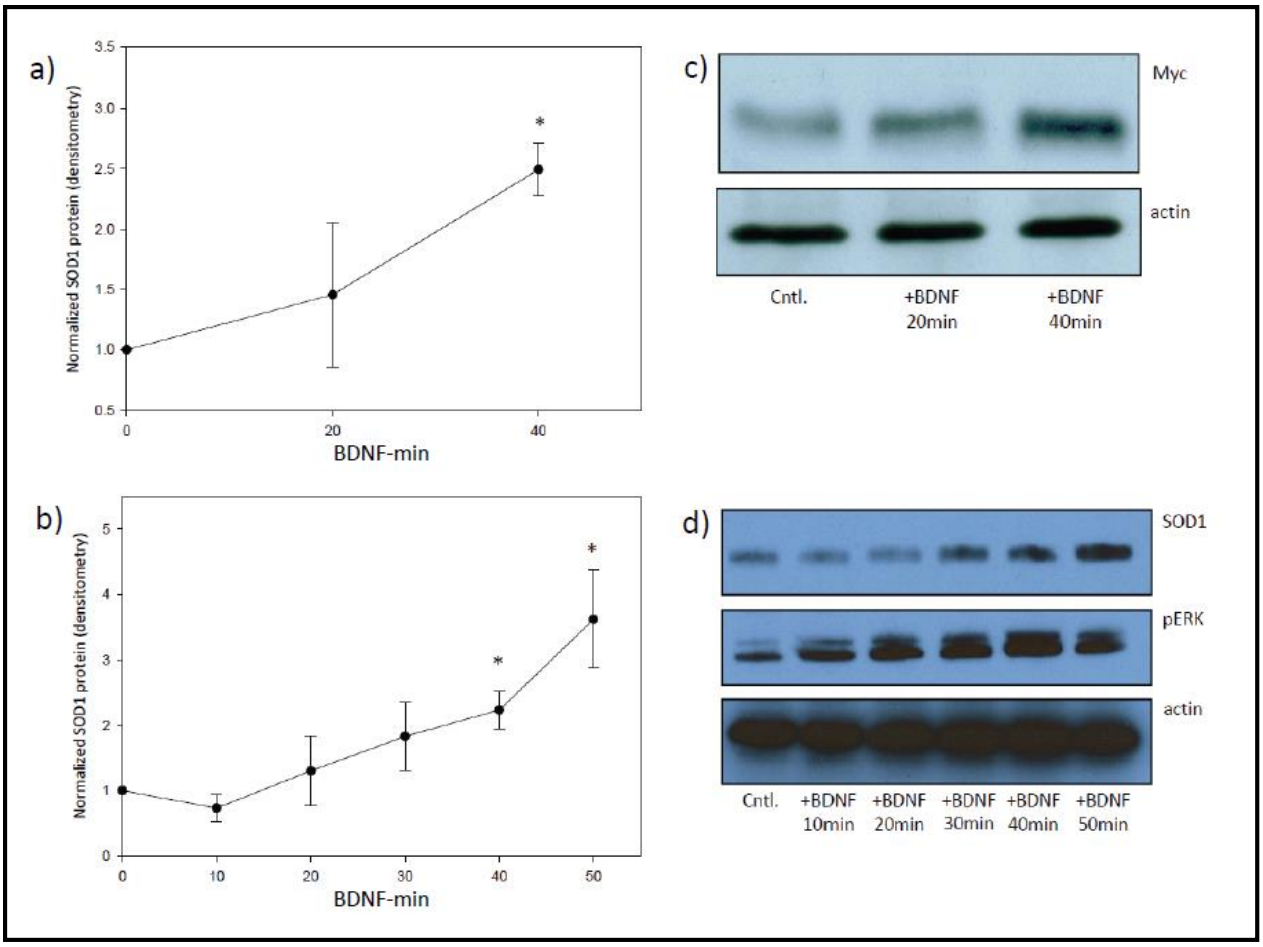


Figure 3-14 Acute BDNF treatment increases SOD1 protein levels. a and c) NSC34 cells were transfected with Myc-SOD1-A4V and treated with BDNF for the indicated times. Treatments were staggered so that all cells were harvested at the same time. Westerns are for anti-myc (hSOD1) and actin. b and d) non-transfected NSC34 cells were treated with BDNF for the indicated times to examine the effect on endogenous SOD1. Westerns are for anti-SOD1 (endogenous), phosphor-ERK, and actin. Increase in SOD1 protein correlates with increases in phosphor-ERK levels. * = p-value > 0.05, (n=4)

3.10 Discussion

These data provide strong evidence that TrkB and its associated pathways are involved in the regulation of SOD1. Both chronic application of BDNF and expression of TrkB significantly reduced SOD1 protein levels. This effect was also present when we expressed RasGRF-1, an effector that acts on signaling components that are known to be activated by TrkB. On the other hand, acute application of BDNF to NSC34 cells produced an increase in SOD1 levels. This suggests that the mode of activation of BDNF-TrkB signaling determines how its target substrates (in this case SOD1) are regulated. Alternatively, the changes in SOD1 in response to TrkB have an initial phase where the protein increases acutely followed by a second phase wherein it returns to baseline levels and then decreases owing to the longer duration of the stimulus.

These effects also appear to be cell-type dependent as expression of TrkB on SOD1 in HeLa cells had no effect and expression of RasGRF-1 affected SOD1 in the opposite direction from that observed in NSC34 cells. This last point has particularly important ramifications for ALS as it has been shown to be a non-cell autonomous disorder, the implication being that different cell types in the CNS may regulate SOD1 expression in disparate manners through similar signaling mechanisms. For example, stimulation of TrkB signaling in astrocytes has been shown to drive degeneration of neurons in a mouse model of multiple sclerosis (Colombo *et al.* 2012). Conversely, application of BDNF and BDNF mimetics to primary cortical neurons has a protective effect after exposure to amyloid-beta (Arancibia *et al.* 2008 and Massa *et al.* 2012). Clearly, these are different disease models, and this could account for some of the differences observed with TrkB, but it nonetheless underscores the idea of cell-dependent signaling outputs.

TrkB has previously been implicated in ALS. TrkB mRNA levels were found to be elevated in post-mortem tissue from ALS patients as early as 1993 (Seebuger *et al.* 1993*).* Mutoh *et al.* more closely examined post-mortem spinal cord tissue from ALS patients and found that TrkB phosphorylation was significantly decreased when compared to age-matched controls. Additionally, they saw that TrkB expression was upregulated at both the mRNA and protein level. Given TrkB's demonstrated involvement in regulating cell survival their findings suggest a compensatory upregulation of TrkB protein to combat the decreased level of phosphorylation. The caveat here is that these studies examined the end-stage of the disease, making it difficult to determine whether or not this data is relevant to pre-symptomatic individuals. Furthermore, these examinations were not specific for SOD1-ALS patients and as such, SOD1 expression was not examined.

As previously mentioned, several clinical trials for neurotrophic factors have been launched with the hope that stimulating cell-survival pathways would ameliorate disease symptoms. In 1999, BDNF was specifically tested as a candidate therapy for ALS. It reached a Phase 3 study group but failed to show any broad clinical efficacy beyond a small subgroup of patients that demonstrated slightly improved survival (Henriques *et al.* 2010*).* A follow-up Phase 1.2 trial using a different mode of delivery supported BDNF as a well tolerated treatment with a daily dosing regimen of 25-1000μg/day, but no conclusions could be drawn regarding efficacy due the small size of the group tested (Ochs *et al.* 2000).

A more recent study examined TrkB's association with ALS via its truncated variant TrkB.T1 in a SOD1 animal model. They show that knockout of T1 improves motor function and delays disease onset in mutSOD1 mice, but had no impact on survival which agrees with previous research that demonstrates improved neuromuscular function with genetic deletion of

T1 (Yanpallewar *et al.* 2012 and Dorsey *et al.* 2012). Additionally there is evidence that misregulation of FL and T1 isoforms of TrkB leaves neurons more susceptible to excitotoxicity which is known to exacerbate degeneration in ALS (Vidaurre *et al.* 2012). Notably though, these studies did not examine any possible effects on SOD1 expression.

T1 is the result of alternative splicing of the TrkB mRNA and while it lacks an intracellular kinase domain it is thought to have its own unique signaling characteristics. Historically though, it has been considered to act as a negative regulator of FL-TrkB by either sequestering ligands or forming heterodimers with FL-TrkB (Fenner 2012). The structure and putative function of T1 would predict that it would behave similar to TrkB-K571N. However, in our cell culture model, expression of T1 reduced SOD1 protein levels in a manner similar to the expression of our FL-TrkB double mutant (Fig. 1, appendix b). T1 is known to be endogenously expressed in our cell line and these results suggest that T1 may be able to modulate SOD1 via its own signaling capacities which are poorly characterized.

In contrast, other recent research suggests that blocking TrkB activity is protective against neurotoxicity. Selective knockout of TrkB in motor neurons of mutSOD1 mice has been shown to extend motor function and survival. Furthermore, TrkB-KO animals displayed decreased levels of aggregated mutSOD1 from spinal cord samples, which is in contrast to our result of decreased hSOD1 from increased expression of TrkB. One possible explanation is that chronic activation or increased expression of TrkB is auto-inhibitory as ERK1/2 signaling is known to contain a negative feedback loop (Bae *et al.* 2009). Another group has demonstrated that inhibiting BDNF signaling and TrkB transactivation protects motor neurons from excitotoxicity (Mojslilovic-Petrovic *et al.* 2006). However, it should be noted that these experiments were conducted in a co-culture setting of spinal motor neurons and astrocytes which

suggests that their effects may be due to inhibitory action within astrocytes, which, as previously mentioned, promote neuronal vulnerability via TrkB activity. An alternative explanation is that the kinetics of TrkB activation that we and others have observed may be responsible for the aforementioned contradictory results and that refining the method of BDNF treatment or TrkB activation may clarify these discrepancies.

Mechanistically, the simplest explanation for TrkB's effect on SOD1 is transcriptional control. The pathways downstream of TrkB are known to regulate several well characterized transcription factors, some of which have been shown to bind specific elements within the human SOD1 promoter. Rojo *et al.* showed that SOD1 protein levels increased in response to expression of Akt in PC12 cells, a rat sympathetic ganglion cell line. This result is consistent with our current observations wherein SOD1 expression increases with acute BDNF treatment which is coincident with increased phospho-Akt (data not shown). Akt is recruited to the cell membrane by binding PIP_3, which is produced by PI3K, a downstream effector of Trk signaling. This group also showed that the observed increase in SOD1 protein occurred via increased mRNA levels, i.e. through transcriptional regulation. Luciferase assays using cloned regions of the SOD1 promoter provided evidence that the increase in mRNA observed with Akt expression was predominantly due to an NfκB site within the promoter, suggesting that Akt-induced nuclear localization of NfκB induced transcription of SOD1 with a subsequent increase in protein. Similarly, Wang *et al.* showed that activation of ERK1/2 either with application of MPP or a constitutively active MEK increased SOD1 protein levels in SY5Y cells, a human neuroblastoma line. Their evidence suggests that this increase was also transcriptionally based as it appeared to be dependent on the presence of Elk-1, a downstream target of ERK1/2 that is known to have a binding site within the human SOD1 promoter. However, as mentioned above, there are several

downstream effectors of TrkB signaling that are known to regulate the translation machinery and BDNF activity in neurons is known to stimulate local translation of synaptically targeted mRNAs. Furthermore, there is some evidence that application of BDNF and chronic elevation of neuronal activity increases ubiquitination of synaptic proteins (Jia *et al.* 2008). Therefore, it is possible that TrkB is regulating SOD1 through these mechanisms as well. Additionally, the data from the acute experiments with BDNF indicate that production of SOD1 protein is increasing within 30 minutes of stimulation. This is a relatively short window for a transcriptional response given the size of the genomic sequence of mouse SOD1 (7.3kb) and other mitigating factors such as assembly of the transcription machinery and chromatin structure, which points again points to alterations in translation rate or protein degradation. However, there are several immediate early genes (IEG) such as cFOS and Arc which known to have very rapid transcriptional responses to neuronal stimuli, so an increase in SOD1 transcription is possible with acute BDNF treatment. Given that BDNF-TrkB signaling is known to affect transcriptional activity and the translation machinery it is also possible that both of these mechanisms are involved in modulating SOD1. Going forward, we will test these potential mechanistic players in order to establish the manner in which SOD1 expression is being altered.

Chapter 4

Mechanism of SOD1 Regulation via BDNF-TrkB signaling

4.1 Introduction

The data to this point provided strong support for the idea that TrkB-BDNF signaling is involved in the expression of SOD1. However, the underlying molecular mechanisms of these effects remain to be elucidated. Evidence from the literature demonstrates that TrkB-BDNF signaling is capable of initiating both transcriptional and translational responses in neurons making these processes the most likely avenues for modulating SOD1 levels. Additionally, experiments in other cell lines that are similar to our NSC34 model provide evidence that SOD1 can be transcriptionally regulated by both ERK1/2 and Akt signaling, both of which are downstream of TrkB. Furthermore, the long half-life of SOD1 (~30hrs.) argues against changes in the UPS leading to changes in SOD1 expression. This suggests that changes in SOD1 mRNA levels are most likely mediating what we see at the level of SOD1 protein. Also, as mentioned previously, some of the results observed in the cDNA screen appeared to be cell and/or promoter specific. Since the rate or extent to which a gene is transcribed is largely dependent on cis-acting elements within the promoter region, this further suggests that the rate of SOD1 transcription is being affected.

There are several simple ways of experimentally testing changes in transcription. First, we can test changes in mRNA levels using quantitative polymerase chain reaction (qPCR), which allows for quantitative measurements of mRNA transcripts across different samples relative to internal controls. The drawback to this method is that on its own it cannot distinguish between changes in transcription or changes mRNA turnover, therefore it should be paired with additional experiments to measure one or both of these processes. One such method would be a reporter construct for the gene of interest (GOI) such as Luciferase which would allow us to directly test the activity of the SOD1 promoter in the presence of TrkB and BDNF. Pairing the

Luciferase assay with qPCR then gives us a very effective means of determining any changes to SOD1 transcription. These experiments alone are insufficient to determine the full extent of the molecular players. As mentioned above, it is possible, given the signaling components involved, that both transcription and translation are being affected within our experimental conditions. Therefore, pulse labeling of SOD1 and experiments using translation inhibitors should also be employed to examine SOD1 protein production at this level.

These experiments would provide a very simple read out of the cellular mechanisms that are responsible for modulating SOD1: 1) A change in mRNA with no subsequent alteration rate of translation points towards a transcriptional mechanism. 2) No change in mRNA with changes in translation rate points to a translation mechanism. 3) Changes in both mRNA and translation rate would suggest that both processes are involved. 4) There is no change to either mRNA or protein production, suggesting alternative responses such as changes in protein degradation.

4.2 The effects of TrkB-BDNF are dependent on the SOD1 5' elements and coding region

Before testing the involvement of transcriptional or translational mechanisms it is necessary to clarify the possible promoter dependence observed in the cDNA screen. The primary expression screen was performed in HEK293 cells with hSOD1 being driven by a CMV promoter. The CMV promoter was used mainly out of necessity because we were expressing a mutant SOD1 (A4V) with extremely low expression in this cell line with the human promoter. In contrast, expression levels with the human promoter were not an issue in the HeLa cell line used for validation. However, some of the effects seen in the primary screen were lost during validation which raised two possibilities: cell-type dependence and promoter specificity. Part of this issue was resolved when we re-tested RasGRF-1 in a cell line of neural origin, NSC34, but

the question of promoter specificity remains open. To test this, we repeated our simple over-expression experiments with TrkB and RasGRF-1 with a CMV promoter driving SOD1 expression. Contrary to the dramatic decrease in SOD1 protein with co-expression of either TrkB or RasGRF-1, expression of CMV-hSOD1 was unchanged (Fig 4.1b). We also saw no change in CMV-SOD1 levels with acute treatment of BDNF whereas we previously observed increases in both SOD1 driven from a human promoter and endogenous SOD1 (Fig 4.1b). These results suggest that our effects are dependent on elements within the native promoters for SOD1.

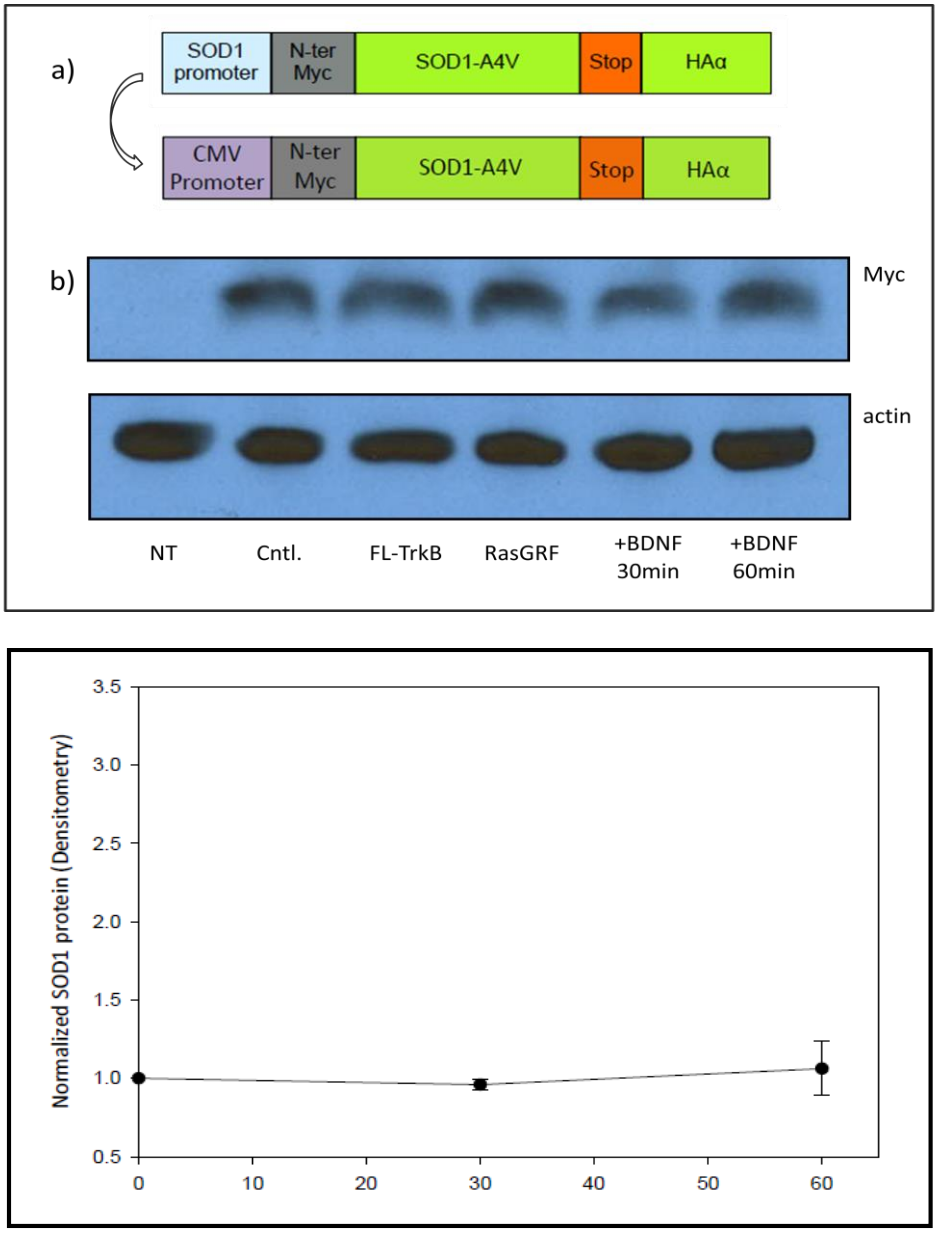


Figure 4.1 Promoter dependence, CMV-hSOD1 – TrkB, RasGRF, acute BDNF treatment. a) To test the involvement of the hSOD1 promoter in the observed effects of BDNF, TrkB, and RasGRF-1, we cloned our N-terminally Myc tagged SOD1 cDNA into an identical vector behind a CMV promoter. b) Representative western blots for anti-Myc (hSOD1) and anti-actin; TrkB, RasGRF-1, and acute BDNF treatment were repeated with the Myc tagged, CMV driven hSOD1. NT= empty vector only, cntl = CMV-SOD1-A4V + empty vector; +TrkB, +RasGRF, +BDNF 30min, +BDNF 60min all contain CMV-SOD1-A4V, (n=3).

To investigate the requirement of the promoter further, we cloned our human SOD1 promoter (hSOD1.prom) into a promoter-less Luciferase (Luc) vector, which is a common method for examining transcription. We then repeated our overexpression experiments with the hSOD1.prom-Luc reporter. There was a slight decrease in Luc signal between control conditions and co-expression with either TrkB or RasGRF-1, but it was not statistically significant and did not match the fold change we observe in SOD1 protein (Fig 4.2, top). Similarly, when we expressed our Luc construct and then treated acutely with BDNF, we observed no change in reporter levels (Fig. 4.2, bottom). These results in tandem with the promoter analysis experiments suggest that the changes that we observed in SOD1 are dependent on the 5' elements and the SOD1 coding region.

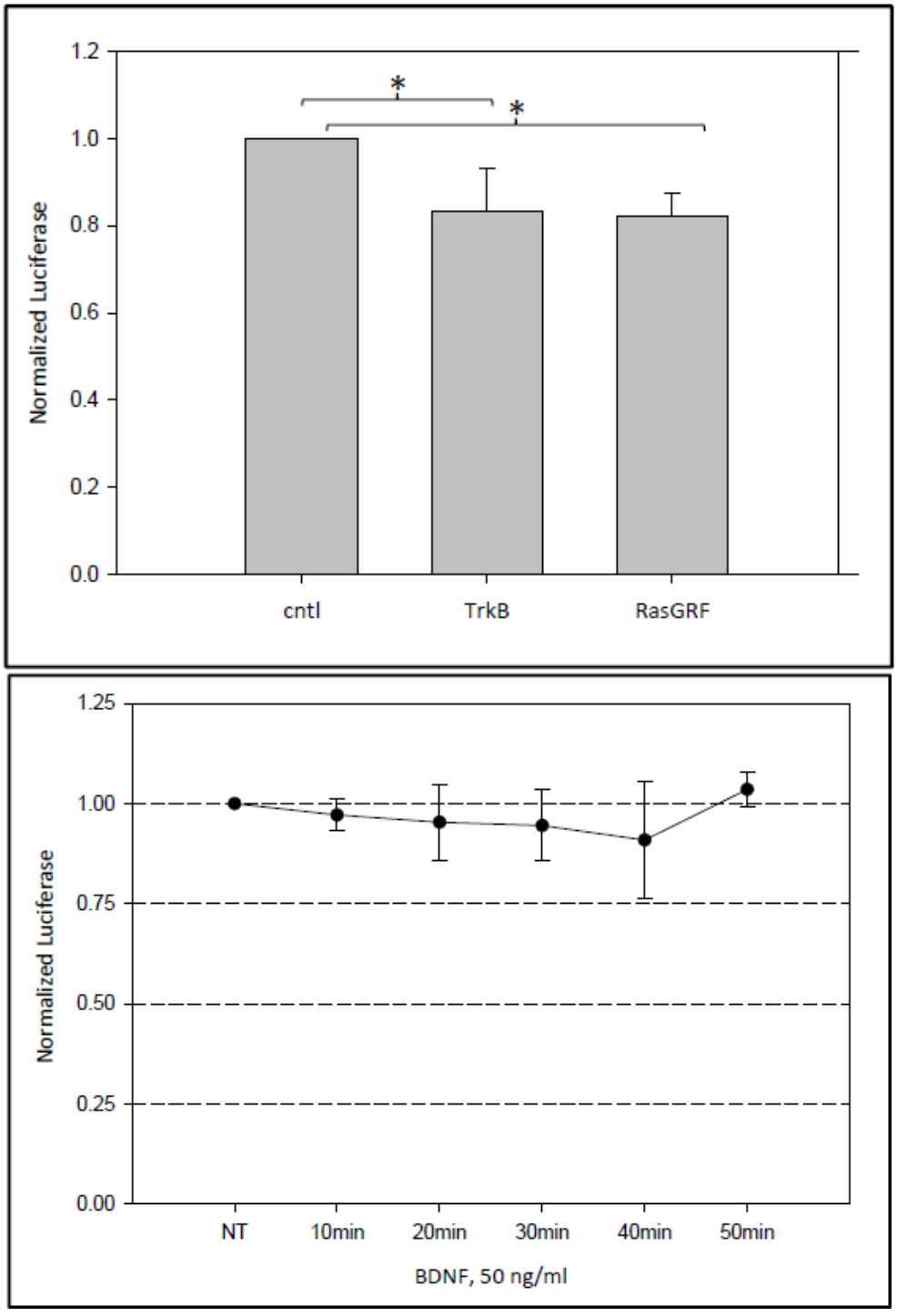


Figure 4.2 Luciferase assays with TrkB, RasGRF-1, and BDNF treatment. Top - activity of the hSOD1.prom was tested with a Luc vector: expression of TrkB and RasGRF-1 do not alter Luc expression driven by the hSOD1.prom. Bottom - acute BDNF treatments were repeated with the hSOD1.Luc construct. No change was observed in Luc reporter levels.

4.3 Changes in SOD1 are independent of mRNA levels

The results from the Luciferase assays and the experiments with different promoters suggested that our effects required the presence of both the 5' and coding regions of SOD1 and points towards a post-transcriptional mechanism. However, this dual requirement also opens the possibility that any alterations to SOD1 under these conditions are mRNA specific. In other words, any changes in the rate of production or turnover at the RNA level are due to the mRNA produced from the promoter-5' UTR-coding region of SOD1. Therefore, we examined SOD1 mRNA levels directly.

To do this we employed qPCR, a quatitative measure of mRNA levels that used a DNA binding dye. Over-expression of TrkB and RasGRF-1 was repeated with our hSOD1 construct and cells were harvested for RNA. Analysis of mRNA levels from these samples again showed a slight decrease with expression of TrkB or RasGRF-1 in a manner similar to what we observed with the Luc assay (Fig 4.3 top). However, as with the Luc reporter, these changes were not significant and do not correlate with what we see at the protein level. Endogenous SOD1 mRNA levels were similarly unchanged (Fig 4.3 bottom). The mRNA data combined with the results from the Luc assays provides evidence that our mechanism is post-transcriptional and independent of mRNA levels, suggesting alterations in the steady state of SOD1 protein, i.e. translation or degradation.

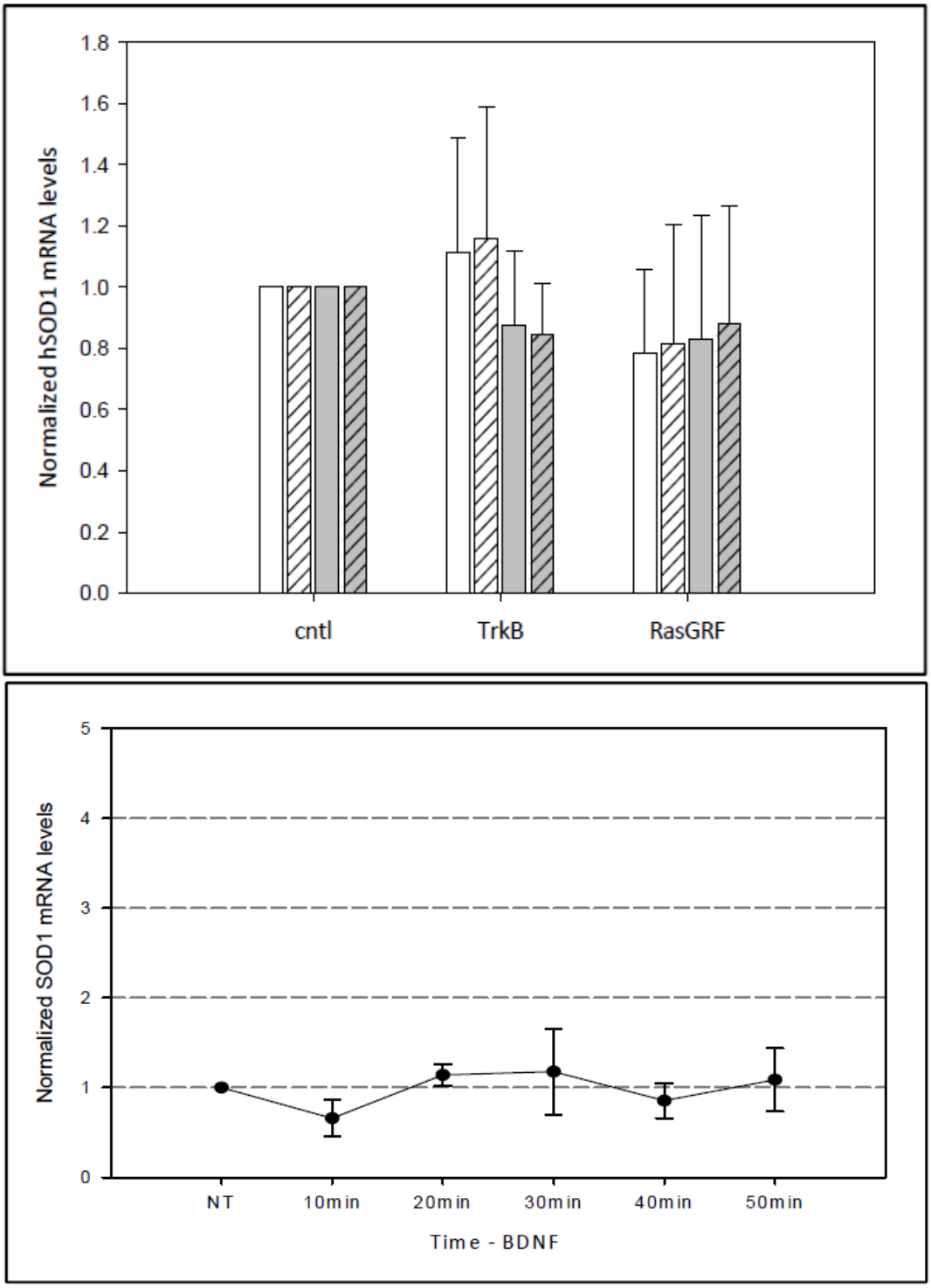


Figure 4.3 SOD1 mRNA levels do not correlate with changes in SOD1 protein levels. Top panel: hSOD1 mRNA levels are unchanged with TrkB or RasGRF-1 expression. Transfections with hSOD1 promoter driven WT (white bars) or A4V (gray bars) SOD1 were repeated with co-expression of TrkB and RasGRF-1. 48hrs after transfection cells were harvested for total RNA

and hSOD1 levels were compared across conditions with internal normalization to β-actin (clear bars) and GAPDH (lined bars) mRNA. Bottom panel: endogenous SOD1 mRNA was examined with acute BDNF treatment and was similarly unchanged at each time point examined.

4.4 Turnover of SOD1 after expression of TrkB or RasGRF is unchanged

While there is little evidence to date that Trk receptors affect proteasomal activity, one recent report showed that BDNF-TrkB signaling enhanced ubiquitination of synaptic proteins, although proteasome activity was unaffected (Jia *et al*. 2008). Furthermore, our data suggest that changes in the rates of translation and/or degradation are mediating the changes in SOD1 protein in response to BDNF-TrkB signaling. To test potential changes in protein turnover, we employed a pharmacological inhibitor of the proteasome, MG132. MG132 is a specific inhibitor of the 26S complex and blocks ubiquitin mediated protein degradation. If expression of TrkB or RasGRF-1 is enhancing ubiquitination and therefore degradation of SOD1, treatment with MG132 should rescue SOD1 protein levels.

We optimized our cell culture and β-gal assay for treatment with MG132. Treatment with MG132 for the final 12-16 hours of a 48hr transfection at a concentration of 3μM produced a significant increase in SOD1.reporter signal with no obvious signs of cell death. Vehicle treated controls showed no difference in SOD1 signal from untreated controls. However, when we repeat this treatment with expression of TrkB and RasGRF-1, there is no detectable increase in SOD1 levels (Fig. 4.4). This result is slightly confusing because control transfections that received MG132 displayed increased SOD1, as expected. Given this, even if enhanced proteasomal degradation is not the principal actor here, we should observe increase in SOD1 signal. One possible explanation is that expression of TrkB or RasGRF-1 is enhancing degradation of SOD1 in a proteasome-independent manner, perhaps through the lysosomal pathway. Alternatively, inhibition of the proteasome has been shown to reduce protein synthesis (Jiang and Wek 2005**)**, so the rate of production of SOD1 with expression of TrkB or RasGRF-1 may be further affected with application of MG132.

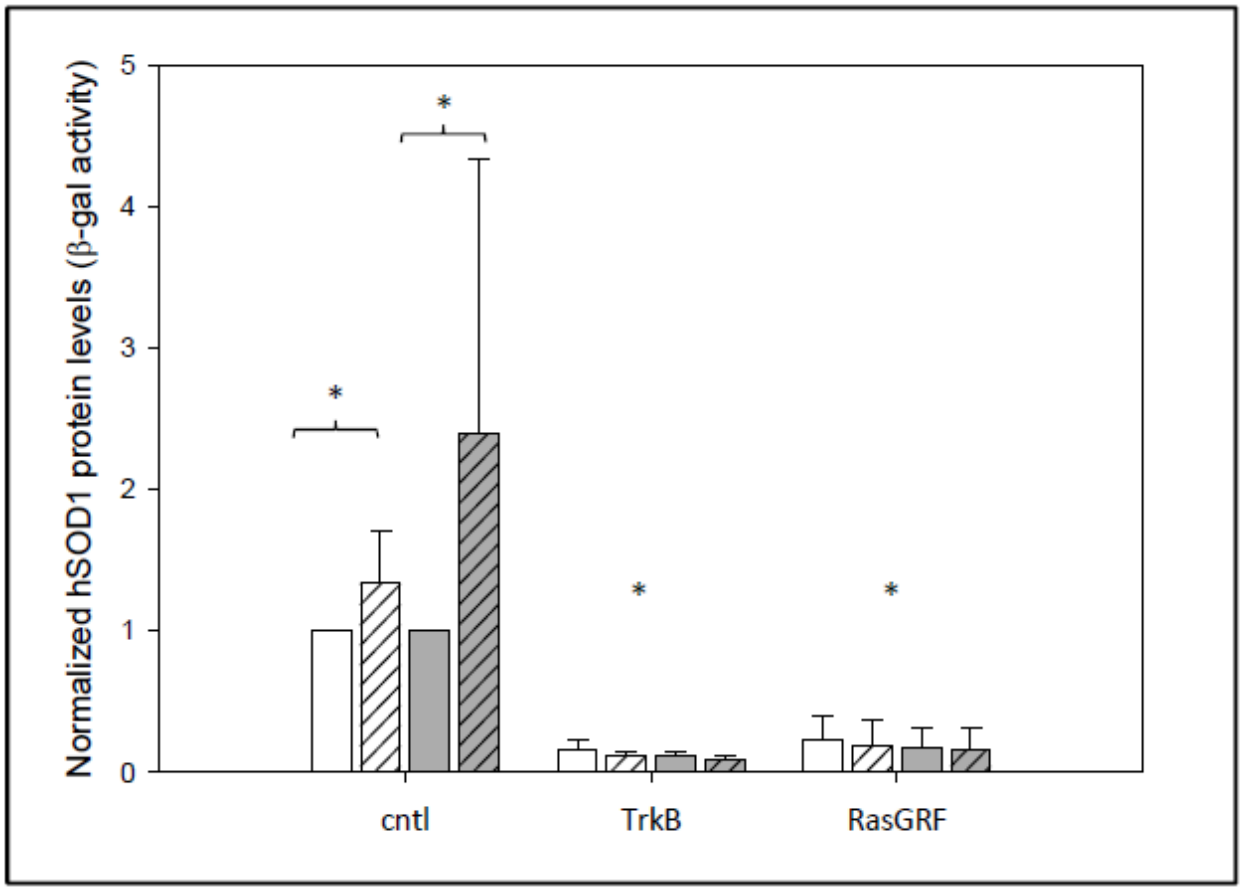


Figure 4.4 Treatment with MG132 in the presence of TrkB or RasGRF-1 expression does not affect expression of SOD1. Cells were treated with 3μM MG132 or equivalent volume of vehicle for the final 12-16 hours of a 48 hour transfection. MG132 treatment increases both WT (white bars) and A4V (gray bars) SOD1 levels in control condition but not when TrkB or RasGRF-1 is expressed, (n=6); clear bars = veh., lined bars = MG132. * = p-value > 0.05

4.5 Increase in SOD1 protein via BDNF treatment is translation dependent

Results from the Luciferase assays and qPCR experiments suggest that the effects of TrkB and BDNF on SOD1 are independent of transcription and mRNA levels. Therefore, the most likely remaining cellular mechanisms are either alterations in translation or changes in protein turnover at the level of the proteasome. As mentioned above, BDNF-TrkB signaling has been shown to be required for neuronal survival and morphogenesis during development. More interestingly, BDNF-TrkB has been shown to play a role in synaptic plasticity and neurotransmitter release (Li *et al*. 1998). BDNF potentiates the release of excitatory glutamate through TrkB-PLCγ/PI3K activity and release of intercellular Ca^{2+}. This in turn directs the insertion of AMPA channels into the post-synaptic membrane, thus enhancing the ability of the cell to respond to subsequent firings, i.e. plasticity. Critically though, it is well established that synaptic plasticity has early and late phases that require both transcription and translation of specific neuronal genes. Because the data to this point suggest that there are no changes in transcription of SOD1, this leaves translation as the most likely remaining mechanism.

To test the translation hypothesis we repeated our acute BDNF treatment. Cells were pretreated with either vehicle (DMSO) or cycloheximide (CHX), an inhibitor of translation, and then treated with BDNF. As previously observed, acute treatment with BDNF increased SOD1 protein levels. However, in cells pretreated with CHX, SOD1 levels were not elevated above control levels and CHX treatment alone did not significantly alter SOD1 expression (Fig 4.5). This data suggests that the increase in SOD1 protein with acute BDNF treatment is translation-dependent.

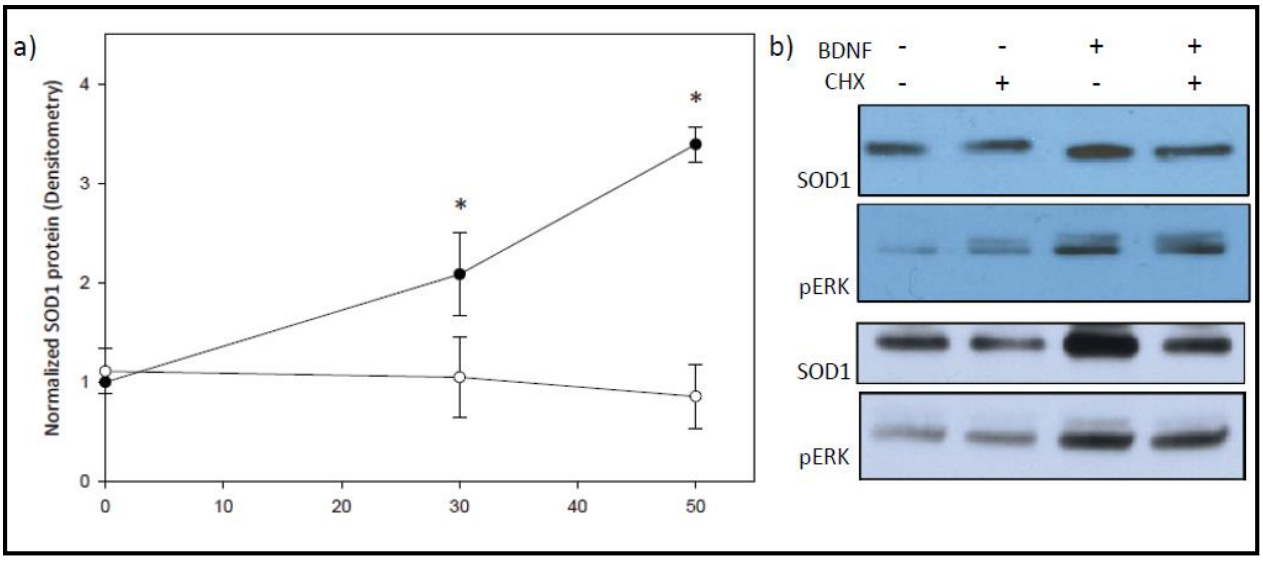


Figure 4.5 Acute BDNF treatment +/- cycloheximide pretreatment. Cells were pretreated with DMSO or cycloheximide and then treated with BDNF for 30 or 50 minutes. SOD1 protein levels increase with BDNF-DMSO but not with BDNF-CHX. a) Quantification of results across BDNF +/- CHX, v=vehicle, c=CHX. b) Westerns for SOD1 and pERK with 30min (top) or 50min (bottom) application of BDNF; BDNF activity was monitored by phopho-ERK. (n=6), * = p-value > 0.05

4.6 Discussion

BDNF's involvement in enhancing transmission and plasticity has previously been shown to be translation dependent (Kang and Schuman, 1996). Later reports clarified the molecular players underlying this dependency by showing that BDNF treatment acted on translation initiation factors 4EBP1 and eIF4E as well as mTOR (Takei *et al.* 2001). Subsequent studies provided further insight by showing that BDNF induced translation of a specific subset of mRNAs during development and that it increased protein synthesis within the dendritic compartment, the site of synaptic plasticity (Schratt *et al.* 2004, Takei *et al* 2004). Both of these processes were shown to be mTOR dependent, involve PI3K-Akt signaling, and act on p70S6K (a.k.a. p70RSK). Interestingly though, there are some neuronal processes downstream of BDNF stimulation that appear to be dependent on specific elements of TrkB signaling as opposed to the entire cascade. ERK1/2 activation has been shown to stimulate the translation machinery in an mTOR-independent manner and outgrowth of filopodia in hippocampal cells is also dependent on ERK1/2 alone (Roux *et al.* 2007 and Alonso *et al*. 2004). Additionally, there have been some disease models with synaptic perturbations wherein mTOR mediated translation is compromised and local translation is ERK1/2 dependent (Osterwiel *et al*. 2010). With this in mind it may be that ERK1/2 activation downstream of BDNF-TrkB is sufficient to promote translation of SOD1. However, it could also be the case that the convergence of these signaling pathways on the translation machinery, i.e. ERK1/2 and Akt-mTOR, is required and they do not function independently in our experimental setting. The specific pathway requirements could be tested using U0126 and rapamycin which inhibit ERK1/2 phosphorylation and mTOR, respectively.

Chapter 5

Summary, Conclusions, and Future Directions

5.1 Summary and Conclusions

The data presented here provide evidence that TrkB-BDNF signaling is involved in the regulation of SOD1 expression. Expression of TrkB or a downstream signaling effector, RasGRF-1, significantly decreases SOD1 protein levels in a motor neuron cell line. Chronic treatment with BDNF, an agonist for TrkB produces a similar effect both in cell culture and in an organotypic culture of mouse spinal cord sections. In contrast, acute treatment with BDNF produces an increase SOD1 protein. In both scenarios, this alteration in SOD1 protein expression appears to be mediated at the level of translation. Taken together, our data support a model wherein TrkB-BDNF signaling bi-phasically regulates SOD1 expression.

Following the data from our screens, our approach was designed to answer two broad questions: 1) What signaling components of TrkB and the MAPK pathways are involved in modulating SOD1? 2) What is the cellular/molecular mechanism that mediates these changes and can they be exploited for therapy? To answer the first question we employed a series of TrkB mutants and pharmacological inhibitors of various components of the MAPK pathways. Data from experiments using the inhibitors alone demonstrated that SOD1 expression could be affected simply by inhibiting these kinases in the absence of stimulation, i.e. under basal conditions. In the case of U0126 and Triciribine, which specifically target MEK1/2 and Akt, treatment slightly reduced expression of SOD1, suggesting that these pathways positively regulate SOD1 expression. Similarly, genetic expression of the TrkB receptor or RasGRF-1, an effector of the Ras-MEK-ERK axis, drastically reduced SOD1 protein levels. However, in the case of TrkB, this effect was dependent on the kinase activity of the receptor as expression of a kinase-dead TrkB, K571N, had no effect on SOD1 expression. This suggests that activation of these pathways is necessary for suppression of SOD1 expression, which is at odds with the

pharmacologic data. One way to reconcile this is through the negative feedback loops within the MAPK pathways. Phosphorylated ERK1/2 is known to feedback and negatively regulate the activity of SOS, an activator of Ras (Kholodenko *et al.* 2000). Similarly, constitutively activated Raf-MEK has been observed to negatively regulate PI3K-Akt signaling (Menges and McCance 2008). Thus, it may be the case that expression of TrkB or RasGRF-1 constitutively activates the MAPK pathways leading to long-term inhibition and suppression of SOD1 expression. It is also important to note that the reduction in SOD1 protein could be achieved with expression of an N-terminally truncated TrkB. As previously mentioned, this truncation is the result of a recently identified splice variant which lacks exons that code for a portion of the cell surface domain. While the putative neurotrophin binding site is intact, this truncation lacks a signal sequence and may not be targeted to the cell membrane. This suggests that TrkB activity in this setting is not dependent on binding of BDNF or other ligands and may occur through transactivation of the receptor (Mojsilovic-Petrovic et al. 2006).

Another interesting observation that emerged from experiments with TrkB mutants was that partial TrkB signaling appeared to be sufficient to mediate the loss of SOD1 expression. Based on our initial findings with U0126 treatment and RasGRF-1 expression we had hypothesized that TrkB was most likely affecting SOD1 expression via the MEK/ERK pathway. This meant that expression of TrkB-Y515F, which is unable to bind the effector proteins for Ras-MEK-ERK and Akt signaling, should block the suppressive effect we observe with WT-TrkB. However, this was not the case. Similarly, expression of a TrkB-PLCγ mutant, Y816F, did not block suppression of SOD1 expression. These results suggested two possibilities: 1) that either one of these signaling axes, ERK/Akt or PLCγ, could mediate these effects on SOD1 or 2) there is redundancy or crosstalk within these pathways that is sufficient to reduce SOD1 expression.

Given the intricacies of receptor tyrosine kinase-MAPK signaling, we feel the latter is the more reasonable explanation. In support of this is the observation that PLCγ signaling stimulates release of calcium from intracellular stores via IP3 and increases in intracellular calcium can activate ERK (McKay and Morrison 2007, Chuderland and Seger 2008). This provides an alternative route by which TrkB-Y515F could activate ERK signaling. In addition, the GTP-exchange activity of RasGRF, which activates Ras-MEK-ERK, is $Ca2^{+}$-calmodulin dependent (Agell *et al*. 2002). Taken together, it may be the case that calcium-dependent activation of MEK-ERK via TrkB-BDNF or RasGRF-1 is a mediator of the effects on SOD1 expression.

The data supports the hypothesis that alterations in SOD1 expression via TrkB and BDNF are at the level of translation. The longer, 'chronic' experiments with TrkB and BDNF provided evidence that modulations in SOD1 protein levels were independent of transcription, mRNA levels, proteasomal degradation, and lysosoml activity (data not shown) which points towards a translational mechanism but will require a pulse-chasae experiment to support this. For the 'acute' treatment with BDNF, SOD1 protein levels were also independent of transcription and mRNA levels. The finding that these effects were post-transcriptional was surprising because the SOD1 promoter is known to contain binding sites for transcription factors that act downstream of the pathways associated with TrkB. However, in this experimental setting, application of a translation inhibitor blocked the effect of BDNF on SOD1, which provides direct evidence that translation is the mechanism by which TrkB-BDNF signaling acutely regulates SOD1 expression.

As mentioned previously, we divided our approach into two aims, one dedicated towards signaling pathways and the other directed towards cellular mechanisms. As discussed above, the signaling experiments suggest that modulation of SOD1 is indeed dependent on activation of

TrkB. Additional analysis opened up the possibility that our effects are calcium/Ras-MEK-ERK dependent. Given that the signaling pathways downstream of TrkB are known to regulate the translation machinery, the observations from the signaling experiments align nicely with the mechanistic data. In total, these data suggest a model in which activation of TrkB signaling produces a transient increase in SOD1 protein levels which is then followed by a later reduction in SOD1 (Fig 5.1). At the time points examined, these changes appear to be due to alterations in translation of SOD1. In the context of the neuronal environment, regulating SOD1 in such a manner may be a response to free-radical production during periods of high neuronal activity. Mitochondrial function is known to be tightly coupled to neuronal viability and function and clearance of excess free-radicals is known to be neuro-protective (Kann and Kovacs 2007). Therefore it may be that SOD1 is increased in response to high neuronal activity and is decreased after oxygen radicals have been returned to basal levels. Interestingly, this may link some of the known pathology of ALS, as both oxidative damage and mitochondrial dysfunction have been observed in motor neurons of ALS models.

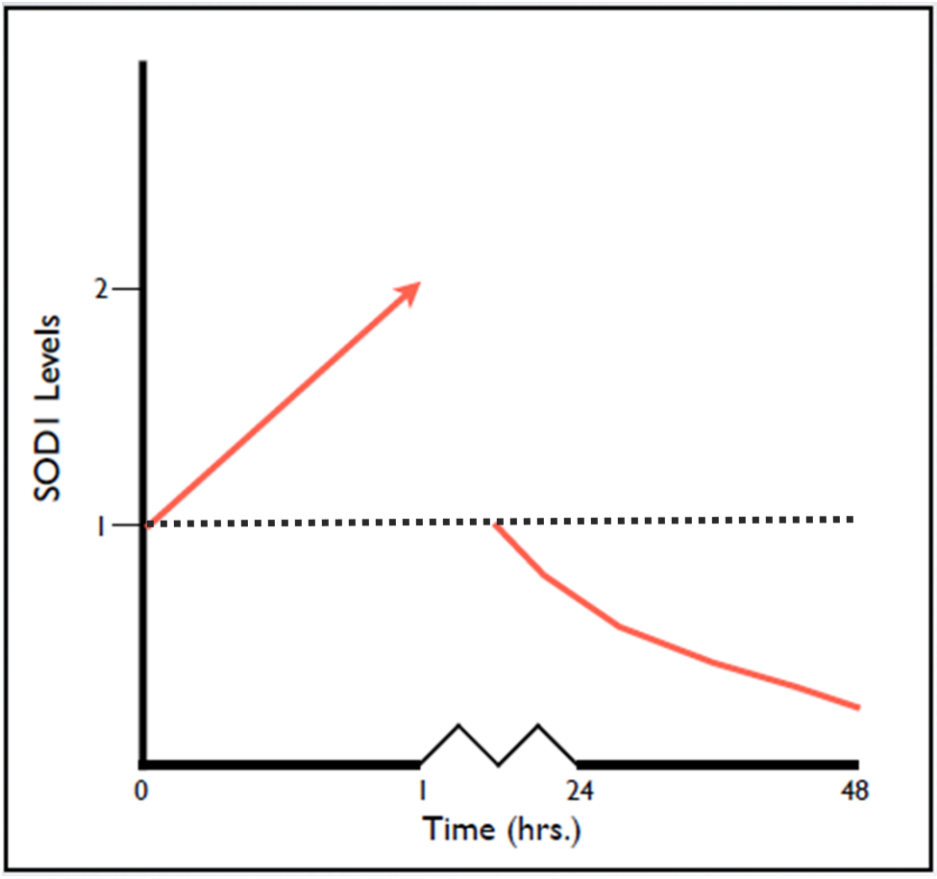


Figure 5.1 Time-course of SOD1 expression in relation to TrkB activation. Activation of TrkB via BDNF leads to an acute increase in SOD1 expression as observed up to one hour. Longer activation of TrkB, via continued treatment with BDNF or expression of TrkB, leads to a return to baseline levels of expression followed by a significant decrease in SOD1 protein 24-48 hours after treatment.

5.2 Relevance for ALS

In considering this data as a whole, it is necessary to consider it within the context of neuronal function and disease. BDNF is known to be released at synapses in an activity-dependent manner and binding to TrkB stimulates both transcription and translation of pro-survival genes and of protein products that are required for maintenance of the synaptic compartment and induction of plasticity. As modeled above, the data suggest that transient activation of TrkB increases SOD1 expression, while longer periods of activity would decrease SOD1 protein levels. One could imagine a scenario where synaptic activity is dysfunctional in a state of disease leading to an extended or chronic activation of TrkB and other synaptic signaling machinery. Indeed, excess release of glutamate and excitotoxicity has been observed as part of the pathogenesis of ALS. The difficulty here is that our model predicts that normal synaptic activity may increase SOD1 expression which would actually contribute to the disease process by making more protein available for aggregation, which is toxic to motor neurons. Furthermore, the onset of a state of chronic activation or glutamate release is likely to occur later in the disease process after some cell death has occurred rendering any effect of chronic TrkB activation on SOD1 moot in this context. The existing literature on TrkB and SOD1 ALS is similarly confusing regarding any potential harmful or protective effect.

Recent work has implicated TrkB-BDNF as being both protective and detrimental to motor neurons. Yanpallewar *et al*. 2012 provided evidence that increased TrkB signaling is protective in a hSOD1 mouse model. By deleting a splice variant of TrkB, T1, that is thought to negatively regulate TrkB, they showed that both symptom onset and motor neuron degeneration were delayed. However, their conclusions were based on behavioral outcomes in mice, and no direct examination of TrkB activity or signaling was presented. Similarly, a more recent report

using pharmacological treatments demonstrated that sustained increases in neuronal excitation and mTOR signaling were protective in a mutSOD1 mouse model (Saxena *et al.* 2013). While they did not examine BDNF or TrkB in this study, BDNF is released during neuronal activity and mTOR is known to be a downstream target of Akt-PI3K signaling in response to TrkB activation. Therefore it seems likely that TrkB signaling is involved in this process. In contrast to these reports, work from Gordon Kalb's lab has suggested that TrkB activity in motor neurons contributes to excitotoxicity and also leaves these neurons more vulnerable to the toxicity of mutSOD1 (Jeong *et al*. 2011). And, as mentioned above, motor neuron-specific knockout of TrkB in mutSOD1 mice delays motor impairments and significantly extends lifespan. Interestingly, in this same study, SOD1 protein was decreased in TrkB-KO animals.

The conflicting nature of these reports and the differences in the manipulations of the signaling pathways make it difficult to assign the direct relevance of TrkB function in SOD1-ALS. This is further underscored in the differences in the systems used for these studies. The study connecting excitability and mTOR was conducted in the G93A Gurney founder lines while the T1.KO experiments were conducted in a similar line backcrossed to C57 mice for several generations. Also, the TrkB-KO animals were generated by crossing a transgenic Cre expresser line to G85R-SOD1 animals. Thus, there are both genetic backgrounds and mutSOD1 variants to consider in interpreting these findings. Additionally, the toxic effect imparted by TrkB to motor neurons was observed in a co-culture setting of astrocytes and motor neurons. While this is obviously a very different experimental system from both the animal models presented above as well as our cell culture model, the primary consideration is the presence of astrocytes, which are known to contribute to the disease process in ALS. If we integrate our findings with the current literature, it seems likely that TrkB function is involved in the disease course of SOD1-

ALS, although it appears that the mechanism of its contribution is highly context dependent. Nonetheless, our data identify TrkB and its associated pathways as a potential means of modulating SOD1 expression. Based on the current literature, this could in turn lead to decreased levels of SOD1 aggregation in a disease model which would ameliorate motor neuron degeneration and perhaps extend life.

5.3 Future Directions

The model presented in Figure 5.1 illustrates the bi-phasic response of SOD1 to TrkB activation. However, we have only examined a limited number of time points within this context. Specifically, there is a sizeable gap between the 2-3 fold increase in SOD1 protein we observe approximately one hour after BDNF treatment and the decrease we observe roughly 24 hours later. One line of experiments would be to simply repeat the BDNF treatment and follow it out to different time points between one and twenty four hours to ascertain where the decrease in SOD1 expression begins. Similar experiments could be performed with single versus titrated doses of BDNF as well as TrkB expression. Previous experiments with hSOD1 showed that its half-life was approximately 30 hours, albeit in a very different cell model than the one we have employed here (Borchelt *et al*. 1994). If we take this figure at face value it means that at least half of the SOD1 protein produced within the initial period of TrkB-BDNF treatment should still be present 24-30 hours later at a time when we typically begin to observe a decrease in SOD1 levels. This suggests that there is an additional mechanism at work, such as increased turnover of SOD1, that contributes to the decrease in SOD1 levels seen at later time points. While we have examined the involvement of the proteasome pharmacologically, we did so only over the last 12-16 hours of 48 hour experiments, primarily because extended inhibition of the

proteasome results in cellular toxicity. While this approach may be impractical for long periods, one way to examine any possible changes in SOD1 turnover for an extended time frame would be with pulse-chase analysis. If we did observe changes in the rate of SOD1 degradation this would mean there is a transient increase in either lysosomal or proteasomal breakdown of SOD1. Thus our mechanism would be due partially to translation and partly to degradation.

Another point to consider is the variation in the SOD1 constructs we have employed here. We have looked at hSOD1 driven off of both a CMV and a hSOD1 promoter, a Luciferase reporter driven by the same hSOD1 promoter, and the endogenous SOD1 elements within our cell system and mouse spinal cord tissue. If we compare these 'constructs' side by side, we see that only the hSOD1 protein being driven from the hSOD1 promoter and the endogenous mouse SOD1 respond to TrkB-BDNF activity (Figure 5.2). As stated previously, this suggest that the native SOD1 5' elements (promoter and 5' UTR) as well as the coding region are required for the effects of TrkB-BDNF. We cannot say that these effects are necessarily promoter specific because the 5' UTR of the CMV-driven SOD1 is different from that in the hSOD1 promoter constructs which contain the human SOD1 5' UTR as well. A simple way to reconcile this would be to clone the SOD1 5' UTR behind the CMV promoter region and re-examine the effect of TrkB-BDNF activity. If a CMV/SOD1.UTR construct responded to TrkB-BDNF activity that would mean that the effects are promoter independent. This then suggests that the elements within the SOD1 5' UTR are dictating the response to TrkB-BDNF signaling, which would support a translational mechanism. If this were the case it is also sensible to consider the secondary structure of the mRNAs produced from these SOD1 variants. Secondary structure predictions of the mRNAs from our experimental SOD1 variants show that the two constructs that behave similarly, endogenous mouse and hSOD1prom-SOD1, also have highly similar

structures (Figure 5.3). Additionally, the translation start sites for these variants appear to be more easily accessible whereas they are tied up in stem-loop structures within the CMV and Luciferase versions. A recent report presented evidence that FMRP, a known regulator of translation, bound SOD1 mRNA, which makes FMRP a candidate effector of SOD1 translation (Bechara *et al*. 2009). This could be examined by overexpressing or knocking down FMRP in a neuronal cell line and observing the effect, if any, on SOD1 protein expression. Taken together, it may be that the mRNA structure of SOD1 and its ability to be efficiently translated or RNA binding proteins that are directed at SOD1 mRNA play a role in the response to TrkB-BDNF.

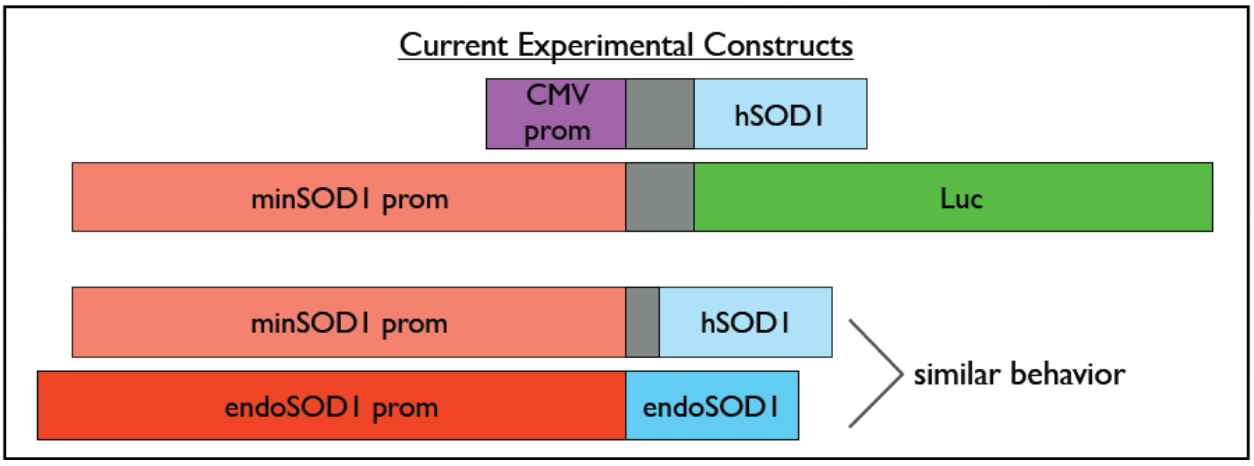


Figure 5.2 Diagram of SOD1 variants used in these experiments. In order from top: hSOD1 cDNA expressed behind a CMV promoter; firefly Luciferase gene driven by our cloned human SOD1 promoter; hSOD1 cDNA with the human SOD1 promtoer; endogenous SOD1 promoter and gene, from NSC34 cells- murine. The latter two forms of SOD1 display similar behavior in response to TrkB-BDNF in our experiments.

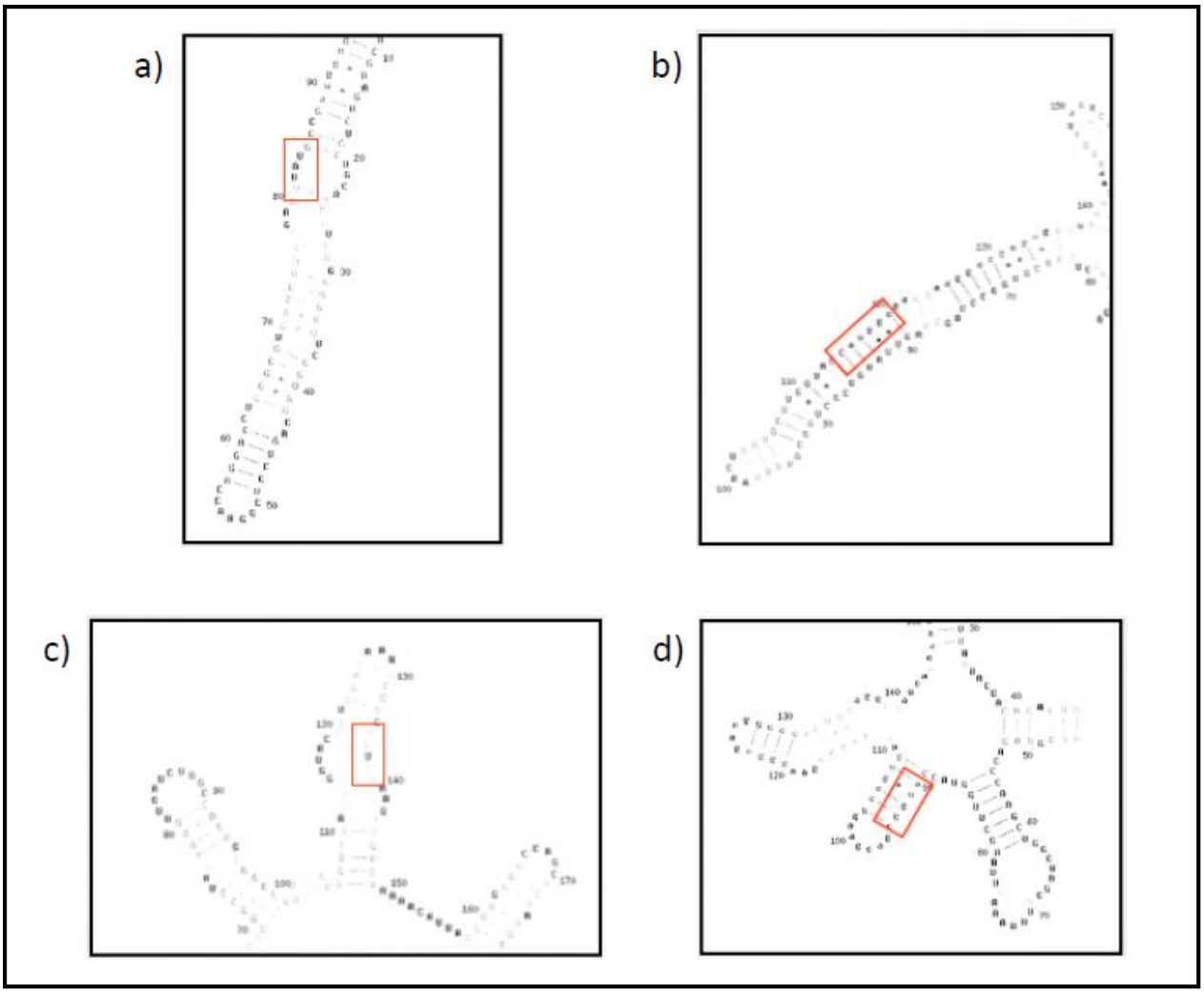


Figure 5.3 Secondary structure prediction of SOD1 variants. a) Endogenous mouse b) hSOD1 5' region with hSOD1 coding region c) hSOD1 5' region with Luciferase coding region d) CMV promoter and 5'UTR with hSOD1 coding region. Predicted structures from A and B are highly similar and respond to TrkB-BDNF activity. Predicted structures from C and D, while similar to each other, have extra stem-loop regions not seen in A and B and do not respond to TrkB-BDNF. Translation start sites are shown in red boxes. Structure prediction from CONTRAfold, Stanford University (Do *et al*. 2006).

Finally, there are additional signaling components to consider. Our data from the short BDNF treatments supports a translational mechanism and in these experiments we used a general inhibitor of translation, cycloheximide. The existing literature on activity-dependent translation in neurons shows that translation in response to BDNF is also dependent on the function of mTOR. mTOR is known to be involved in an array of cellular processes ranging from cell growth and proliferation to protein synthesis. With this in mind, repeating our short BDNF treatments +/- rapamycin, an mTOR specific inhibitor, would reveal whether our effects are mTOR dependent. Furthermore, since mTOR activity in neurons is primarily the result of Akt-PI3K activation, blockade of Akt should produce a similar effect as inhibition of mTOR for acute treatments with BDNF.

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Appendix A

Materials and Methods

Cell Culture

All experiments were conducted using NSC34 cells, a fusion of a mouse spinal motor neuron and a neuroblastoma (Cashman et al. 1992). Cells were maintained at 37°C, 5% CO_2 and in Dulbecco's modified eagle's medium (DMEM) 11965 (Invitrogen) with 10% (fetal bovine serum) FBS, 1% penicillin/streptomycin. Cells were passed typically every 3-4 days at ~70% confluence.

DNA Constructs

WT and A4V SOD1-HA constructs containing an α-fragment of the β-galactosidase (β-gal) gene with either a CMV or a 2.2kb human SOD1 promoter and the ω-fragment β-gal construct were described previously (Somalinga et al. 2011). Myc-tagged SOD1 constructs were made by inserting the Myc sequence at the 5' end of the SOD1 coding sequence and a stop codon in front of the HAα sequence. Constructs for TrkB and RasGRF-1 are as previously described (Somalinga et al. 2011). Additional constructs for full length (FL), Y490F, Y785F, and K560N TrkB were kindly provided by Dr. Christopher Cowan and were described previously (Hale et al. 2011). The TrkB double mutant (Y490F/Y785F) was generated by site directed mutagenesis on the Y490 position of the TrkB-Y785F construct using QuikChange II (Stratagene). Primers for mutagenesis were as follows: Fwd, 5'-3' GCGTCGCCCGTCTTCCTGGACATCCTAGG; Rev, 5'-3' CCTAGGATGTCCAGGAAGACGGGCGACGC.

Over expression and β-gal Assay

Cells were seeded to experimental plates at a density of $2x10^5$/ml. After seeding, cells were incubated for 18-24 hrs, media was exchanged, and then cells were transfected. Over expression experiments of TrkB variants and RasGRF-1 used 1µg/ml of hSOD1 and 0.5µg/ml of

TrkB or RasGRF-1. A pcDNA3.1 vector lacking a gene insert was transfected in control wells to normalize total DNA content. Polyethylenimine (PEI) was used as a transfection reagent in a ratio of 4:1, µl PEI: µg DNA, and stock concentrations of PEI were 1µg/µl. For the β-gal assay, transfections were carried out similarly with the addition of 0.5µg/ml of ω-fragment per well. Cells were then incubated for an additional 48 hrs. post-transfection. Following incubation, media was aspirated and cells were lysed in cold 1x reporter-lysis buffer (Promega) + Complete Mini, EDTA free protease inhibitor (Roche) for 45-60min. at 4°C on a rocker. Whole cell lysates (WCL) were then collected and total protein concentration was estimated by UV-spectroscopy at 595nm with the Bradford method. WCLs were then pelleted for 15min. at 4°C, 16,000g. For β-gal assays, protein concentration measurements were performed after centrifugation on soluble fractions. Supernatants were then separated, pellets were resuspended in equal volumes of 1x lysis buffer, and both fractions were prepared with Lamelli sample buffer with heating at 90-100°C for 5-10min. For the β-gal assay, supernatant fractions were removed and then normalized to 1µg/µl protein based on absorbance at 595nm. 3x25µl of each sample was transferred to a black, flat-bottom 96well plate and 50µl of FDG substrate was added to each well. Plates were then read for fluorescence in a SpectraMax M5 (Molecular Devices), with excitation at 490nm and emission at 525nm.

Antibodies and Western Blotting

Anti-Myc (clone 9E10), 1:1000 (Santa Cruz Biotech); anti-HA (clone 16B12), 1:2000 (Covance); anti-actin, 1:50.000 (Millipore); anti-SOD1, 1:2000 (Calbiochem); anti-SOD1, 1:2000 (Protein Tech); anti-hSOD1 (#2770), 1:2000 (Cell Signaling); anti-TrkB, 1:2000 (BD Biosciences); anti-pERK, anti-ERK 1:2000 (Cell Signaling). All protein samples were run on denaturing PAGE gels and transferred to 0.45µm nitrocellulose (Whatman) or PVDF

(Millipore) membranes. Membranes were blocked in 0.05% TBST-5% milk for 1-2hrs. at room temperature. Primary antibodies were incubated either for 1hr at room temperature or overnight at 4°C. Secondary antibodies were used at a concentration of 1:30.000 except for Bio-Rad anti-sheep, ~1:70.000, and incubated at RT for 1hr. All wash steps were 3x6min. Blots were developed using ECL Plus system (GE Healthcare) and analyzed using either Storm Imager or ImageJ.

Luciferase Assay

The 2.2kb human SOD1 promoter was amplified by PCR from the WT-SOD1-HAα construct and cloned into the promotorless firefly luciferase vector [luc/neo] pGL4.17 (Promega) . Primers for PCR were as follows: Fwd, 5'-3' ACCTGAGCTCGCTAGCAGAATCACTTGAACC; Rev, 5'-3' CCGAGGCCAGATCTCGCCATAACTCGCTA. Co-transfections of the human SOD1 promoter (hSOD1p) Luc vector and modulators were performed as described above. Following transfection and incubation, media was aspitrated and cells were briefly trypsinized and then resuspended in media. Cell counts were taken for each sample and cells were transferred in triplicate to 96well plates, with transfer volumes normalized by cell number. Total volume per well was then brought to 100µl and 100µl of a 1:5 dilution of BrightGlo reagent (Promega) was added to each well. 5 minutes after reagent addition, plates were read on a SpectraMax M5 for luminescence with all wavelengths read. For experiments with BDNF treatment, cells were trypsinized, resuspended in media, normalized by cell count, and transferred to 96well plates 24hrs post-transfection. Cells were then allowed to recover at 37°C, 5% CO_2 for approximately 6 hours prior to treatment and luminescence measurements.

RNA isolation, reverse transcription, and qPCR

Total RNA was isolated from cell culture experiments using NucleoSpin RNA II (Macherey-Nagel) and RNA concentrations were estimated by spectrophotometry at 260 and 280nm. Reverse transcription reactions were done in 50µl volumes according to High Capacity cDNA RT kit (Applied Biosystems). qPCR analysis was then carried out in a 384 well format with triplicates for each sample for either human or endogenous SOD1 and β-actin and GAPDH. Primer sequences for qPCR are as follows:
hSOD1 - Fwd 5'-3' GGATCGAGGGAAGGATTTCAGA, Rev 5'-3' GGCACGTCATAAGGGTAGTC; endogenous mouse SOD1 - Fwd 5'-3'GATGAAGAGAGGCATGTTGGA, Rev 5'-3' TGTACGGCCAATGATGGAATG; β-actin - Fwd 5'-3' GGCTCCGGCATGTGCAAAG, Rev 5'-3' CTTCTGACCCATTCCCACCA; GAPDH - Fwd 5'-3' TGAGGCCGGTGCTGAGTAT, Rev 5'-3' GGCTCCACCCTTCAAGTG.

Pharmacologic Reagents

BDNF (Alomone Labs) was used at 10-100ng/ml and was optimized for each independent cell culture stock. MG132 was used at 3.125uM final concentration. Cycloheximide (Sigma) was used at 100uM final concentration. Lyophilized BDNF peptide was resuspended in MQ-H2O and stored at -80C. All other compounds were dissolved in DMSO and stored at -20C, except for CHX, stored at 4C.

Appendix B

Additional Figures

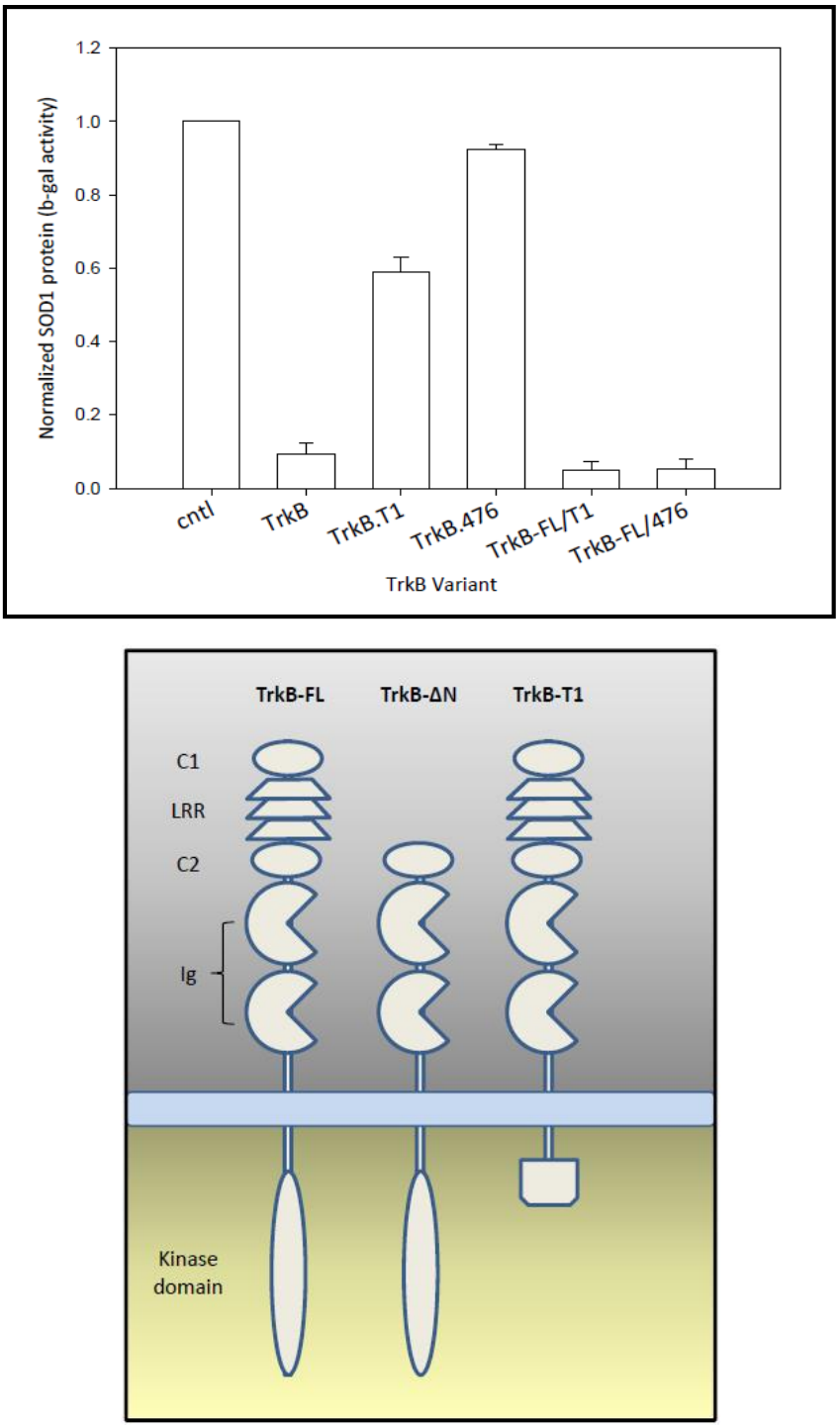

Figure S1 Expression of TrkB.T1 and truncated TrkB. T1 is an alternate splice variant of TrkB; TrkB.476 is a truncated WT-TrkB produced via mutagenesis that matches T1 in amino acid number but retains the WT sequence. These variants were expressed to test the possible contribution of T1 specific signaling on regulation of SOD1 expression.

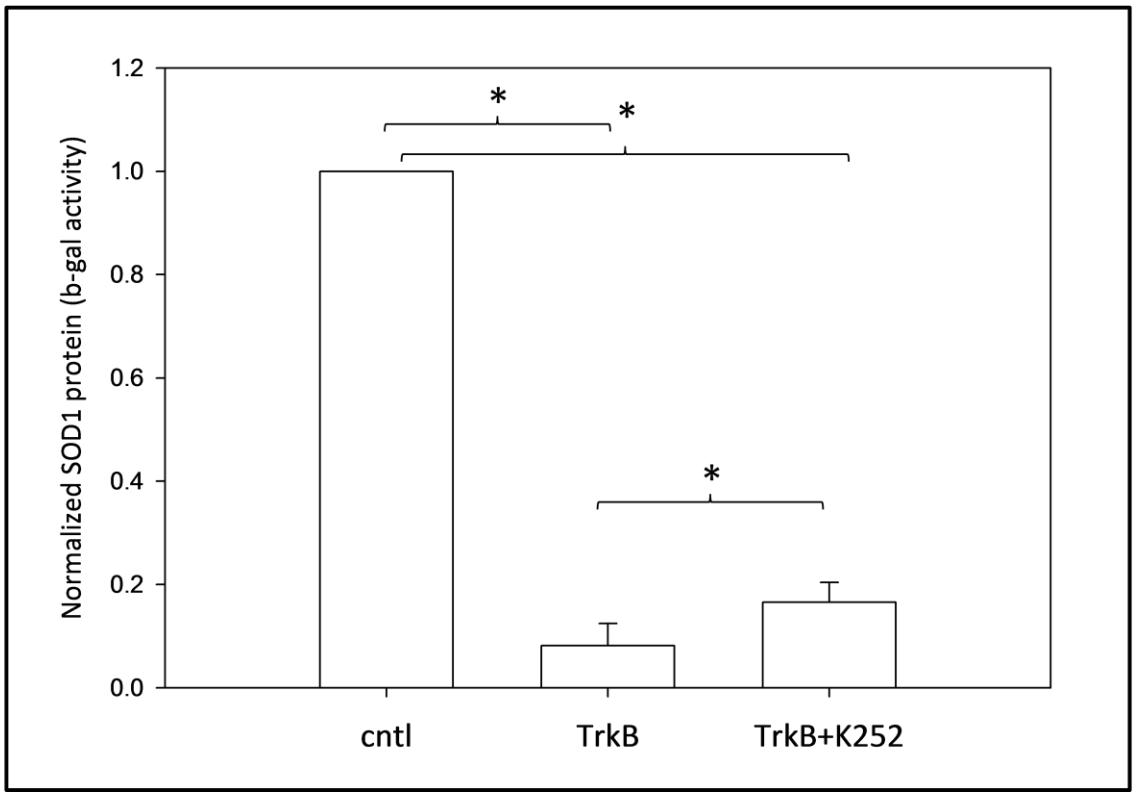

Figure S2 Treatment with K252a. K252a is known to inhibit tyrosine kinase activity and was tested to see if it could block the effects of TrkB on SOD1. O/N treatment with K252 produced some rescue of SOD1 but failed to return it to control levels (n=8). * = $p > 0.05$

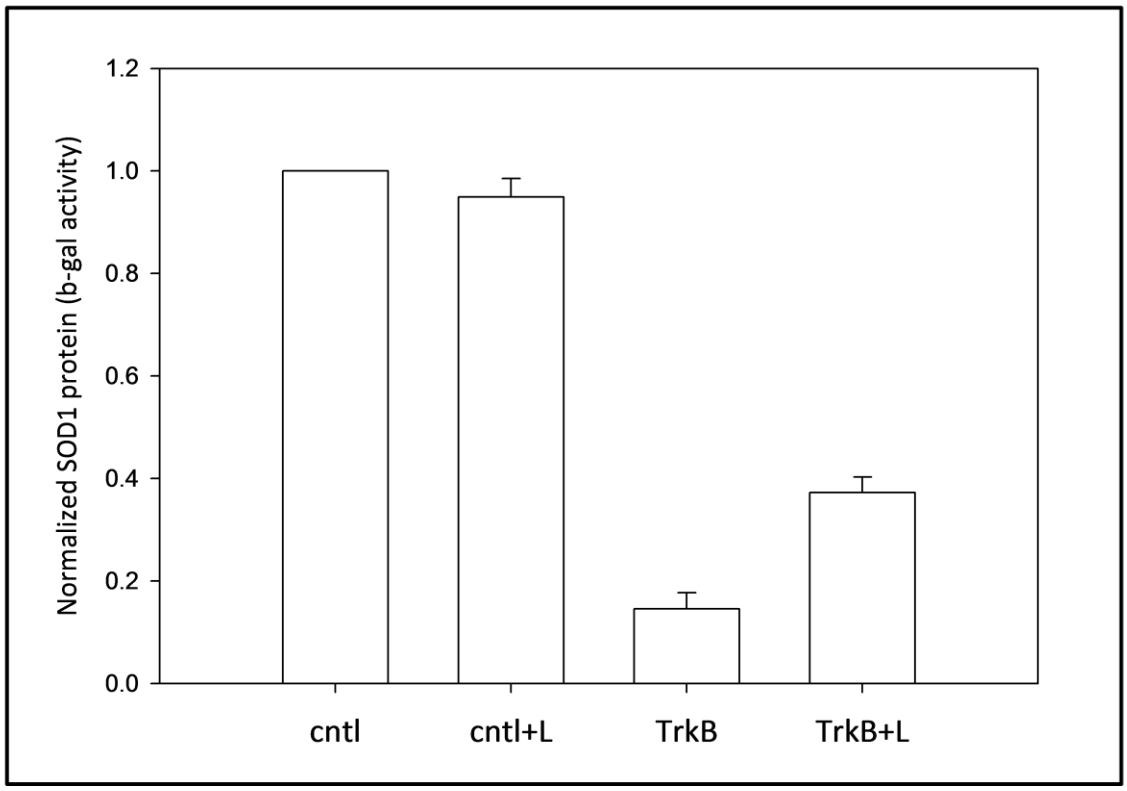

Figure S3 Treatment with Lestaurtinib (LS). LS is an inhibitor with a higher degree of specificity for Trk receptors than K252a. O/N treatment has a similar effect as K252a although more pronounced.