TrkB Isoforms Differentially Modulate Amyloid Precursor Protein Metabolism

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DEDICATIONS

I dedicate my thesis to all the intelligent people that did not have the possibility to pursue a PhD, to my mother, to Damiano, Susanna, Gabriele and to A.F.

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I thank my family for instilling in me the curiosity and persistence needed to complete my PhD.

I thank my family and all my friends for being there when I was down.

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

ABSTRACT

Alzheimer's disease (AD) is a complex neurodegenerative disease determined by the combination of environmental and genetic factors. The prevalence of the disease is rapidly increasing with the aging of the population in the Western world. The main genetic contributors to the disease are still unclear and no cure is available yet. The complete picture of the molecular pathologic mechanisms involved in the disease are also not resolved yet. The current hypothesis is that the disease is caused by accumulation of a toxic fragment of amyloid precursor protein (APP) called Aβ in the neurons. This accumulation causes synaptic dysfunction and neuronal death.

Our study aims at investigating the role of TrkB in modulating APP metabolism. TrkB is a receptor expressed on neurons and important in synaptic function, neuronal survival and long-term potentiation. This receptor has a functional relevance in AD and a genetic relevance since some studies associated single nucleotide polymorphisms on this gene to AD. We hypothesized that TrkB isoforms can affect APP metabolism in different ways and we tested this hypothesis in different human cell lines.

We found that TrkB isoforms can alter APP glycosylation and processing and that the TrkB isoforms can interact with each other and alter their effect on APP. We also showed for the first time that TrkB SHC, one of the TrkB truncated isoforms, regulates the full-length isoform differently from TrkB T.

Our work demonstrates the potential importance of TrkB in AD pathogenesis and lays ground for designing better AD therapies based on the natural TrkB ligand BDNF (Brain Derived Neurotrophic Factor).

1. CHAPTER 1: INTRODUCTION

Alzheimer's disease (AD) is a complex neurodegenerative disease determined by the combination of environmental and genetic factors. The prevalence of the disease is rapidly increasing with the aging of the population in the Western world. The environmental factors that contribute to the incidence of this disease are common to pathologies: age, lifestyle components, such as diet and exercise regime (Maslow, 2010). These factors generally affect the health state of an individual and are not surprisingly related to AD as well. Education levels are also important in determining susceptibility to this disease (Maslow, 2010) suggesting that brain function depends on more than just a healthy body state. Recommendations on healthier life styles might be useful to prevent the occurrence of the disease at earlier age. Nevertheless the main genetic contributors to the disease are still unclear and no cure is available yet. The complete picture of the molecular pathologic mechanisms involved in the disease are also not resolved yet. The current hypothesis is that the disease is caused by accumulation of a toxic fragment of amyloid precursor protein (APP) called Aβ in the neurons. This accumulation causes synaptic dysfunction and neuronal death.

Current studies are aimed at gaining better understanding of AD. The focuses of AD research are:

1. The investigation of genetic factors associated with the disease (genetic association studies, genome wide association studies, family based studies).

2. The identification of molecular interactors and modulators of amyloid precursor protein involved in synaptic function and neuronal metabolism.

3. The identification of markers for early detection of AD and of appropriate therapeutic targets.

Obviously all these aspects are interrelated and important.

Our study aims at investigating the role of TrkB in modulating APP metabolism. TrkB is a receptor expressed on neurons and important in synaptic function, neuronal survival and long-term potentiation. This receptor has a functional relevance in AD and a genetic relevance since some studies associated single nucleotide polymorphisms on this gene to AD.

The introduction that follows establishes the rationale for our study and provides an overview of the current knowledge on TrkB receptor function in relation to APP.

1.1 *NTRK2* and Alzheimer's Disease

1.1.1 Genetic Association of *NTRK2* with Alzheimer's Disease

The major risk factor for late onset AD is age while the respective contributions of environmental and genetic factors remain elusive (Maslow, 2010). Studies on twins have suggested a greater influence of genotype rather than environmental factors for the development of the AD (Pedersen et al., 2004; Gatz et al., 2006). Despite this, the majority of the risk-associated genes remain to be confirmed (Bertram and Tanzi, 2008). To date, the only genetic factor that has been consistently implicated with increased AD risk is the *APOE-ε4* allele (Lehmann et al., 2001; Bertram and Tanzi, 2008).

In an attempt to identify genes associated with AD many researchers are conducting case-control studies that compare the genotypes of AD subjects with those of age-matched controls. Comparisons might regard polymorphisms and alleles in specific genes or the whole genome (Genome-Wide Association Studies) to assess association of genes with the diseased phenotype (Bertram and Tanzi, 2008). These studies often lead to contrasting findings regarding the same gene or significant associations that are difficult to replicate (Lehmann et al., 2001; Bertram et al., 2007a). Defining association of a gene with AD is difficult because: each genetic study is based on samples of subjects that can be limited in size and stratification. Moreover, environmental factors are still an important component in AD and dissecting the interaction between genes and environment is particularly challenging.

Finally, some genetic risk factors might be detectable by genetic association studies only when present in combination with another gene. Also they can be in linkage disequilibrium with other possible genetic risk factors thus complicating the repeatability of case-control studies (Lehmann et al., 2001). Therefore meta-analysis studies that utilize data pooled from each individual case-control study prove useful in defining or negating associations in a manner more relevant to the general population (Bertram et al., 2007b; Bertram et al., 2007a).

Genetic association studies indicate that the region 9q22 on the long arm of chromosome 9 shows a "suggestive association" with AD (Blacker et al., 2003). Some studies show even a stronger association when subsets of the population studied are removed from the analysis (Kehoe et al., 1999; Pericak-Vance et al., 2000; Myers et al., 2002) and when heterogeneity in the population is accounted for (Blacker et al., 2003). A study on the National Institute of Aging Late Onset families found two polymorphisms significantly associated with AD. The first, rs1143025 is located on 9p22.3 and is in an intronic region of a zing finger transcription factor ZDHHC21. The second, rs720974, is not associated with a gene and is found on the short arm of chromosome 9 therefore outside of our locus of interest (Lee et al., 2008b).

A genome-wide study involving more subjects, found again a suggestive evidence for association of the locus 9q22 with AD (Hamshere et al., 2007). The same 9q22 locus has been significantly associated with AD (Perry et al., 2007) even when taking into account the linkage that is dependent on the gene *UBQL1*, which has been significantly and repeatedly associated with AD (Chuo et al.; Bertram et al., 2005; Kamboh et al., 2006; Slifer et al., 2006). In this genomic region, there is

evidence for at least one more significantly associated locus. A possible candidate to explain the additional association is *NTRK2* (localized at approximately 87.3 CM). *NTRK2* encodes the tyrosine kinase receptor TrkB that is the Brain Derived Neurotrophic Factor (BNDF) receptor and is important for neuronal differentiation and function. The receptor is present in humans in at least three splicing isoforms (Ferrer et al., 1999; Stoilov et al., 2002; Hartmann et al., 2004; Webster et al., 2006).

Based on the previous results that SNPs on *NTRK2* display a trend of association with late onset AD (p=0.06) (Perry et al., 2007), Chen et al. systematically analyzed SNPs within the *NTRK2* candidate gene. The purpose was to narrow down the chromosome locus that is genetically associated with AD (Chen et al., 2008). The authors used as a sample population a group of 796 individuals belonging to 203 of the NIMH-ADGI families (National Institute of Mental Health-Alzheimer's Disease Genetics Initiative). Chen et al. found a significant association of some SNPs in the *NTRK2* gene even when correction for ApoE-ε4 and gender was applied. Also these SNPs did not seem to be in linkage disequilibrium with a causative gene. It must be noted that 5 families which were not Caucasian, like the remaining samples, had to be included in the analysis to increase sample numbers, even if excluding these from the analysis did not significantly alter the results (Chen et al., 2008). This would suggest that ethnic background does not affect the frequency of the polymorphisms. No single SNP was found to be significantly associated with the disease but significance was achieved when the SNPs were grouped together with adjacent ones. When SNPs rs1624327 (SNP 4) and rs1443445 (SNP 5) were clustered they showed association (p=0.012) with AD. The same was true for SNPs rs1443445 (SNP 5) and rs3780645

(SNP 6) (p=0.038). Stronger association was found when clustering SNPs 4,5 and 6 (p=0.009) and marginal association was found for the cluster 5,6 and 7 (rs6559840, p=0.045)(Chen et al., 2008). The significant association observed could be explained by the position of the analyzed SNPs in sequences potentially important for splicing regulation. In particular, SNP 4 is located close to exonic splicing enhancers (ESE) but SNPs 5 and 6 are located several thousands of base pairs from any splicing site. SNP 5, though, is located in a CpG island, generally associated with gene transcription regulation. An alternative explanation is that these SNPs are in linkage disequilibrium with other SNPs that are found in proximal intronic regions and that might be involved in splicing regulation (Chen et al., 2008). For example SNP 6 was found to be in linkage disequilibrium with a SNP, rs2289658, located in a sequence 6 bp distal from the junction of intron 19 and exon 20 (Chen et al., 2008). Exon 20 is the first encoding the tyrosine kinase intracellular domain in the TrkB FL transcript. The SNPs analyzed are mainly found in intronic regions, suggesting that more work is necessary to identify the role of TrkB isoforms, generated by alternative splicing, in AD. In particular, the relative abundance of the transcripts could be compared in AD patients and control. Splicing regulation of *NTRK2* might be important in the pathology of AD. The work of Chen et al. suggests that the SNPs on *NTRK2* should be tested for association with AD by haplotypes since they tend to segregate together and show significant association when tested that way. A stronger association of individual SNPs in *NTRK2* with LOAD might be dependent on the sample population. In fact a non-haplotype study has found significant association between SNP rs2289656 in *NTRK2* and LOAD (Cozza et al., 2008). This SNP is located at the

intron 17 exon 16 junction. Interestingly, exon 16 encodes for the terminal part of the TrkB truncated isoform and is only present in that isoform. This study adds to the evidence suggesting the importance of alternative splicing regulation of TrkB isoforms in the pathogenesis of AD.

Other studies attempting to establish a correlation between SNPs in *NTRK2* and AD have investigated SNPs in other regions of the gene. SNP rs1212171 in the 5' UTR of the gene, was not found to be significantly associated with AD both in a Finnish study including 375 AD patients and 460 control subjects (Vepsalainen et al., 2005) and by a study on 418 AD patients and 249 controls sharing the same family environment but not genetically related, from the United Kingdom (Li et al., 2008). While the non-association result is consistent between two different large populations that do not overlap, it would be interesting to see if a non-Caucasian population would yield different results. In fact, SNPs in the 5' UTR are more likely to be associated with general stability of the *NTRK2* RNA transcript rather than regulation of alternative splicing and the latter does not seem to differ between non-Caucasians and Caucasians. Expression levels of TrkB protein influenced by RNA transcript stability might differ in different ethnic backgrounds and partially explain the ethnic-based susceptibility to AD. The Finnish study also investigated association of 2 other SNPs, in the 5' UTR region, failing to find a significant association (Vepsalainen et al., 2005; Li et al., 2008). Li et al. instead, investigated the association of SNPs in the intronic regions intercalating exons that are found in the TrkB FL isoform, the only one that has tyrosine kinase activity. They did not find association between SNPs in introns 19, 20 and 22.

The importance of SNPs in the non-translated regions of *NTRK2* might be dependent on the alteration of microRNA target sites. Also in this case it might be more apparent when microRNAs show ethnic-based differential expression levels. *NTRK2* full-length isoforms contain putative target sites for several microRNAs: 200b/c, 521, 429, 298, 888, 93624, 199a, 215, 551a 551b (MirBase Database).

Significant genetic association between intronic SNPs in the gene *UBQLN1* and LOAD has been established (Bertram et al., 2005). Therefore it is reasonable to think that SNPs in intronic sequences of other genes might be significantly involved in AD by altering the splicing and/or transcriptional regulation.

Alteration of RNA transcripts stability and levels, both microRNA dependent and independent, probably reflects in TrkB protein level alteration and possibly in different isoforms ratio. It remains to be determined how these proteins influence the main pathogenic mechanisms of AD. TrkB isoforms levels could cause developmental alterations contributing to AD risk later in life or could directly affect Amyloid Precursor Protein metabolism or other proteins, such as Tau, that have been established to be causative in AD pathogenesis. Recently, a genome wide association study suggested that proteins involved in protein cellular trafficking like TOMM40 are major genetic contributors in AD (Hong et al., 2010). This suggests that trafficking could be an important mechanism of AD pathogenesis. Our results suggest that TrkB might be involved in APP trafficking and glycosylation pointing to a possible mechanism of action of this receptor in AD.

After performing genetic association studies, it becomes necessary to show the biological relevance of the suggested associated genes with the pathology. This can

be done at two different levels. The first is through *in vitro* and *in vivo* laboratory experiments exploring the molecular mechanisms responsible for the observed association. This approach can help identifying new therapeutic targets and also be useful to test drugs. The second approach, is the observation of the relationship between the genetic variables under study and phenotypic characteristics of AD patients. In this case, the phenotypic markers can be used for diagnositic purposes (Biffi et al., 2010).

Table 1.1: *NTRK2* SNPs that have been studied for association with late-onset AD. MCC: Multiple Comparison Correction; NS: Non Significant; *: Initially used to establish the importance of studying genetic association of NTRK2 SNPs with AD. Numbers in parenthesis (For example (1)) indicate the denomination of the SNPs in the original study that is being cited. **: Haplotype analysis study. ---: SNPs analyzed as haplotype.

SNPs	***Bp change***	***Location/Intron***	***p-value***	***Isoform***	***Reference***
rs4412435	C/T	22	0.06	FL	(Perry et al., 2007)
Not specified		5	NS		
rs1212171	A/G (C/T in NCBI)	5' UTR	NS		(Vepsalainen et al., 2005)
rs1187326	A/G	5' UTR	NS		
rs1187327	A/G	5' UTR	NS		
rs1212171	G/G G/A A/A	5' UTR	NS		(Reiman et al., 2007)
rs1443445	C/C C/T T/T	19	NS	SHC	
rs1212171	G/G G/A A/A	5' UTR	NS		
rs1443445	C/C C/T T/T	19	NS	SHC	(Li et al., 2008)
rs3780645	T/T T/C C/C	20	NS	FL	
rs4578034	C/C C/T T/T	22	NS	FL	
rs1047856	T/T T/A A/A	Exon 8 (extracell domain)	NS	ALL	(Cozza et al., 2008)
rs2289656	T/T T/C C/C	Boundary exon 16 intron 17	0.0036	T	
rs893584 (1)*	A/G	5	~0.05	ALL	
rs1778931 (2)	A/G	8	NS	ALL	
rs7048278 (3)	A/G	14	NS	ALL	
rs1624327 (4)	C/T	16	0.009	T	
rs1443445 (5)	C/T	19	0.009	SHC	
rs3780645 (6)	C/T	20	0.009	FL	
rs6559840 (7)	C/T	22	NS	FL	(Chen et al., 2008)**
rs4412435 (8)*	C/T	22	0.06	FL	
rs4578034 (9)	C/T	22	NS	FL	
rs12001219 (10)	C/T	22	NS	FL	
rs11795386 (11)	C/T	22	NS	FL	
rs10512159 (12)	C/T	22	NS	FL	
rs4142909 (13)	C/T	22	NS	FL	
rs17418241 (14)	C/T	22	NS	FL	

1.1.2 TrkB/BDNF signaling regulation during development, aging and disease

Expression of *NTRK2* has been found to be localized to the central nervous system during embryonic development and in adult mice (Klein et al., 1990b). In the mammalian brain TrkB and TrkB T are developmentally regulated: strong expression of TrkB is observed during rat embryonic development and TrkB T is elevated in the post-natal period when the majority of the inter neuronal connections are formed (Fryer et al., 1996). TrkB FL and TrkB T expression decreases with age in human hippocampus while TrkB T remains at constant levels through life in the temporal cortex. The TrkB ligand, BDNF, shows the opposite trend: remains constant in the hippocampus but decreases in the temporal cortex (Webster et al., 2006). TrkB shows higher expression levels in the hippocampus in infants rather than in adults while BDNF levels do not significantly differ (Tang et al.).

All trans-retinoic acid (ATRA) regulates TrkB expression during embryonic development and is also involved in the maintenance of adult brain functions (Lane and Bailey, 2005). Retinoic acid receptors are expressed in discrete parts of the brain and are important for memory formation in the hippocampus and adult neurogenesis in the dentate gyrus (Katsuki et al., 2009). ATRA can activate transcription not only of TrkB but also of PS1, PS2 and APP (Lane and Bailey, 2005). ATRA also increases expression of the ADAM10 gene (Prinzen et al., 2005). Interestingly, up-regulation of the presenilins and APP is generally associated with increased degeneration while ADAM10 is protective. Since ATRA treatment results in beneficial effects in AD

context, it has been tested as a therapy in AD models. The pathways mediated by ATRA activation must be over-all protective.

Activation of the retinoic acid receptors causes increase in BDNF/TrkB mediated signaling that decreases inflammation in a mouse model of Parkinson disease when the dopaminergic neurons are subjected to injury or inflammatory challenge (Katsuki et al., 2009). Treatment of Alzheimer's disease mice with retinoic acid decreased Aβ load and improved cognitive function. The effect seemed to be related to a decreased activation of glial cells and decreased inflammation (Ding et al., 2008). ATRA has also been shown to decrease iNOS synthesis and production of pro-inflammatory cytokines in activated microglia (Katsuki et al., 2009). Therefore, in AD, up-regulation of TrkB expression via ATRA treatment or RARs agonists (like Am80) could improve neuro-degeneration by decreasing the inflammatory response. It remains to be determined if this effect is at least partially mediated by TrkB signaling. The effect of RAR agonists seems to be specific for RAR-α and β receptors and not general RXRs, moreover, expression of RAR-α and β is prevalent in neurons while only few microglial cells show RAR-β expression, astroglial cells do not show expression of either RAR α or β (Katsuki et al., 2009). It is known that TrkB isoforms are differentially expressed on neurons and glial cells. In particular TrkB FL is mainly expressed in neurons while TrkB T is mainly expressed on glial cells. If ATRA increases the transcription of the *NTRK2* gene, it will do so not-specifically in all these cell types and the splicing regulation that yields different TrkB isoforms protein levels will allow up-regulation of TrkB FL in neurons but not in glial cells. It is possible that ATRA mediated up-regulation of TrkB FL in neurons

enhances their survival and that other signaling pathways regulate the anti-inflammatory activity of ATRA. If pro-inflammatory activity of glial cells is partially mediated by TrkB T expression and signaling, when TrkB FL is up-regulated on neurons, BDNF will bind on neuronal cells and not on glial cells. This could result in inhibition of glial cell pro-inflammatory pathways.

The developmental regulation of ATRA and TrkB and their importance in neuronal functions suggests that the mechanisms activated by their signaling might be important in AD and possible therapies.

Post-mortem analysis of TrkB full length and BDNF expression in AD versus age matched controls revealed that both TrkB FL and BDNF levels are decreased in AD tissues while TrkB T is increased in AD versus non AD controls. Interestingly, neurons with higher numbers of neurofibrillary tangles showed lower expression of TrkB (Ferrer et al., 1999). On the contrary, TrkB T expression was found to be higher in AD brains than in controls, especially in the reactive glial cells (Allen et al., 1999; Ferrer et al., 1999). This finding is in agreement with the possible previously described role of TrkB signaling in inflammation. More recently, another study has shown that TrkB expression levels decrease as cognitive impairment increases in AD patients brains (Ginsberg et al., 2006). This study showed that, in the basal nuclei, TrkB FL expression levels were higher in non-demented and mildly cognitive impaired patients but much reduced in severely cognitive impaired patients. Another study showed that Aβ generation in Tg2576 mice impairs BDNF-TrkB mediated axonal transport in neurons (Poon et al., 2009). Moreover Aβ42 accumulation in neurons that are over-expressing APP^{Swe} has been shown to decrease the BDNF

mediated degradation of TrkB receptor (Almeida et al., 2006). These data suggest that Aβ can negatively affect TrkB FL signaling. On the other hand, interruption of NGF/BDNF signaling in primary neurons induces an increase in Aβ production and increased PS1 levels (Matrone et al., 2008).

In contrast with these findings, one report does not find a significant difference in TrkB FL mRNAs expression levels among AD or control brains. BDNF and TrkB mRNA levels were measured in laser-captured neurons from AD brains or controls. There was also no significant reduction in TrkB immuno-reactivity in neurons that showed Aβ immuno-reactivity (Fujimura et al., 2009). The fact that this report is in disagreement with previous studies might depend on the sampling of the neurons by laser capture and also by the lack of discrimination between TrkB FL and TrkB T mRNA. Total TrkB levels might remain stable but isoform levels might explain neurologic dysfunctions.

All these results suggest that TrkB FL signaling is generally down-regulated in AD context.

Consistent with these observations, increased TrkB FL/BDNF signaling and pharmacological treatments or life styles, like an healthy diet and exercise, that increase TrkB expression and BDNF production seem to be beneficial in AD context (Peng et al., 2007; Nichol et al., 2009) (Cho et al.; Ding et al., 2008; Komulainen et al., 2008; Bousquet et al., 2009; Katsuki et al., 2009; Nichol et al., 2009). Expression of BDNF is increased by omega 3 fatty acids in the diet and the increased levels of BDNF cause an increase in TrkB expression and mRNA levels (Bousquet et al.,

2009). TrkB and BDNF also protect cells from apoptosis by decreasing caspase 3 activation in cells that receive cytotoxic insults (Nguyen et al., 2009).

The levels of BDNF in AD patients have also been measured in different studies. Some groups report an increase in BDNF levels in AD or mildly cognitive impaired patients compared to controls (Angelucci et al.). Others report a decrease in BDNF levels caused by Aβ aggregates (Peng et al., 2009). Whatever the cause of these contrasting reports, a role for TrkB isoforms in the pathogenesis could be supported in either case. If increased BDNF levels are found concomitantly with decreased TrkB FL and increased TrkB T levels, then the signaling activated will be mainly mediated by TrkB truncated isoforms. These isoforms are located on glial cells, which can mediate inflammation. If BDNF decreased levels are associated with normal TrkB FL/T levels the BDNF mediated signaling necessary for neuronal function could be insufficient due to the lower ligand levels.

In conclusion, TrkB plays an important role during development and in the adult brain and alterations in TrkB/BDNF signaling seem to be involved in AD pathology. Therefore elucidation of the mechanisms underlying the role of TrkB in AD will be important to design therapeutic strategies.

1.2 *NTRK2* gene and TrkB protein

1.2.1 TrkB isoforms structure and functions

TrkB (tyrosine receptor kinase B) was first identified in 1989 for homology with the already known TrkA, the receptor of Nerve Growth Factor (NGF) (Klein et al., 1989). Expression levels of this receptor where found to be high in the central nervous system and many primary transcripts were isolated suggesting the presence of different isoforms. Soon after, another isoform, TrkB T, lacking the tyrosine kinase activity, was isolated (Klein et al., 1990a). The third isoform, TrkB SHC was identified much later (Stoilov et al., 2002). More isoforms have been identified recently (Luberg et al., 2010) but our work will focus on the three aforementioned isoforms.

NTRK2, the gene encoding TrkB, has a complex genomic organization and it is expressed in 3 different splicing isoforms in rat and mouse (Kumanogoh et al., 2008) and at least five isoforms in humans (Stoilov et al., 2002; Luberg et al., 2010). The numerous TrkB isoforms expressed in the human brain differ for short sequences but can be grouped, based on their intra-cellular domains, in two full-length isoforms (TrkB FL), two intermediate isoforms (TrkB SHC) and one truncated isoform (TrkB T). Each of these can bind BDNF in the conserved extra-cellular domain (encoded by exons 5-15) but differ in the cytoplasmic domain (Figure 1.1). BDNF binds to the IgG like domains of the TrkB receptors on the extra-cellular portion. Isoforms that lack the leucine-rich region in the extra-cellular portion have also been recently identified

(Luberg et al., 2010). These N-truncated isoforms are probably not transported to the cell surface lacking the signal sequence but include all the intracellular functional domains of their non N-truncated counterparts (Luberg et al., 2010). We are interested in investigating the role of the intracellular functional domains. The longest TrkB isoforms, TrkB FL (822-838 amino acids in length) contain a tyrosine kinase, a SHC binding, and a PLC-γ binding domain in the cytoplasmic portion. The intermediate length isoforms, TrkB SHC (537-553 amino acids in length) contain only a SHC binding domain (located on exon 19) while the truncated TrkB isoform, TrkB T (477 amino acids in length) does not contain any known functional domains in the cytoplasmic domain that is encoded by exon 16 (Figure 1.1) (Klein et al., 1990a; Middlemas et al., 1991; Stoilov et al., 2002)

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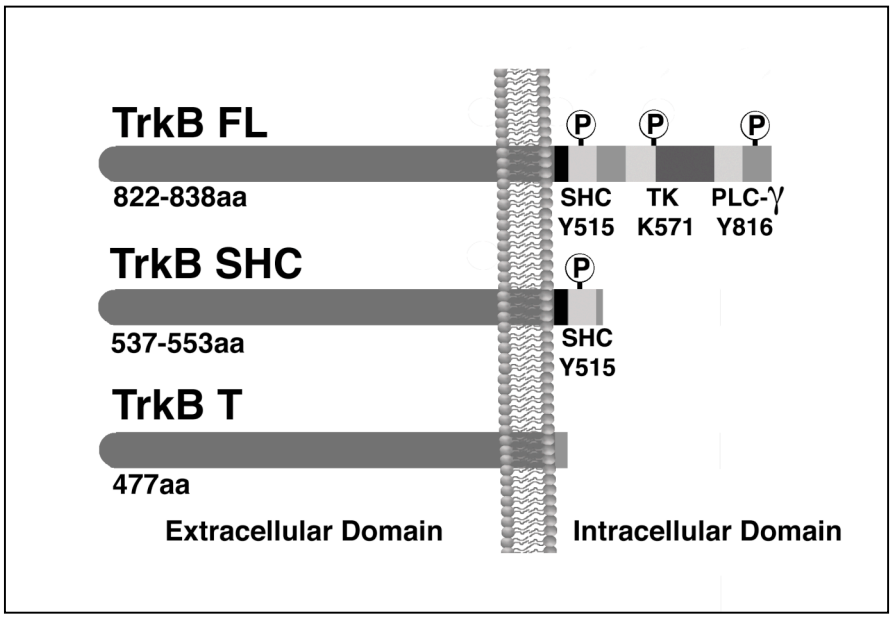


Figure 1.1: Schematic representation of TrkB isoforms highlighting the differences in the intracellular domains.

In the central nervous system, TrkB regulates synaptic plasticity, neuronal differentiation, neuronal proliferation and development (Dechant and Barde, 2002; Nagappan and Lu, 2005; Reichardt, 2006; Minichiello, 2009). The importance of TrkB in neuronal development is underlined by the fact that mice with a targeted disruption of the *NTRK2* locus develop until birth but die at P1 (Klein et al., 1993). They have dramatic deficits in the sensory and motor systems which affect their feeding behavior (Klein et al., 1993). Mice lacking BDNF display major movement and balance defects caused by major neuronal loss in the vestibular ganglion, many do not survive past the second post-natal week (Ernfors et al., 1994).

During development TrkB isoform expression has different roles: TrkB T seems to promote synapse destruction while phosphorylated TrkB FL promotes synaptic formation. Correct balancing of these isoforms allows the correct innervation and synaptic connections to be formed during brain development (Sherrard et al., 2009).

TrkB is important in cancer as an oncogene. Up-regulation of TrkB FL and its ligand, BDNF, is related to increased cell proliferation. TrkB is a marker of high malignancy and chemotherapy resistance in neuroblastomas while TrkA is associated with more positive outcomes (Thiele et al., 2009). It is probably for this reason that activated TrkB FL levels at the cell surface are tightly regulated. After binding to its ligand TrkB FL is rapidly endocytosed and degraded through the proteosome (Sommerfeld et al., 2000). The rapid decrease in TrkB FL surface levels is mediated by a juxta-membrane domain in the protein and is not observed in TrkA after binding of NGF (Sommerfeld et al., 2000). Instead, the truncated, non-catalytically active, TrkB T, it is not subjected to the same rapid degradation.

TrkB is subjected to different regulation and has different functions that do not over lap with TrkA despite the very similar structure of the two receptors.

1.2.2 TrkB full-length functions and signaling pathways

TrkB FL is the only isoform with a tyrosine kinase domain. Upon binding to BDNF it dimerizes and auto-phosphorylates. There are several phosphorylation sites in the intracellular domain some of which act as a docking site for adaptor proteins and effector proteins (Reichardt, 2006). The activated receptor can trigger intracellular signaling through several pathways (Figure 1.2).

Binding of SHC on tyrosine 515 mediates activation of PI3K/AKT survival pathways and activates transcription through ERK down-stream signaling targets (Figure 1.2) (Minichiello, 2009). Since SHC contains both PTB (phospho tyrosine binding) and SH domains it is also possible that binding of SHC occurs on the non-phosphorylated docking site through the SH domains. The SHC binding site is important for the activation of MEK/IP3K that signal downstream and activate ERK1/ERK2 pathways, which mediate survival and axonal growth (Atwal et al., 2000). This pathway is mediated by Ras. The PLC-γ binding site is not required for these functions (Atwal et al., 2000). Signaling downstream of SHC binding to TrkB FL mediates the formation of conditional fear in mice (Musumeci et al., 2009).

A PLC-γ binding site is located on tyrosine 816. Phosphorylation of the receptor on that residue is necessary since PLC-γ employs only PTB domains for binding. PLC-γ signaling is mediated by IP3 (Inositol-tri-Phosphate) and DAG (diacyl glycerol). IP3

allows release of intracellular Ca2+ and activates calmodulin and calmodulin dependent kinases, which can then activate transcription via CREB phosphorylation. DAG activates PKC (phospho kinase C) that can phosphorylate many substrates (Figure 1.2) (Minichiello, 2009).

Trks can also undergo a neurotrophin independent activation. In this case the trans-activation can be dependent on a G-protein coupled receptor (Rajagopal et al., 2004). Once the G-protein coupled receptor is activated by its ligand (for example the neuropeptide pituitary adenylate cyclase-activating polypeptide (PACAP)(Lee et al., 2002), adenosine or synthetic adenosine agonists (Lee and Chao, 2001). The activation of TrkA through this alternative route triggers phosphorylation of the same residues that are phosphorylated by the neurotrophin binding but happens on a longer timescale (over an hour versus seconds of neurotrophin stimulation). The active receptors, also, are found associated to the Golgi membrane and are immature (non completely glycosylated). Fully glycosylated receptors do not get activated by GPCR agonists. Disruption of the Golgi via Brefeldin A treatment abolished the activation of this pool of activated immature receptors. These experiments were performed both in PC12 cells and primary neurons. Transactivation of the TrkA receptor through this pathway causes activation of PI3K but not of MAPK pathway (Rajagopal et al., 2004). Activation of Trks independent of neurotrophin binding has been observed even when the receptors are ectopically over-expressed. Over-expression of the TrkB receptor in HEK293 cells can cause auto-activation of the receptor in the Golgi (Schecterson et al.). This auto-activation causes Golgi fragmentation impeding complete glycosylation of the receptor and of other proteins (Schecterson et al.).

These observations are very similar to the ones made with GPCR-mediated activation of Trks and were dependent on the tyrosine kinase activity of the receptor (Schecterson et al., 2010). Interestingly ectopic expression of TrkB FL in MDCK cells showed that, while TrkB T was uniformly distributed in the cells, TrkB FL was not present on the apical side of the cells (Kryl et al., 1999). This suggests that localization of the TrkB isoforms is different and might be dependent on TrkB FL auto-activation and residency in the Golgi.

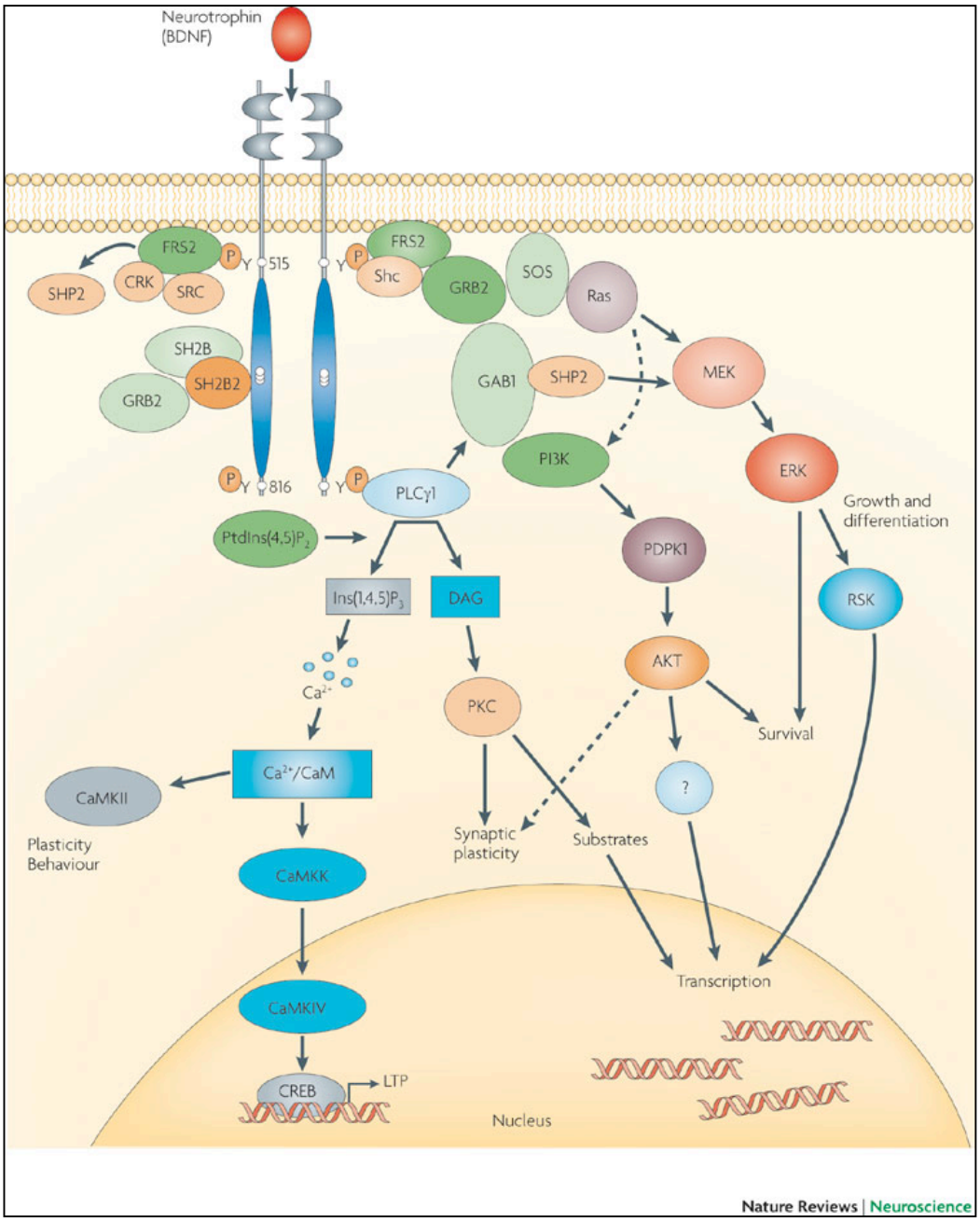


Figure 1.2: **TrkB FL signaling pathways elicited by BDNF binding of the receptor**. The figure is from Minichiello 2009. Note that BDNF mediated activation of TrkB FL occurs in 15 seconds and involves surface receptors. BDNF independent activation of TrkB FL occurs in hours and engages receptors localized inside cellular

compartments such as the Golgi (see text for references and details) (Minichiello, 2009). Figure used with permission granted by Nature Publishing Group.

1.2.3 TrkB truncated functions and signaling pathways

In humans there is only one isoform of TrkB truncated, TrkB T, (homologous of TrkB T1 in mouse and rat), the other non-catalytically active TrkB isoform is TrkB SHC that contains a SHC binding site (Stoilov et al., 2002).

In mouse and rat there are two TrkB truncated isoforms, TrkB T1 and T2, both expressed in neurons. TrkB T1 and T2 have 12 amino acids of their short intracellular domains in common with TrkB FL while 11 and 9 amino acids respectively are isoform specific (Baxter et al., 1997). The two short non-conserved domains are necessary for BDNF induced intracellular signaling of the two truncated isoforms in cell lines (Baxter et al., 1997). This intracellular signaling is probably dependent on kinases since it can be prevented by kinase inhibitors (Baxter et al., 1997). TrkB T1 displays a largely non over-lapping patter of expression with TrkB FL while TrkB T2 is expressed in the same central nervous system regions and motor neurons as TrkB FL suggesting a regulatory role of this isoform on TrkB FL. TrkB T1 is mainly expressed on the glial cells (Armanini et al., 1995). Interestingly, both TrkB T1 and T2 were able to promote dendritic filopodia growth in hippocampal neurons (Hartmann et al., 2004).

Co-culture experiments of SH-SY5Y neuroblastoma cells with 3T3 NIH fibroblasts over-expressing TrkB T1 suggested that TrkB T1 could act as a BDNF scavenger and thus reduce neurite sprouting on the TrkB FL expressing neurons (Fryer et al., 1997). This possible mechanism of action suggests that TrkB T

expression on non-neuronal cells could obstacle neuronal regeneration in neurodegenerative diseases.

TrkB T co-expressed in PC12 cells expressing TrkB FL, was found to inhibit cell survival probably by inhibiting TrkB FL signaling via dimerization (Haapasalo et al., 2001). TrkB T was also shown to mediate BDNF independent neurite outgrow in N2a neuronal cells and 3T3 fibroblasts (Haapasalo et al., 1999). The authors find that deletion of the extra-cellular domain eliminates this effect while deletion of the intracellular domain of the TrkB T receptor does not. It has to be noted that the deletion mutant used eliminates the IgG like domains that have been shown to mediate binding of the neurotrophin (Arevalo et al., 2001). Moreover co-expression of TrkB FL antagonized the TrkB T process sprouting via a tyrosine kinase dependent mechanism (Haapasalo et al., 1999). Another study also found the same TrkB T mediated, BDNF independent, process sprouting effect in hippocampal neurons (Hartmann et al., 2004). These two studies both demonstrate that some TrkB T effects are independent of BDNF signaling. Hartman et al. also found that p75 was necessary for TrkB T mediated process growth. In p75-/- neurons or neurons co-expressing p75 without its intracellular domain the TrkB T induced filopodia outgrowth was inhibited (Hartmann et al., 2004).

TrkB T can activate intracellular Ca^{2+} currents in glial cells independently from TrkB FL but upon BDNF stimulation. TrkB T was found to be expressed mainly on glial cells that were able to generate Ca^{2+} currents upon BDNF stimulation (Rose et al., 2003). Interestingly, these responses were dependent on PLC-γ activation but not on tyrosine kinase activity. Glial cells from mutant mice TrkB FL-/- were still

able to generate the Ca^{2+} currents demonstrating that TrkB T functions independently of TrkB FL (Figure 1.3). The effect seemed to be dependent on activation of a G-protein upstream of PLC-γ. Treatment with a G-protein agonist was able to sustain the Ca^{2+} evoked currents after the first application of BDNF or ATP but this effect could not be blocked by conventional antagonists of G-proteins. The authors conclude that the G-protein mediating the signal is insensitive to the blockers used (Figure 1.3). Confirming these findings that TrkB is able to elicit signaling pathways, further studies demonstrated that TrkB T can promote proliferation of neuronal progenitors in neurospheres (Tervonen et al., 2006) and direct the differentiation of neuronal stem cells to glial differentiation through PLC-γ and protein G mediated signaling (Cheng et al., 2007). Moreover the TrkB T isoform has been shown to regulate TrkB FL kinase activity *in vivo*. Mice lacking TrkB T but not TrkB FL display increased aggression and weight gain compared to control littermates, even if learning and memory are not affected. The dendritic arborization of some neuronal populations is affected as well (Carim-Todd et al., 2009).

TrkB T has also been demonstrated to bind a 61KDa protein that was named TrkB Truncated Interacting Protein (TTIP) the physiologic function of this protein is still unknown (Kryl and Barker, 2000).

In summary, several physiological roles have been proposed for TrkB T: molecular scavenger of BDNF limiting its availability to TrkB FL (Fryer et al., 1997), dominant negative dimer partner of TrkB FL (Haapasalo et al., 2001), independent activator of intracellular signaling pathways that regulate TrkB FL signaling for example to control neurite sprouting and neuronal proliferation (Haapasalo et al.,

1999; Haapasalo et al., 2002; Rose et al., 2003; Hartmann et al., 2004; Tervonen et al., 2006; Cheng et al., 2007). All these data suggests that TrkB T homodimers have an independent signaling function or maybe that BDNF mediates binding of TrkB T with p75 as observed (Hartmann et al., 2004).

1.2.4 TrkB SHC functions and signaling pathways

The TrkB SHC truncated isoform of TrkB has been recently identified in human cells (Stoilov et al., 2002), there are no homologous counterparts in rodents. Surprisingly, this isoform has not been widely investigated. There is only one study which investigated the cellular distribution of TrkB SHC, TrkB FL and T in N2a neuroblastoma cells (Haapasalo et al., 2002). This study shows that while TrkB FL is distributed mainly on proximal processes, soma, granular structures and to a lower extent on the membrane, TrkB T is distributed on the membrane and on the distal processes (Haapasalo et al., 2002). TrkB SHC co-localizes with TrkB FL in the soma and granular structures but is also found in distal processes and on the membrane, displaying a distribution that is intermediate between the other two isoforms (Haapasalo et al., 2002). The TrkB T isoform was able to reduce TrkB FL surface levels while TrkB SHC and a TrkB FL kinase inactive mutant were able to increase those levels (Haapasalo et al., 2002). This suggests that TrkB SHC and T have different regulatory roles on TrkB FL, which are probably mediated by the SHC binding site (Figure 1.3).

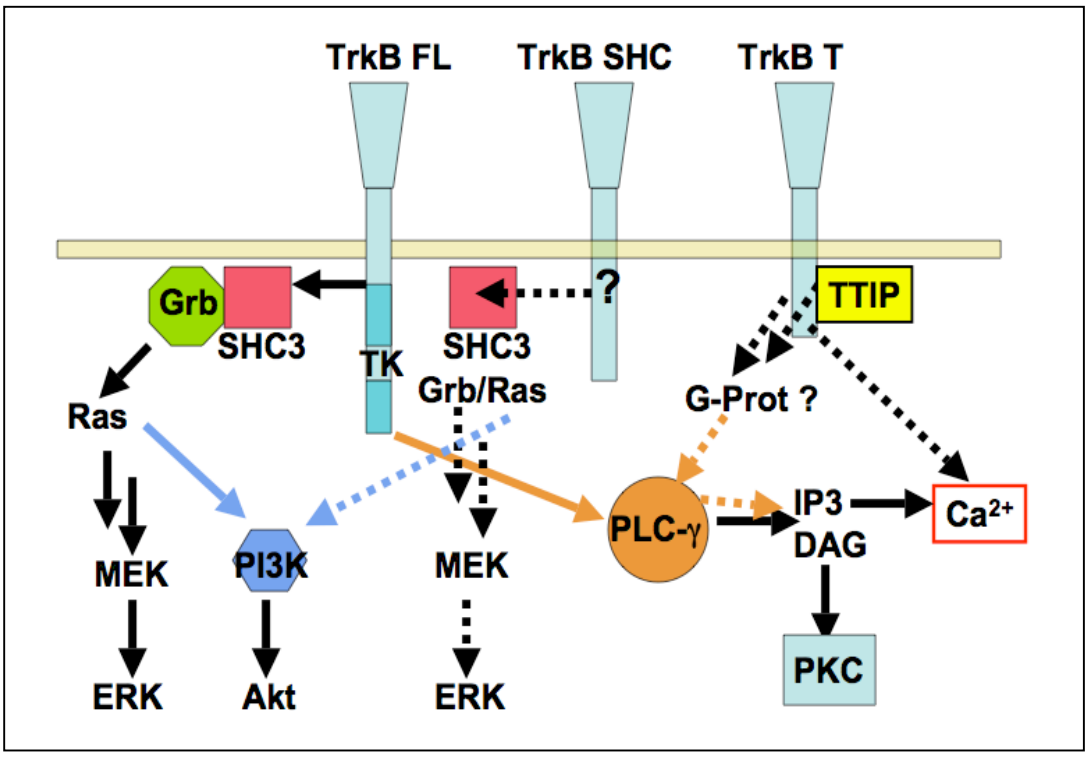


Figure 1.3: Established and hypothesized signaling pathways activated by the three TrkB isoforms. Note that the TrkB FL activates many signaling pathways that have been confirmed by different studies. TrkB SHC contains a SHC binding site that could mediate the activation of MEK/ERK and Akt pathways. These relationships have not been established or investigated. TrkB T has been shown to activate intracellular Ca^{2+} current even if the exact mechanism is not clear; a G-Protein seems to be involved in this pathway. TrkB binds to Truncated TrkB Interacting Protein but the role of this interaction has not been elucidated. Solid lines indicate established pathways; dashed lines indicate hypothesized or not fully understood pathways.

1.3 TrkB and APP: established and possible interactions

1.3.1 Amyloid hypothesis and Alzheimer's Disease molecular pathogenesis

The Amyloid Precursor Protein (APP) is a glycosylated type I trans-membrane protein ubiquitously expressed in three isoforms: APP_{770}, APP_{751} and the neuronal specific APP_{695}. The primary function of this protein has not been identified to date but APP is implicated in many cellular processes: cell adhesion, differentiation, proliferation and intracellular signaling (De Strooper and Annaert, 2000). The proteolytic cleavage of APP is considered the main pathological event in AD (Figure 1.4). APP is mainly cleaved by α-secretases, β-secretases and γ-secretases (multiprotein complexes) (Figure 1.4). The α-secretases activity is attributed to Alpha-disintegrin and metalloproteinases family members (ADAM9, 10 μand 17). The β-secretase, BACE1 (Beta APP Cleaving Enzyme1) is responsible for production of the neurotoxic Aβ fragment. Both α and β secretases produce soluble N-terminal fragments (sAPPα and sAPPβ) that are released in the extra-cellular space and membrane bound C-terminal fragments (C83 and C99) that are subsequently cut by γ-secretases (Figure 1.4). The γ-secretase activity, PSEN1 and PSEN2 (Presenilin 1 and 2) releases the p3 fragment from the α-CT stub and the Aβ peptide from the β-CT. At the same time intracellular fragments are released that have nuclear signaling functions (De Strooper and Annaert, 2000). During AD pathogenesis APP cleavage is shifted towards the production of the Aβ42 fragment that is lipophilic and aggregates forming oligomeric species that are highly toxic (Ma and Nussinov, 2002;

Mamikonyan et al., 2007). Aggregation of the Aβ peptide starts inside neurons, glial and endothelial cells and eventually spreads to the extra-cellular environment. Here, Aβ accumulates in the characteristics amyloid plaques that are found in AD brains (Turner et al., 2004). In EOAD Aβ peptide formation is increased by mutations in APP (for example the Swedish mutation: K595N and M596L) or in the presenilins (Turner et al., 2004).

Stimulation of α-secretase cleavage of APP is considered a therapeutic approach because it prevents the formation of the Aβ peptide (Fahrenholz and Postina, 2006).

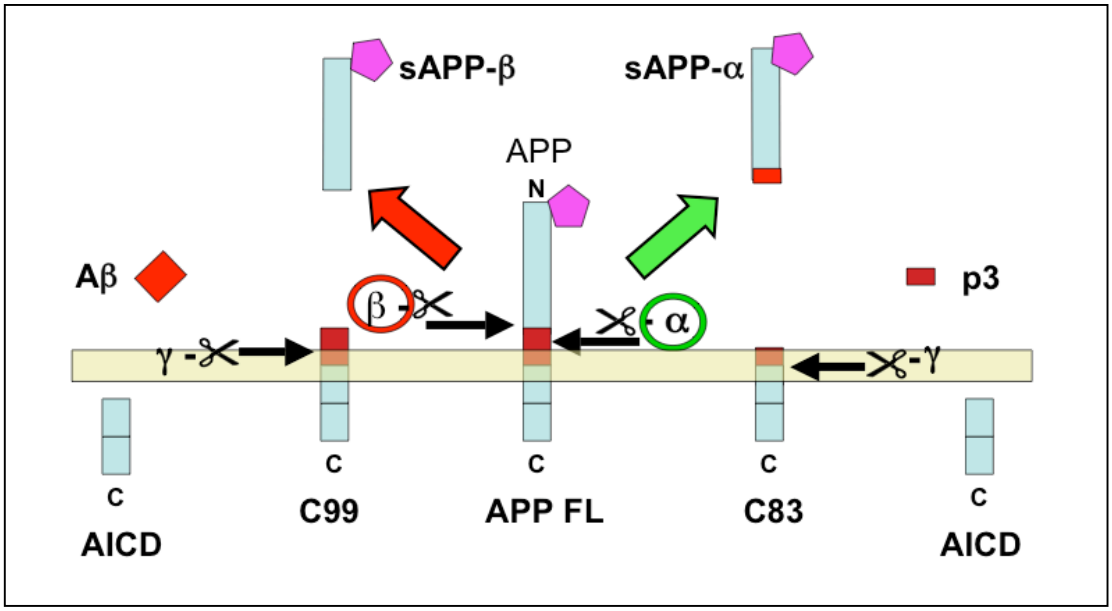


Figure 1.4: **Schematic representation of the main proteolytic processing of Amyloid Precursor Protein.**

1.3.2 TrkB as modulator of APP transcription

Treatment of SH-SY5Y neuroblastoma cells with retinoic acid (RA) promotes their differentiation to neuronal phenotype with process sprouting (Ruiz-Leon and Pascual, 2003). RA treatment induced up-regulation of TrkB and also increased the levels of APP, interestingly, BDNF treatment of SH-SY5Y cells further increased APP levels (Ruiz-Leon and Pascual, 2003). These transcriptional effects occurred in 48 hours suggesting an indirect effect of BDNF/TrkB signaling on APP promoter activity but were blocked by K252a the tyrosine kinase inhibitor suggesting the importance of the tyrosine kinase activity of the receptor in mediating transcriptional activation (Ruiz-Leon and Pascual, 2003). The use of site directed mutants of TrkB FL indicated that the APP transcriptional effect is due to activation of the Ras/MAPK and PI3K/Akt pathways downstream of the SHC binding and tyrosine kinase activity of the receptor respectively (Figure 1.5) (Ruiz-Leon and Pascual, 2004). Importantly these effects were independent of AP-1 transcription factor activation, which has binding sites on the APP promoter (Ruiz-Leon and Pascual, 2001).

1.3.4 TrkB as modulator of APP processing, ADAM10 and BACE

Consistent with these findings, RA induced TrkB up-regulation and BDNF stimulation of the TrkB receptor in SH-SY5Y cells. RA and BDNF treatments of these cells increase APP production and shift the APP cleavage pathway towards α-secretase, as demonstrated by quantification of sAPP-α (Figure 1.5) (Holback et al.,

2005). In addition, impairment of O-glycosylation of APLP1, a protein that is homologous of APP, was observed in cells treated with BDNF (Holback et al., 2005). The increase in sAPP-α levels seems to be dependent on both increased APP transcription and increased α-secretase processing of APP, in fact C99 levels were decreased (Holback et al., 2005). It could be hypothesized that, TrkB activation of PLC-γ producing DAG, which is the endogenous agonist of PKC, increases the amount of active ADAM that cleaves APP (Figure 1.5). This mechanism of action has not been demonstrated yet and the previously mentioned studies did not elucidate a mechanism of action by measuring levels of ADAM10 or BACE.

The regulation of APP main processing enzymes, BACE and ADAM10, is a possible way in which TrkB could affect APP processing. There are several possible regulation mechanisms of the secretases: transcriptional, glycosylation and trafficking. TrkB has been shown to affect other proteins at all these different levels. TrkB affects transcription (Ruiz-Leon and Pascual, 2001; Groth and Mermelstein, 2003), glycosylation of proteins (Schecterson et al., 2010) and trafficking (Cassens et al.; Colley et al., 2009) and the secretases could be included in the list of proteins affected. Below we briefly review the secretases metabolic pathways that could be affected by TrkB.

Many transcription factors are downstream of BDNF/TrkB FL signaling. For example CREB and AP-1 both bind the BACE promoter (Sambamurti et al., 2004) and are both activated by TrkB. NFAT4c (also known as NFAT3), the neuronal-specific form of the nuclear factor of activated T-cells, has also been shown to be activated downstream of PLC-γ activation through IP3 and PKC mediated signaling

(Groth and Mermelstein, 2003). Interestingly, while the activation of NFAT1 through ionomycin has been related to increased BACE1 levels, NFAT4 (also known as NFAT3c) was not affected by this treatment (Cho et al., 2008). This suggests that TrkB FL signaling could be specifically activating pathways that do not lead to increased β-secretase cleavage of APP. The ADAM10 promoter is responsive to retinoids but it does not contain any binding site for transcription factors down-stream of TrkB (Prinzen et al., 2005).

ADAM10 and BACE are both N-glycosylated and the proteolytic activity has been related to the glycosylation state (Capell et al., 2000; Walter et al., 2001; Escrevente et al., 2008; Vanoni et al., 2008).

BACE1 fully glycosylated migrates at 66KDa while the holo protein migrates at 46KDa. BACE1 that resides in the endoplasmic reticulum is 58KDa showing only partial glycosylation (Huse et al., 2000). BACE is a highly stable protein with a half life of 16 hours and matures to the fully glycosylated form in only thirty minutes (Huse et al., 2000). It localizes to the endosomes and cell surface, not to lysosomes. The signal sequence needed to localize BACE at the endosomes is DDXXLL, when this is disrupted BACE1 localizes mainly to the cell surface. BACE can be active even if not glycosylated (Vanoni et al., 2008). Finally, BACE is synthesized as a pro-protein displaying a peptide of 24 amino acids that is necessary for its transport outside the ER and gets cleaved in the Golgi but does not seem to significantly inhibit its enzymatic activity (Benjannet et al., 2001).

In conclusion, BACE1 mediated APP cleavage could be modulated mainly by TrkB trafficking effects since neither glycosylation/cleavage are fundamental in

determining BACE1 activity. Therefore, localization of BACE1 is probably going to be the main determinant of APP processing modulation by TrkB. In fact, BACE1 is only active in the endosomes. Also, since its half-life is long, the only limiting factor in generating APP fragments is the co-localization of BACE1 and APP. Supporting the importance of BACE1 localization, siRNA mediated knock-down of SHC, that binds to TrkB, and Fe65 was able to reduce CTFs and Aβ production (Xie et al., 2007). While SHC knock-down also decreased BACE1 levels, Fe65 knock-down did not affect BACE levels (Xie et al., 2007). The authors suggest that the reduced APP processing observed is mainly due to altered APP trafficking within the cells.

ADAM10, ADAM17 and ADAM9 have been implicated in the α-secretase cleavage of APP (Allinson et al., 2003). They are integral glycoproteins membrane type I. In addition to transcriptional and translational regulation, there are three main ways of ADAMs activity regulation: first the conversion of the zymogens to the active form (maturation process). This process requires proprotein-convertases that are Ca^{2+} dependent endoproteinases (Endres et al., 2003). Second, the phosphorylation and the activation of signaling pathways mediated by adaptors at the intracellular domain. Third the intra-cellular trafficking.

ADAMs intracellular domain can be phosphorylated or have binding sites for adaptor proteins like SHC3 (Allinson et al., 2003). ADAM9 and ADAM10 display constitutive and regulated (PKC induced) α-secretase activity while ADAM17 only displays regulated activity (Endres et al., 2003). α-secretase cleavage can be increased by DAG activation of PKC by binding to its C1b domain (Endres et al., 2003). ADAM17 (TACE: Tumor Necrosis Factor α Converting Enzyme): is

converted to its mature from by furin and PC7 both substrates of PKC (Endres et al., 2003). ADAM10 is regarded as the main α-secretase activity involved in processing of APP in AD. ADAM10 activity depends on the pro-domain but not on the disintegrin domain (Fahrenholz et al., 2000). Trafficking and sub-cellular localization of ADAMs are important for ADAMs activity and therefore APP processing (Borroto et al., 2003; Marcello et al., 2007). In conclusion, TrkB could modulate ADAM activity by a PLC-γ mediated pathway leading to PKC activation and by trafficking effects.

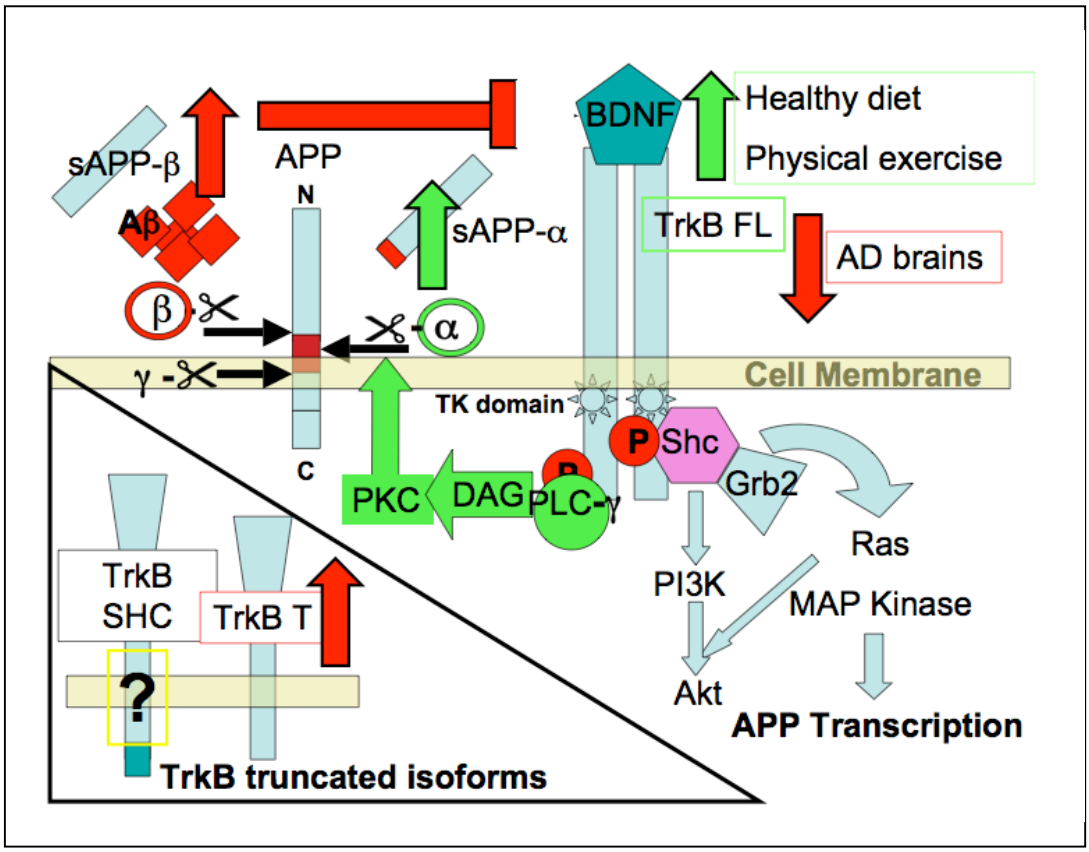


Figure 1.5: Some of the interactions between TrkB isoforms and APP. Aβ is known to down-regulate TrkB/BDNF signaling and TrkB/BDNF signaling stimulation is generally associated with beneficial outcomes in Alzheimer's disease. The role of the truncated isoforms is not well established. TrkB T seems to promote degeneration while the role of TrkB SHC is unknown. Red arrows/boxes indicate factors that promote degeneration while green labeled objects are associated with beneficial factors.

1.3.4 TrkB as modulator of APP trafficking/maturation

1.3.4.1 Trafficking of TrkB isoforms to the cell surface and degradation

Trafficking of TrkB receptor at the cell surface is regulated by many mechanisms and probably regulates trafficking of neighboring proteins as well. The TrkB isoforms display different surface levels and their trafficking is probably differently regulated.

Expression of TrkB receptor at neurites with rapid kinetics is regulated by Ca^{2+} influx and membrane potential. Phosphorylation of the receptor at Ser 478 by Cdk5 and the juxtamembrane domain of TrkB are essential for activity dependent membrane localization of TrkB (Zhao et al., 2009).

TrkB T is present on the surface of the neurons when the neurons are unstimulated while TrkB FL is greatly up-regulated when cells are stimulated by an electric current. Blockers of membrane potential are able to prevent the differences (Du et al., 2000). The process is dependent on the activity of Ca^{2+}/calmodulin dependent kinase II. In agreement with these findings, it has been shown that surface expression of TrkB isoforms is regulated by BDNF. A 15 seconds treatment of primary hippocampal neurons with BDNF caused a rapid increase in cell surface levels of TrkB FL suggesting that there is an intracellular pool of receptors that is stored in vesicles inside the cells and is released to the surface when BDNF bound receptor is internalized. TrkB receptors that lack the tyrosine kinase activity can regulate TrkB FL surface expression: TrkB T decreases the surface levels of TrkB FL while TrkB

SHC increases it (Haapasalo et al., 2002). This suggests that isoform interactions are important in regulating surface levels of the receptor and also a role for the SHC binding site in the trafficking of TrkB. It is interesting to see how the two different truncated isoforms can affect differently TrkB FL. These results underline the importance of defining the roles of the truncated isoforms as they can have very different effects on the TrkB FL. Moreover the fact that TrkB isoforms can regulate each other expression at the surface suggests that they might regulate the surface transport and trafficking of other proteins as well. For example, SHC has been shown to mediate TrkB induced transport of the channel Kv1.3 without BDNF mediated activation of the TrkB receptor (Colley et al., 2009). Interestingly APP has binding sites for SHC as well (Tarr et al., 2002). Recently, TrkB has also been shown to modulate surface levels of the inwardly rectifying K (+) channel Kir3.3 via NCAM interaction (Kleene et al.). This suggests that not only adaptor proteins could be involved in TrkB mediated APP trafficking, but that even a direct APP TrkB interaction should be tested. Based on the aforementioned data, we therefore hypothesized that TrkB might modulate APP trafficking and that TrkB isoforms might do so in different ways.

Previous research on tyrosine kinase receptors has shown that internalized receptors are transported to multi-vesicular bodies (MVB) and, depending on their tyrosine kinase activity, are localized in the vesicles in the lumen portion of the MVB or at its periphery on the membrane. The different localization in the MVB corresponds to a different recycling pattern: the tyrosine kinase deficient receptors are transported back to the plasma membrane while the tyrosine kinase active receptors

are transported to lysosomes and degraded (Ullrich and Schlessinger, 1990). The hypothesis for this difference in sorting of the receptor is that the tyrosine kinase active receptors can interact with sorting proteins, maybe by phosphorylating them. The inactive receptors, instead, do not interact with other proteins and are then segregated from the degradation pathway.

A recent microarray study on the C1 hippocampal region of AD and non demented control brains has shown that early endosomal markers such as Rab5/7 are up-regulated in AD while they are not in controls. They also found a down-regulation of both TrkB T and FL in the same brain region that was a consequence of the endosomal markers up-regulation. In fact, over-expression of rab5 in fibroblasts was able to reduce TrkB levels as measured by both Western blot and qPCR. There was no regulation of TrkB on rab5 expression levels (Ginsberg et al., 2010). Rab5 was also found in vesicles transporting APP from endosomes to the TGN. This retrograde trafficking decreases Aβ production and increases N-O-glycosylated APP half-life in the cells (Vieira et al.). An APP mutant mimicking T655 phosphorylation is preferentially retrogradely transported from the endosomes to the TGN showing that this trafficking is phosphorylation mediated (Vieira et al.). Phosphorylation of APP has been previously involved in regulating APP processing (Lee et al., 2003). TrkB has been shown to bind and phosphorylate NCAM on tyr734 after being activated by its ligand BDNF (Cassens et al.). This demonstrates that TrkB can phosphorylate in trans intramembrane proteins and could theoretically do the same on APP even if this has never been shown. Moreover it is intriguing that rab5 is associated with both TrkB and APP containing vesicles, it is possible that trafficking of these proteins is

regulated by the same mechanisms and maybe is partially dependent on their interaction.

1.3.4.2 *Trafficking and intracellular processing of APP*

TrkB trafficking regulation is complex and involves specific compartments, APP trafficking regulation is also complex and very important in the pathogenesis of AD. APP retention at the cell surface via a dynamine dominant negative that blocks efficient endocytosis, increased both the production of C83/99 and sAPP-α/Aβ demonstrating that APP trafficking is very important in determining levels of its processing products (Chyung and Selkoe, 2003). As detailed below, there are overlapping patterns between TrkB and APP intracellular trafficking. These over-laps suggest that TrkB and APP might regulate each other trafficking or at least be regulated by the same factors. Elucidation of these interactions and factors is going to be fundamental in furthering the knowledge of APP biology.

It was initially postulated that APP cleavage only occurs when APP, fully glycosylated, is transported to the cell periphery in neurons but recent evidence shows that APP epitopes containing the N-terminal or C-terminal domains are segregated in different cellular compartments (Muresan et al., 2009). This finding suggests that APP cleavage occurs in different cell compartments and the localization of the fragments is tightly controlled. Fragments containing the N-terminus of APP localized preferentially at the tip of the neurites while the C-terminus containing fragments localized in the cell soma. The Aβ containing fragments also labeled

mainly the cell soma. Further study of the vesicles showed that Aβ containing fragments were for the majority not co-localized with CTFs containing fragments (Muresan et al., 2009). Phosphorylated species of APP preferentially localize at the neurites (in particular at the center of the growth cone of neurites) and are mainly CTFs and not full length APP (Muresan et al., 2009). The phosphorylated forms of APP also localized at the periphery of the growth cone while the non–phosphorylated, probably APP FL and N-terminal fragments, localized to vesicles at the center of the growth cone and acetylated microtubules (Muresan et al., 2009). From an interactome study it is clear that different fragments of APP, localized to different cellular compartments interact with proteins that are specific to the cytoplasm, the membrane or the extra-cellular space (Bai et al., 2008)

APP CTFs and AICD are collected in the intra luminal vesicles of multi-vesicular bodies and exocytosed in exosomes. Treatment with alkalizing agents impedes degradation of these APP fragments in late endosomes and allows their accumulation (Vingtdeux et al., 2007). Interestingly, the experiments showing that alkalizing agents allow accumulation of CTFs and AICD in exosomes were performed in SH-SY5Y cells differentiated with retinoic acid. Retinoic acid induces TrkB up-regulation in SY5Y cells and TrkB FL has been shown to be associated with multivescicular bodies after BDNF stimulation (SH-SY5Y cells produce basal levels of BDNF). In particular, tyrosine kinase active receptors are found inside the luminal vesicles (just as APP CTFs and AICD) while the tyrosine inactive receptors are associated with the membrane of the multi-vesicular bodies and recycled to the cell surface. These findings suggest that APP and TrkB are found in the same cellular compartments and

this co-localization might be, at least partially, regulated by TrkB isoforms expression levels and activation.

The fact that TrkB can interact with several membrane and adaptor proteins, suggests that it might affect APP indirectly. For example, the expression of *SORL1* has been shown to be increased by BDNF treatment in primary mouse neurons (Rohe et al., 2009) while in AD patients *SORL1* expression is decreased. The *SORL1* gene encodes for the SORLA (sortilin related) receptor. This belongs to the family of the vacuolar sorting domain-containing receptor proteins 10, the LDLR family and the fibronectin type III repeats protein. Polymorphisms in this gene have been associated with late onset AD in several populations. SORLA controls the trafficking of APP from the trans-Golgi network and early endosomes. Over-expression of SORLA decreases APP processing by localizing APP to the Golgi (Andersen et al., 2005). BDNF treatment of primary neurons increases SORLA expression levels and decreases Aβ formation (Rohe et al., 2009). The SORLA-mediated effect on APP is not observed in SORLA deficient mouse primary neurons demonstrating that the TrkB/BDNF effect is SORLA dependent. It is interesting to notice how in SORLA deficient animals TrkB T has lower levels of expression while it has higher levels in BDNF treated SORLA+ cells. The effects on APP do not seem to be mediated by the downstream signaling of the receptor (Akt and ERK do not differ between SORLA+ or – cells).

Enzymatic complexes that process APP are also subjected to trafficking regulation and can regulate APP trafficking. The γ-secretase complex has been shown to be targeted to the cells surface (Kaether et al., 2002). At the same time, impaired γ-

secretase cleavage of APP decreased APP re-internalization without affecting β-secretase expression levels at the cell surface (Kaether et al., 2002). This data suggest that processing enzymes can affect APP trafficking through the cleavage itself. It remains to be determined what exactly causes the observed effect. It might be a feedback mechanism regulation due to the APP cleavage products.

1.3.4.3 APP glycosylation: role in trafficking and intracellular processing

APP is both O- and to a lower extent, N-glycosylated. O-glycosylation accounts for the majority of the gel shift, from 91KDa to 110KDa, that is observed between non-glycosylated APP and N-O glycosylated APP (Weidemann et al., 1989). O-glycosylation of APP occurs in the Golgi compartment (Tomita et al., 1998). N- and O-glycosylated APP695 over-expressed in SH-SY5Y neuroblastoma cells is found on the surface of the cells and in the trans-Golgi and post-Golgi compartments. N-glycosylated forms are found in the endoplasmic reticulum and in the cis-Golgi compartment (Tomita et al., 1998). Glycosylation of APP is important for its processing and surface transport. There can be several ways in which glycosylation of APP can be modified. The most obvious include altering its trafficking between the compartments where the glycosylation occurs (both with interacting proteins or chemicals) and disruption of the Golgi structure. Other less characterized mechanisms include molecules involved in synaptic activity. We report some examples for these different mechanisms below.

Over-expression of wild type syntaxin 1A abolished glycosylation of APP and inhibition of α and β processing as measured by dramatically decreased levels of C99 and C83 (CTFs) (Khvotchev and Sudhof, 2004). Further analysis of APP glycosylation, in presence of the different syntaxins, showed that wild type syntaxin induces impairment of APP N- and more dramatically, of O-glycosylation. Interestingly, when BACE1 was co-expressed with APP and truncated syntaxin, there was an increase in CTFs levels while this increase was not observed when wild type syntaxin 1A was expressed. Moreover, analysis of Aβ, revealed that in wild type syntaxin transfected cells, Aβ intracellular levels were lower when BACE1 was co-expressed and there was no increase in Aβ levels in the conditioned media (Khvotchev and Sudhof, 2004). This result underlines what already stated earlier: increased BACE1 levels do not necessarily correlate to increased APP processing. In fact, if APP and BACE1 are physically separated processing cannot occur. While having opposite effects on CTFs production, syntaxin and its truncated mutant both eliminated sAPP from the culture media (Khvotchev and Sudhof, 2004). Another example of a protein affecting APP glycosylation is TMEM59, a trans-membrane protein that is resident in the Golgi. TMEM59 over-expression results in APP retention in the Golgi (even if no direct interaction with APP was observed) and impaired N- and O-glycosylation of APP (Ullrich et al.). TMEM59 seems to act as a general inhibitor of glycosylation since it was also able to inhibit N-glycosylation of BACE1 and the prion protein. The authors do not describe any effects on C83/C99 production but observe a decrease in α and β sAPP levels that was not due to loss of

BACE1 or ADAM10 activity suggesting, once again, a trafficking mediated effect (Ullrich et al.).

Brefeldin A (a molecule that inhibits APP O-glycosylation by disrupting the Golgi) treatment increased Aβ generation in the ER compartment. Moreover, APP was not properly localized to the cell surface (Chyung et al., 1997). This study underlines once again how APP glycosylation, trafficking and processing are intimately related and influence each other.

NMDA receptor activation increases APP α-secretase processing while decreasing Aβ40 intracellular and extracellular levels (Hoey et al., 2009). The over-expression of the NMDA receptor also caused decreased APP O-glycosylation (Hoey et al., 2009). These results are interesting because they relate receptors involved in synaptic transmission to APP processing. Moreover, they show that proteins with a specific physiologic role that is unrelated to glycosylation, can affect APP glycosylation.

Auto-activation of the TrkB receptors in the Golgi has been shown to cause both an accumulation of immature (not fully glycosylated) forms of the receptor and at the same time a decreased glycosylation of other proteins. The proteins that are not fully glycosylated are then not localized at the cell surface (Schecterson et al.).

2. CHAPTER 2: TrkB isoforms differentially affect AICD production through their intracellular functional domains

Abstract

We report that *NTRK2*, the gene encoding for the TrkB receptor, can regulate APP metabolism, specifically AICD levels.

Using the human neuroblastoma cell line SH-SY5Y, we characterized the effect of three TrkB isoforms (FL, SHC, T) on APP metabolism by knock down and over-expression.

We found that TrkB FL increases AICD mediated transcription and APP levels while it decreases sAPP levels. These effects were mainly mediated by the tyrosine kinase activity of the receptor and partially by the PLC-γ and SHC binding sites. The TrkB T truncated isoform did not have significant effects on APP metabolism when transfected alone, while the TrkB SHC decreased AICD mediated transcription. TrkB T abolished TrkB FL effects on APP metabolism when co-transfected with it while TrkB SHC co-transfected with TrkB FL still showed increased APP levels.

In conclusion we demonstrated that TrkB isoforms have differential effects on APP metabolism.

2.1 Introduction

Alzheimer's Disease (AD) is a neurodegenerative disorder that will affect 15 million people in the US alone in the next ten years (Wancata et al., 2003; Alzheimer's, 2010). The most common form of the disease is the late onset (LOAD) that affects people older than 65. LOAD is caused by a complex interaction of risk factors including age, genetics and environmental factors, such as level of education, diet and physical activity (Munoz and Feldman, 2000; Khvotchev and Sudhof, 2004; Gatz et al., 2006; Scarmeas et al., 2009; Ramesh et al., 2010). To date, the ApoE-ε4 allele is the only genetic allele that has been unequivocally associated to increased risk of late onset AD (Corder et al., 1993; Bertram et al., 2007a; Bertram et al., 2010). The majority of the genetic risk factors for AD are still unknown. Genetic linkage, association and genome wide association studies are underway to identify genes and genomic loci involved in AD pathogenesis (Carrasquillo et al.; Jun et al.; Lipinski et al.; Naj et al.; Zetzsche et al.; Myers et al., 2000; Hardy and Selkoe, 2002; Blacker et al., 2003; Bertram and Tanzi, 2005; Bertram et al., 2007a; Rogaeva et al., 2007; Lee et al., 2008a; Brown et al., 2010; Hong et al., 2010; Reitz et al., 2010). The hope is that these studies will provide a further understanding of the disease processes, new early diagnosis markers and possible ways of therapeutic intervention.

We used a previously described cell-based functional screen (Zhang et al., 2007) to identify putative Amyloid Precursor Protein (APP) metabolism regulators on chromosome 9q22. This locus has been genetically linked to AD (Hamshere et al., 2007). One of the genes on this locus, *NTRK2*, has been here investigated as a potential modulator of APP metabolism.

NTRK2 encodes the Tropomyosin Related Kinase B or Tyrosine Receptor Kinase B (TrkB), which is the specific receptor of the Brain Derived Neurotrophic Factor (BDNF) (Middlemas et al., 1991). There are three major TrkB isoforms derived by alternative splicing (Figure 2.1) (Stoilov et al., 2002; Luberg et al., 2010). All the isoforms we investigated share an extra-cellular BDNF binding domain and differ in their cytoplasmic domain (Figure 2.1) (Stoilov et al., 2002). Two splice variants encode full length receptors, TrkB full length (FL), that contain a tyrosine kinase domain, a SHC binding domain and a PLC-γ binding domain in the intra-cellular portion (Middlemas et al., 1991; Atwal et al., 2000). Two are shorter receptors, TrkB SHC, that contain only a SHC binding site in the intracellular portion and the remaining isoform is a truncated receptor, TrkB T, that does not have any known intracellular functional domain (Figure 2.1). Recently, isoforms differing in the extra-cellular domain have also been identified (Luberg et al., 2010).

It is known that TrkB can affect APP metabolism. In fact, TrkB FL increases APP promoter transactivation and promotes accumulation of sAPP-α in the media of TrkB expressing cells (Ruiz-Leon and Pascual, 2001, 2003, 2004; Holback et al., 2005). Conversely, Aβ has been found to reduce TrkB FL/BDNF levels and to impair TrkB mediated signaling (Olivieri et al., 2003; Tong et al., 2004; Poon et al., 2009; Zeng et al., 2010). These results suggest a dynamic and mutual influence of TrkB/BDNF signaling and APP. The three neurotrophin receptors TrkA, TrkB and TrkC but not $p75^{NTR}$ are down regulated in AD brain samples (Ginsberg et al., 2006). Moreover, Trks down regulation seemed to be a good marker of progressively severe cognitive impairment (Ginsberg et al., 2006). Finally TrkB T knock-down in a mouse

model of Down syndrome, which also displays AD symptoms, rescued neuronal death (Carrasquillo et al.). However, case control and genome-wide association studies of *NTRK2* single nucleotide polymorphisms (SNPs) found no significant association with AD (Vepsalainen et al., 2005; Perry et al., 2007; Reiman et al., 2007; Chen et al., 2008; Cozza et al., 2008; Li et al., 2008). Only one family based study found genetic association of *NTRK2* haplotypes and AD (Chen et al., 2008).

All these results suggest that TrkB and its isoforms affect APP metabolism and could play a role in the pathogenesis of AD. Nevertheless, the effects of TrkB on APP metabolism have not been widely investigated, especially the role of the different TrkB isoforms.

We hypothesized that the TrkB truncated isoforms would have different effects on APP metabolism dependent on the ability of binding SHC of the TrkB SHC isoform. We also hypothesized that the different functional domains on the TrkB FL cytoplasmic region would be responsible for some of the previously described APP metabolism effects. In particular, we hypothesized that TrkB FL activation and binding to PLC-γ activates PKC and therefore increases ADAM10 activity.

To test these hypothesis, we used a SH-SY5Y neuroblastoma cell line over-expressing APP as a fusion protein with the yeast transcription factor Gal-4 (Zhang et al., 2007). This cell line expresses low endogenous levels of TrkB and BDNF. To characterize the role of TrkB FL and truncated isoforms we knocked down and over-expressed the isoforms and measured APP FL levels and proteolytic products using Western blots and luciferase assays. We found that TrkB FL increases AICD-Gal4 mediated luciferase activity. TrkB T does not alter the luciferase activity while TrkB

SHC decreases the luciferase activity compared to control. For the first time we show a different effect of the TrkB isoforms on APP. We determined that the functional domain responsible for this effect on TrkB FL is the tyrosine kinase domain and that the SHC binding and PLC-γ binding domains also contribute to increase the AICD mediated luciferase activity in combination with an active tyrosine kinase site. We also found that the SHC binding site on TrkB SHC is responsible for the decreased AICD mediated luciferase activity. BDNF stimulation of the TrkB exogenously expressed receptors amplified the APP metabolism effects and co-transfection of the TrkB truncated isoforms with TrkB FL alters its effect on APP metabolism.

2.2 Materials and Methods

2.2.1 Constructs and site directed mutagenesis

Four shRNA containing plasmids specific for *NTRK2* were obtained from the pSM2 retroviral Library of the Drexel RNAi Resource Center purchased from OpenBiosystems. The constructs ID numbers are: NTRK2.1: 1920; NTRK2.2: 2295; NTRK2.3: 29734; NTRK2.4: 30795. We also used APP (ID 39147) and luciferase targeting shRNA (RHS1705) as positive controls and a scrambled shRNA sequence (non-silencing, NS, RHS1707) as a negative control. The TrkB full length and truncated GFP fusion constructs and the GFP-F control over-expression plasmid were kindly donated by Dr. Eero Castren (University of Helsinki, Finland) and were previously described (Haapasalo et al., 1999; Haapasalo et al., 2002). Site-directed

mutagenesis (Stratagene, Quikchange mutagenesis kit) was utilized to generate point mutants on the TrkB full length receptor functional domains. The mutant amino acid indicated refers to the amino-acidic sequence of TrkB. Therefore TrkB FL K571M indicates the tyrosine kinase dead receptor since it is mutated on the ATP binding site; TrkB FL Y515F indicates the receptor mutated on the SHC binding site; TrkB FL Y816F indicates the receptor mutated on the PLC-γ binding site (Minichiello, 2009). Note that in some literature the TrkB mutants are referred to with the numbering of the amino-acidic sequence of TrkA, the NGF receptor, that has functional sites in common with TrkB and therefore are referred to as K560M, Y490F and Y785F respectively (Middlemas et al., 1991; Atwal et al., 2000). TrkB SHC indicates the other human truncated isoform (isoform d and e, NCBI Gene NM_001018064₊2 and NM_001018066₊2). TrkB SHC was obtained by insertion of the exon 19 followed by a STOP codon after the SHC binding site on the TrkB FL constructs. After obtaining the TrkB SHC isoform by insertion, the SHC binding site was mutated on that isoforms using the same primer sequence employed for the TrkB FL mutant on the SHC binding site (TrkB Y515F).

Mutagenesis was carried out according to manufacturer's instructions and the primers employed are reported in the table below (the bolded sequences represent the mutations/insertion):

Table 2.1: Primer sequences used to obtain the point mutations and the sequence insertion on the TrkB FL receptor. The mutated bases are represented in bold.

Primers	Sequence 5' to 3'
TrkB.Y515F.F	GTCATTGAAAACCCCCAGT**T**CTTCGGTATCACCAACAG
TrkB.Y515F.R	CTGTTGGTGATACCGAAG**A**ACTGGGGGTTTTCAATGAC
TrkB.Y816F.F	GCGTCGCCCGTCT**T**CCTGGACATCCTAG
TrkB.Y816F.R	CGCAGCGGGCAGA**A**GGACCTGTAGGATC
TrkB.Shc.F	CTCAAGCCGGACACA**TGGCCCAGAGGTTCCCCCAAGACCGCCTGATAGTAA**TTTGTTCAGCACATC
TrkB.Shc.R	GATGTGCTGAACAAA**TTACTATCAGGCGGTCTTGGGGGAACCTCTGGGCCA**TGTGTCCGGCTTGAG
TrkB_K571M.F	GGTGGCCGTGA**T**GACGCTGAAGG
TrkB_K571M.R	CCTTCAGCGTC**A**TCACGGCCACC
TrkB.SHC.	GTCATTGAAAACCCCCAGT**T**CTTCGGTATCACCAACAG
TrkB.SHC.	CTGTTGGTGATACCGAAG**A**ACTGGGGGTTTTCAATGAC

Successful mutations were identified by sequencing and one clone per construct was transformed in *E.coli* (DH5-α competent cells, InVitrogen). Transformed bacteria were selected on 100 μg/ml ampicillin LB-agar plates and liquid cultures were grown overnight at 37 °C. Bacterial cultures were miniprepped (MiniPrep Kit, Quiagen) and used for transfection after DNA quantification.

2.2.2 Cell culture and transfection

SH-SY5Y cells stably transfected with UAS-Luciferase and APP-Gal4 described before (Zhang et al., 2007) were maintained in DMEM (Gibco) supplemented with 10% FBS, penicillin-streptomycin and 200 μg/ml G418 (Gibco).

To assess the effects of TrkB knock-down or over-expression on AICD-Gal 4 mediated luciferase we used the following transfection protocol previously described (Zhang et al., 2007). Briefly, one day before transfection cells were plated in 96-wells plates at approximately 40-50% confluency. The day of transfection media was removed from the cells and replaced with transfection media: 100 μl of serum free DMEM media containing 2 μg/well Arrest-In (Open Biosystems) and 0.2 μg/well plasmid DNA. The pSM2 plasmids referred to as NTRK2.1 through 4 were transfected in the cells. Cells were also transfected with shRNA targeting APP, luciferase and a control shRNA that contains a scrambled sequence that does not target any human gene. In addition a mock transfection, containing only Arrest-In was performed to control for selection effectiveness. 6 replicate wells per shRNA constructs and mock control transfection were set up for each independent experiment. The transfection media was left on the cells for 8 hours and then replaced with complete media. 48 hours after transfection, transfected cells were selected with 4 μg/ml puromycin (Sigma) in 10% FBS DMEM with 200 μg/ml G418. Puromycin selection was used to preserve transiently transfected cells. The media was changed every 48 hours and cell death was monitored and compared to the mock-transfected control. Once all the cells in the mock control wells were dead, surviving cells, in the

shRNA transfected wells, were split and transferred to another 96-well plate and a 24 well plate. Cell lysates were collected from 60-80% confluent 96-wells 11-13 days after transfection in 100 μl Glo Lysis Buffer per well (Promega). Lysates were used immediately post collection or frozen prior to performing Steady Glo luciferase assays (Promega). The luciferase assays were performed as per manufactures' instructions. ShRNA mediated knock-down effectiveness was monitored by comparing the luciferase signal of the non-silencing control shRNA with the APP targeting shRNA. After assessing successful knock-down, luciferase data for the experimental shRNA targeting NTRK2 was collected and analyzed. In parallel 24-well plates and 12-well plates were seeded with the same cells that had been assayed for luciferase signal, and collected for Western Blot analysis.

The same transfection procedure was followed for the over-expression experiments but lysates were collected 48 hours after transfection and transfection efficiency was monitored in fluorescence microscopy, no antibiotic selection was performed in this case.

2.2.3 Western Blotting Procedures

Conditioned media was collected from the cells (48 hours after transfection) in eppendorf tubes and centrifuged at 14,000 rpm for 10 minutes at 4 °C (Beckman Coulter, Microfuge 22R). The resulting supernatant was collected and 142 μl were mixed with 33 μl of 4X Reducing loading buffer (InVitrogen) supplemented with 0.4% β-mercapto-ethanol (Sigma). These samples were heated at 70 °C for 10

minutes. The remaining conditioned media was stored frozen at -20 °C for later Western Blot analysis.

Whole cell lysates were collected (48 hours post transfection) by lysing the cells with ice-cold radio immuno-precipitation (RIPA) buffer (150mM NaCl**,** 1% NP40**,** 0.5% DOC**,** 1% SDS**,** 50mM Tris, pH 8.0) supplemented with Halt cocktail of protease and phosphatase inhibitors (ThermoScientific). Cell lysates were sonicated in an ice-cold water bath sonicator for 6 minutes then centrifuged 20 minutes at 4 °C at 14,000 rpm. The resulting supernatants were collected and protein concentration measured with a BCA protein concentration kit (Pierce) according to manufacturer's instructions.

Western blot samples were prepared at a final concentration of 1-2 μg/μl in 4X reducing loading buffer (InVitrogen) and heated at 70 °C for 10 minutes. 15-25 μg of total protein/well were separated on 4-12% Tris-Glycine midi gels (InVitrogen) in MES-SDS running buffer (InVitrogen) and run at 190 mVolts for 45 minutes. The separated proteins were transferred to PVDF FL membranes (Millipore) in a Semi-Dry transfer apparatus (AA Hoefer TE77X) for 3 hours at 125 milli Amp per gel.

Membranes were blocked one hour at room temperature using Licor blocking buffer then probed overnight with primary antibodies diluted in Licor blocking buffer at 4-25 °C. Membranes were then washed for 5 minutes 4 times with 0.1% Tween (Sigma) in PBS. One last wash was performed with PBS to rinse off the detergent. After washing, membranes were incubated in the dark with the appropriate secondary antibody IRDye (Licor) diluted in Licor blocking buffer for one hour. Again membranes were washed as above and finally rinsed with PBS. Membranes were scanned on an Odyssey InfraRed scanner (Licor) at appropriate intensities and images

acquired. Band intensities were quantified with the provided in-built software (Licor) and always normalized to the actin loading control. When conditioned media was analyzed the signals were normalized to the protein concentration of the corresponding lysates.

2.2.4 Antibodies

Detection of TrkB-GFP tagged constructs utilized mouse anti GFP antibody (1:1000, Living Colors, Clontech); detection of APP full length and C- terminal fragments utilized A8717 rabbit antibody (1:2000, Rb, Sigma); detection of sAPP 22C11 utilized mouse antibody (1:1000, Millipore); detection of sAPP-α 6E10 utilized mouse antibody (1:1000, Covance); detection of actin A5441 utilized mouse antibody (1:15,000, Sigma). The secondary antibodies: IRDye700 anti mouse antibody (1:15,000) and IRDye800 anti rabbit antibody (1:15,000) were obtained by Licor.

2.3 Results

2.3.1 NTRK2 knock-down decreases AICD-mediated luciferase activity

We applied our functional screening method (Zhang et al., 2007) to all the genes in the linkage region on chromosome 9 that displays a high likelihood of disease score for AD (Blacker et al., 2003). This screening is conducted in SH-SY5Y cells stably transfected with a luciferase reporter driven by the yeast UAS promoter

and APP fused to Gal4. When APP is cleaved by the secretases the AICD-Gal4 domain is released and can activate the transcription of the luciferase reporter. Variations in AICD mediated luciferase activity are measured. Since changes in AICD-mediated luciferase activity can occur through a variety of mechanisms affecting APP, this is an effective and general way of identifying regulators of APP metabolism (Zhang et al., 2007). We targeted *NTRK2* with 4 different shRNA constructs. Three shRNAs targeted all the TrkB isoforms (NTRK2.1-3) and one (NTRK2.4) targeted all the isoforms except the TrkB T. We also transfected a non-silencing scrambled shRNA (CTRL) that does not target any human gene as a negative control and a shRNA targeting APP as a positive control. Of the four transfected constructs NTRK2.1-3 decreased AICD-mediated luciferase to the same extent of the APP targeting shRNA compared to the CTRL shRNA (Figure 2.2A). The fourth construct, NTRK2.4 targeting all TrkB isoforms except TrkB T, consistently caused cell death (data not shown). This result suggests that *NTRK2* can affect APP metabolism and that the isoforms have different roles since down-regulation of all the isoforms except TrkB T was lethal. Therefore we investigated the effect of the single isoforms in the same experimental model.

We transiently transfected individual TrkB isoform over-expression constructs in the cells and measured AICD-mediated luciferase activity. We found that there was no difference in AICD-mediated luciferase activity between TrkB T and the GFP-control while TrkB FL significantly increased luciferase activity ($p=0.01$) and TrkB SHC significantly decreased it ($p=0.01$) (Figure 2.2B). These results demonstrate that TrkB isoforms have different effects on APP metabolism. Moreover we show that

there is a difference between the isoforms TrkB SHC and TrkB T even if both of them lack the tyrosine kinase activity.

2.3.2 Two functional domains of TrkB FL (tyrosine kinase and PLC-γ binding domains) and the SHC binding domain on TrkB SHC determine the effect on AICD mediated luciferase activity

TrkB T did not alter AICD-mediated luciferase activity compared to the GFP-F control, while TrkB SHC decreased it and TrkB FL increased it. We hypothesized that the intracellular domains of the TrkB SHC and TrkB FL are responsible for the effects observed. To determine which domain was responsible for this effect, we mutated each cytoplasmic functional domain individually.

We generated a mutant of the TrkB SHC isoform that cannot bind SHC (Y515F). We transfected this mutant and the other TrkB wild type isoforms, in SH-SY5Y-APP-Gal4 cells and measured AICD-mediated luciferase activity. We observed that TrkB FL Y515F (SHC binding site mutant) does not significantly alter luciferase activity compared to TrkB T but significantly increased it compared to the TrkB SHC wild type isoform ($p<0.001$) (Figure 2.3A). Therefore, disrupting the SHC binding site on the TrkB SHC isoform impairs its ability to decrease AICD-mediated luciferase activity.

We then generated mutants on the three functional sites of the TrkB FL. We mutated the SHC binding site (Y515F) to generate a mutant that cannot bind SHC. Then we mutated the ATP binding site (K571M) to generate a TrkB FL tyrosine kinase inactive receptor (Middlemas et al., 1991; Atwal et al., 2000). Similarly, we

disrupted the PLC-γ binding site by introducing the mutation Y816F. We also generated a double mutant that is tyrosine kinase inactive and does not bind SHC (TrkB Y515F/K571M). We then transfected these TrkB FL mutant constructs in SH-SY5Y-APP-Gal4 cells. We measured the AICD mediated luciferase activity and compared it to TrkB FL wild type (Figure 2.3B).

The TrkB Y515F mutant (preventing SHC binding) did not significantly alter AICD-mediated luciferase activity compared to TrkB FL (Figure 2.3B). The TrkB FL K571M (tyrosine kinase inactive) significantly decreased luciferase activity compared to TrkB FL ($p=0.0006$). TrkB FL Y816F, (preventing PLC-γ binding) also significantly decreases luciferase activity compared to TrkB FL ($p<0.0002$). The double mutant TrkB Y515F/K571M (preventing SHC binding and tyrosine kinase inactive) significantly decreased luciferase compared to TrkB FL ($p<0.002$) but did not differ from the tyrosine kinase inactive TrkB K571M (Figure 2.3B).

In summary, the TrkB FL mediated increase on luciferase activity is mainly dependent on the tyrosine kinase activity and PLC-γ binding, while the decrease caused by the TrkB SHC isoform is dependent on the SHC binding site. In fact, either inactivation of the tyrosine kinase activity (TrkB K571M) or prevention of PLC-γ binding (TrkB Y816F), decreased AICD mediate luciferase signal approximately to the same extent of TrkB T, 50-70% compared to TrkB FL (Figure 2.2B and Figure 2.3B). While TrkB SHC decreased AICD-mediated luciferase activity about 90% compared to TrkB FL, its mutant, TrkB SHC Y515F, only decreased it to the extent of TrkB T, about 70% (Figure 2.3A).

2.3.3 NTRK2 knock-down decreases APP FL levels

The effects we observe on AICD-mediated luciferase activity can occur through many different mechanisms: decreased APP transcription, increased APP degradation, decreased APP cleavage, destabilization of AICD and trafficking that affects APP localization. Anything that decreases AICD levels will be reflected in a decrease in luciferase activity. The most immediate way of decreasing AICD levels is to decrease APP levels. To determine if *NTRK2* knock-down decreased APP levels, we tested if APP levels were altered. We transfected the *NTRK2* targeting shRNA, a CTRL shRNA, and an APP targeting shRNA as a positive control. As an additional control we used shRNA targeting the luciferase gene: this construct accounts for over-expression of shRNA that have to be processed by the endogenous RNAi machinery. We then measured APP protein levels by Western blot (Figure 2.4A). We found that knock-down of all the TrkB isoforms causes a significant decrease in APP FL levels ($p<0.05$) (Figure 2.4B) and we concluded that decreased APP levels might be at least partially responsible for the observed reduction in luciferase activity.

2.3.4 TrkB FL over-expression increases APP FL levels and AICD Gal4 levels

Based on the previous knock-down results, we then hypothesized that over-expression of TrkB FL causes increased AICD-mediated luciferase activity by increasing APP FL levels. We transfected the TrkB isoforms in the cells, performed Western blot analysis and quantified APP FL levels in cell lysates. Transfection of TrkB FL significantly increased APP FL levels compared to TrkB T ($p=0.03$) and TrkB SHC transfections ($p=0.008$) (Figure 2.5A). There was no difference in APP levels between TrkB T and TrkB SHC transfected cells (Figure 2.5A).

We then verified that AICD-Gal4 levels in TrkB FL transfected cells correlated to the observed increase in luciferase activity. AICD-Gal4 is the intracellular domain of APP that is generated by γ-secretase cleavage, translocates to the nucleolus and activates transcription. We found that, as expected, TrkB FL showed increased AICD-Gal4 levels compared to TrkB T. TrkB SHC, instead, caused an expected decrease in AICD-Gal4 levels compared to TrkB T (Figure 2.5B). Interestingly, we consistently observed TrkB FL lower levels compared to TrkB T and TrkB SHC in our Western blot analysis (Figure 2.5A).

2.3.5 TrkB FL over-expression decreases sAPP levels without altering C83 levels

To assess changes in APP proteolysis we measured APP C-terminal fragments (CTFs) and sAPP levels upon TrkB transfection. CTFs include both C83 and C99.

C83 and C99 are generated by the cleavage of APP by α-secretase and β-secretase respectively. In our cell line we measure C83-Gal4 and C99-Gal4 levels since APP over-expressed is a fusion protein with Gal4. These fragments are the precursors of AICD that is released in the cytoplasm by γ-secretase cleavage (Kamenetz et al., 2003; Cao and Sudhof, 2004). While C83 and C99 are membrane bound fragments of APP, the soluble N-terminal fragment of APP, sAPP, generated by α/β-secretase cleavage is released in the extra-cellular environment. In SH-SY5Y cells, the β-secretase cleavage occurs at a lower extent than α-secretase cleavage. Therefore, the majority of the luciferase signal observed is due to AICD-Gal4 generated from C83-Gal4 γ-secretase cleavage. If the AICD-Gal4 fragment levels are increased, as measured by luciferase and Western blot, then the levels of its precursor C83-Gal4 should also be increased.

We then tested the hypothesis that C83-Gal4 and sAPP levels are increased by TrkB FL over-expression and decreased by TrkB SHC. However we could not detect a difference in C83-Gal4 levels among the cells transfected with the different TrkB isoforms (Figure 2.5C).

Since C83-Gal4 levels did not differ upon transfection of the different isoforms, we hypothesized that sAPP levels should also be constant. We tested this hypothesis by measuring sAPP levels in the conditioned media of the cells and normalizing it to the total APP levels of the corresponding cell lysates. Surprisingly, TrkB FL decreased sAPP levels compared to TrkB T ($p=0.01$). TrkB SHC showed a non-significant difference in sAPP levels compared to TrkB T (Figure 2.5D). We hypothesized that the decrease in sAPP levels mediated by TrkB FL might be due to a

decrease in APP glycosylation, which is fundamental to allow APP transport to the cell surface, where the majority of sAPP is generated. We then measured the ratio of APP glycosylated (mature, APPm) to APP non-glycosylated (immature, APPim) in cells transfected with the different isoforms and we noticed an unexpected increase in APP mature to immature ratio in cells transfected with TrkB FL compared to TrkB T (Figure 2.5E, p=0.03). TrkB SHC did not significantly alter APPm/im ratio compared to TrkB T.

2.3.6 γ-secretase inhibition increases sAPP levels independent of TrkB isoforms

The effects seen on sAPP levels and APP glycosylation suggest that TrkB isoforms might be involved in altering APP trafficking and therefore its cleavage. It is known that APP γ-secretase cleavage affects its trafficking and viceversa. In particular, decreased γ-secretase cleavage increases sAPP levels (Kaether et al., 2002). We then tested if TrkB isoforms effects on sAPP levels were affected by γ-secretase impaired cleavage. We found that TrkB isoforms had the same effects on sAPP levels in both L-685 or vehicle treated cells (Figure 2.6A and 2.6B). TrkB FL caused a significant decrease in sAPP levels compared to TrkB T while TrkB SHC did not alter sAPP levels compared to TrkB T. Then we checked if γ-secretase impaired cleavage was able to increase sAPP levels independently of TrkB isoform transfected. We found that γ-secretase inhibition was able to increase sAPP levels independent of TrkB isoform transfected (p=0.0002, Figure 2.6C). Therefore the

effects on sAPP levels caused by TrkB isoforms and γ-secretase activity impairment are independent of each other.

2.3.7 BDNF treatment of TrkB isoforms does not significantly alter their effects on APP metabolism

All three TrkB isoforms studied here can bind BDNF (Reichardt, 2006). Moreover it has been previously shown that TrkB FL BDNF-mediated intra-cellular signaling can alter APP metabolism (Ruiz-Leon and Pascual, 2001, 2003, 2004; Holback et al., 2005). We hypothesized that application of exogenous BDNF would stimulate the TrkB FL mediated effects on APP FL and proteolytic products levels. We then tested this hypothesis applying BDNF to cells transfected with TrkB isoforms and measured the levels of APP FL by Western blot. We found that short term (10 minutes) BDNF application increases APP FL levels in cells transfected with the TrkB T or TrkB SHC isoforms and to a greater degree in cells that had been transfected with TrkB FL (Figure 2.7). Longer BDNF application on TrkB FL transfected cells did not increase APP FL levels.

2.3.8 Co-transfection of the TrkB isoforms modulates TrkB FL mediated effects on APP metabolism

It had been previously shown that TrkB T has a dominant negative effect on TrkB FL (Haapasalo et al., 2001). We hypothesized that co-transfection of TrkB T with TrkB FL would eliminate the TrkB FL effects on APP metabolism observed when we transfect TrkB FL alone. Moreover we hypothesized that co-transfection of the TrkB SHC with TrkB FL would also have dominant negative effect on TrkB FL. Finally we hypothesized that co-transfection of TrkB FL with TrkB Y515F or TrkB

Y816F would not significantly alter the effects seen on APP since they seem to be primarily mediated by the tyrosine kinase domain and not by the SHC binding domain. For this reason we also hypothesized that co-transfection of TrkB FL with TrkB K571M (TrkB FL/K571M) would have the same effect as the co-transfection of TrkB FL and TrkB T (TrkB FL/T).

Consistent with our hypothesis, TrkB FL/T co-transfection did not increase APP FL levels, nor did co-transfection of TrkB FL/K571M, the tyrosine kinase inactive mutant (Figure 2.8). Also as expected there was very little difference between the APP FL levels in cells transfected with TrkB FL/Y515F, TrkB FL/Y816F and TrkB FL/FL. Surprisingly, co-transfection of TrkB FL/SHC increased APP FL levels compared to TrkB FL/T co-transfection but not compared to TrkB FL/FL (Figure 2.8).

We also hypothesized that BDNF treatment of the co-transfected cells would affect the effects on APP of the transfected isoforms. Surprisingly BDNF treatment did not significantly alter the effects of the co-transfected TrkB receptors.

In summary both truncated isoforms were able to decrease APP FL levels compared to TrkB FL/FL transfection; TrkB T to a greater extent than TrkB SHC. The tyrosine kinase inactive receptor decreased APP FL levels to the same extent of TrkB FL/ T co-transfection while TrkB FL/Y515F and TrkB FL/Y816F co-transfection did not alter APP FL levels compared to TrkB FL/FL.

2.4 Discussion

TrkB is a neurotrophic receptor that is involved in synaptic plasticity and long term potentiation (Minichiello, 2009; Thiele et al., 2009; Yoshii and Constantine-Paton, 2010). Interestingly, APP transcription is up-regulated by TrkB and Aβ impairs TrkB/BDNF signaling (Ruiz-Leon and Pascual, 2001, 2003, 2004; Holback et al., 2005). This evidence is only based on the TrkB FL receptor and there is no investigation of the two additional TrkB splicing isoforms encoded by the *NTRK2* gene. Some single nucleotide polymorphisms on *NTRK2* that might be associated with AD, are mainly found in proximity of splicing regulating regions suggesting that alternative splicing of the gene might be important in AD pathogenesis (Chen et al., 2008). TrkB T, the shortest isoform, has been shown to mediate neuronal cell death in a mouse model of trisomy 21 which develops AD symptoms (Dorsey et al., 2006). This experimental evidence points to a possible role of the TrkB isoforms in AD pathogenesis. The TrkB SHC isoform has not been extensively studied. We therefore investigated the role of the TrkB isoforms on APP metabolism in SH-SY5Y cells over-expressing an APP-Gal-4 fusion protein that can transactivate a luciferase reporter gene. This system monitors changes in APP metabolism that are reflected in altered AICD-mediated transcription of the luciferase gene (Zhang et al., 2007).

We found that knock-down of all TrkB isoforms in SH-SY5Y-APP-Gal4 cells caused a decrease in AICD-mediated luciferase activity. This decrease is probably due to a decrease in APP levels observed in cells with *NTRK2* knock-down. We hypothesize that decreased APP levels in this system are mainly due to increased APP

degradation caused by altered trafficking in absence of TrkB. Transcriptional down-regulation of APP might be partially responsible for the decreased signal observed in the Western Blot but that is only possible for the endogenous APP. In fact the endogenous *APP* gene is under the physiologic transcriptional regulation while the APP-Gal4 over-expressed is under CMV promoter regulation. A general increase in transcription caused by TrkB FL should be accounted for by the normalization to the other transfected reporter, renilla luciferase. In agreement with the knock-down data, over-expression of TrkB FL increased APP full length levels compared to TrkB T while TrkB SHC did not affect APP levels. This suggests that the modulation of APP full length levels is due to the tyrosine kinase active receptor and not to the truncated isoforms.

Knock-down of all the TrkB isoforms except the TrkB T lead to cell death and this is consistent with the finding that TrkB T is one of the causes of neuronal death in a mouse model of trisomy 21 (Dorsey et al., 2006).

To discriminate between the effects of the different isoforms, we over-expressed one isoform at a time and measured the resulting AICD-mediated luciferase activity. As a control, we employed a GFP expression vector (GFP-F) that includes a farnesylation sequence that targets GFP to the cell membrane. This is a better control for a membrane bound receptor than a cytoplasmic GFP. Interestingly, we observed a different effect of the isoforms: as expected TrkB FL increased luciferase activity, while no difference was observed between TrkB T and GFP-F control transfected cells. TrkB SHC induced a decrease in AICD-mediated luciferase activity. We hypothesize that the decrease in AICD-mediated luciferase activity

induced by TrkB SHC might be mediated by binding of SHC adaptor proteins. Binding of adaptor proteins to TrkB and possibly to APP, might decrease the endocytosis of APP decreasing its β-secretase cleavage (Lichtenthaler, 2006). The luciferase assay described here has been found to be particularly sensitive in detecting decreased β-secretase processing (Hoey et al., 2009) and that can be the cause of the decrease in luciferase activity that we observe, at least with co-transfection of the TrkB SHC isoform.

Western blot analysis of the cell lysates showed, in agreement with the luciferase findings, an increase and AICD-Gal4 levels in cells transfected with TrkB FL and a decrease in cells transfected with TrkB SHC compared to TrkB T. We did not find a correspondent increase in C83-Gal4, the precursor of AICD-Gal4. This discrepancy could be due to the different half life of these fragments and to the low sensitivity of Western blots as a detection system. sAPP levels were instead decreased by TrkB FL transfection while we expected an increase, or no change, like we detected for C83-Gal4. This finding suggests that altered trafficking of APP might be also contributing to affect the APP fragment levels. In fact, APP cleavage is strongly dependent on its localization. Supporting this hypothesis, is the fact that TrkB isoforms differently affect the APPm/im ratio. APP glycosylation is dependent on the correct trafficking between endoplasmic reticulum and Golgi apparatus and can affect APP processing as well. Therefore if TrkB isoforms affect glycosylation and/or trafficking of APP they might cause different APP cleavage patterns. APP trafficking and processing are tightly linked in fact impaired γ-secretase cleavage can increase surface APP cleavage (Kaether et al., 2002). TrkB isoforms are affecting these

processes. Inhibition of γ-secretase cleavage of APP did not change the effects on sAPP levels of the TrkB isoforms but was still able to increase sAPP levels, when compared to control, independent of TrkB isoform transfected. This shows that APP trafficking is still regulated through common mechanisms independent from the TrkB isoform transfected.

Our data demonstrates differential effects of the TrkB isoforms on AICD-mediated transcription and APP showing that TrkB SHC behaves differently from both TrkB FL and TrkB T. It has been previously demonstrated that BDNF application does not improve the cognitive function in a trisomy 21 mouse model because TrkB T is up-regulated. Knock-down of TrkB T rescues neuronal death suggesting that TrkB T contributes to this phenotype possibly by inhibiting binding of BDNF to TrkB FL (Dorsey et al., 2002; Dorsey et al., 2006). Since the three isoforms are co-expressed on neurons, the binding of BDNF to TrkB FL will be limited by the levels of the truncated TrkB T and TrkB SHC. Therefore, a better understanding of the individual TrkB isoforms and their signaling role will improve the therapeutic potential of BDNF or BDNF agonists.

Experimentally, we found that the detected protein levels of TrkB FL were much lower than TrkB T and TrkB SHC levels. We can exclude effects due to plasmid copy number in the cells since we used equimolar amounts of plasmid DNA that account for differences in plasmid size. We can also exclude differences in transcription levels due to plasmid promoters since the TrkB SHC plasmid was obtained from the TrkB FL encoding construct by insertion mutagenesis. The difference in expression levels of the TrkB isoforms is highly reproducible suggesting

that there might be a tight regulation of the expression levels of the TrkB FL. TrkB FL is stored in intracellular vesicles that rapidly fuse to the cell membrane upon BDNF stimulation of the cells (Haapasalo et al., 2002). This causes a fast BDNF mediated phosphorylation of the receptor and initiates intracellular signaling (Du et al., 2003). After this spike of activity TrkB/BDNF complexes are rapidly endocytosed and degraded (Sommerfeld et al., 2000). TrkB FL high expression levels increase malignancy in neuroblastomas reinforcing the idea that regulatory mechanisms of TrkB expression and signaling are necessary to maintain homeostasis (Douma et al., 2004; Thiele et al., 2009). TrkB FL expression is also decreased by chronic BDNF stimulation of H4 neuroblastoma cells while TrkB T levels remain almost constant (Rohe et al., 2009). We therefore hypothesize that, in our model system, TrkB FL levels are controlled by mechanisms that cannot be overcome by TrkB FL over-expression and that BDNF expressed by the cell line might be one of the causes of this down-regulation.

To determine which functional domain and pathway was mediating the TrkB effect, we over-expressed the TrkB isoforms in the same cell line and monitored AICD-mediated luciferase activity. We found that the tyrosine kinase domain of TrkB FL was responsible for the increased luciferase effect because a mutation of the ATP binding site (K571M) in the tyrosine kinase domain abrogated the luciferase activity increase. The mutation of the PLC-γ site (Y816F) also induced a significant decrease in luciferase activity. We hypothesize that this effect is due to lack of PLC-γ activation which produces DAG (Diacyl Glycerol) that is an activator of PKC, a protein that mediates ADAM10 activation (Lammich et al., 1999). Moreover, PLC-γ

mediates release of Ca^{2+} from intracellular storage and this might activate some transcription factors increasing overall APP levels. The fact that there is a difference between the TrkB K571M mutant and the TrkB Y816F PLC-γ binding site mutant suggests that not all the effect is due to lack of PLC-γ activation (Figure 2.3B).

The SHC binding site on the TrkB FL receptor did not seem to be involved in mediating increased AICD luciferase activity since the TrkB Y515F mutant did not differ from the TrkB FL isoform in increasing AICD-mediated luciferase activity. Also the AICD-mediated luciferase signal in cells transfected with the double mutant TrkB K571M/Y515F did not differ from the cells transfected with the TrkB K571M mutant suggesting that there is no additive effect in eliminating both signaling pathways. This does not completely exclude a role for the SHC binding domain. In fact, the binding of SHC might occur more efficiently when the site is phosphorylated so that, when phosphorylation is prevented, the small change in luciferase signal is not detectable in our experimental system. Supporting this hypothesis is a non-significant decrease of AICD-mediated activity caused by the TrkB Y515F mutant compared to TrkB FL.

We find a significant effect of the elimination of the SHC binding site on the TrkB SHC isoform, this mutant induces the same luciferase activity signal of the TrkB T. This finding suggests that binding of SHC to the TrkB SHC isoform might mediate signaling pathways independently of phosphorylation. Importantly, we demonstrate that there is a difference in signal transduction between the two truncated TrkB isoforms and that they act on APP mediated transcription. Moreover, we identify the SHC binding domain as responsible for the difference in signaling

mechanism between TrkB T and SHC. Mutation of the binding site for SHC adaptor proteins on the truncated TrkB SHC isoform increases AICD-mediated luciferase signal while the same site mutated on the TrkB FL is involved in a decreased luciferase signal. This contrasting result suggests that interaction between the same proteins and specific TrkB isoforms mediates different signaling pathways.

The cell line used, SH-SY5Y, expresses basal levels of TrkB receptors and BDNF (Stoilov et al., 2002), the endogenously expressed BDNF can promote dimerization and activation of the over-expressed receptors. Also, BDNF independent activation of TrkB FL receptors has been previously demonstrated (Schecterson et al., 2010) and we hypothesize that both BDNF independent and dependent activation co-exist in our experimental system. Endogenous TrkB receptors might also be up-regulated or down-regulated in response to exogenous TrkB expression.

To assess the effect of TrkB BDNF dependent activation we added exogenous BDNF on the transfected cells. BDNF is hypothesized to activate the receptors by mediating their dimerization (Reichardt, 2006). In our experimental system BDNF treatment did not significantly alter the effects of the TrkB isoforms on APP FL levels. It has been observed before that TrkB FL over-expression can cause receptor auto-activation (Schecterson et al., 2010). Our data suggests that a similar mechanism occurs in our experimental system. The close proximity of the over-expressed receptors on the membrane probably allows dimerization and activation of the receptors independently from BDNF so that even when BDNF is added to the system any additional effect on TrkB activation is not detectable.

We mentioned above that SH-SY5Y cells express basal levels of the TrkB receptors. To investigate the role of TrkB isoforms interaction on TrkB FL mediated signaling, we co-expressed exogenous TrkB FL with truncated isoforms and mutated variants. Co-transfection of the TrkB FL with the truncated T and SHC isoforms or the tyrosine kinase inactive mutant abrogated the increase in APP FL levels induced by TrkB FL. Interestingly TrkB FL/SHC co-transfection had higher APP FL levels than TrkB FL/T co-transfection. This result points to a possible difference between the two TrkB truncated isoforms in the regulation of the TrkB FL catalytic receptor. The fact that in the co-transfection experiments TrkB FL/SHC showed increased APP FL levels compared to TrkB FL/T also suggests TrkB SHC does not have a dominant negative effect on TrkB FL. Maybe this is due to recruitment of SHC adaptor protein on its binding site in TrkB FL/SHC heterodimers since the SHC binding site of TrkB SHC could be phosphorylated in trans by TrkB FL. Interestingly, TrkB K571M, functions, likewise TrkB T, as a dominant negative suggesting that even if the SHC binding site is not affected, two tyrosine kinase sites are necessary to induce the observed effects, at least on a TrkB FL isoform. Supporting this, co-transfection of TrkB FL/Y515F had similar effects on APP FL to TrkB FL/FL co-transfection. TrkB Y515F was less effective in inducing an increase in APP FL levels than TrkB FL. This suggests that the tyrosine kinase activity of the receptor is very important in increasing APP levels but that two functional SHC binding sites in a heterodimer are more efficient than one. TrkB FL/Y816F co-transfection was indistinguishable from TrkB FL single transfection suggesting that PLC-γ signaling is not crucial in determining increased APP FL levels. BDNF treatment of the co-transfected cells

seemed to accentuate the effect of TrkB FL on APP FL levels. For example it increased APP FL in cells co-transfected with TrkB FL/T but not in cells co-transfected with TrkB FL/K571M. On the contrary, TrkB FL/Y515F co-transfection seemed to cause lower APP FL levels when BDNF was applied. It is intriguing to think that when TrkB isoforms are all represented in the cell, like in this experimental setting, BDNF might promote homodimerization versus heterodimerization. The issue of preferential homo versus hetero interaction of TrkB isoforms has not been investigated so far and it would be important to address.

Conclusions

This work demonstrates the difference between TrkB truncated isoforms and TrkB FL in affecting APP processing and APP levels. Not only do the truncated isoforms have a different effect when transfected alone, they were also able to modify the TrkB FL effects when co-transected with it. This finding points to the importance of the TrkB isoforms in the pathogenesis of AD. In fact all the isoforms are present on neurons and other cell types of the CNS. The proportion of TrkB FL to TrkB T and TrkB SHC is then important to determine signal transduction. Since all the isoforms bind BDNF in the extra-cellular domain, a therapeutic approach that uses BDNF biomimetic drugs might not be as effective as if only TrkB FL was expressed. This is assuming that only TrkB FL mediates the beneficial effects in AD context. In fact, truncated isoforms could scavenge the drugs, activate alternative non-beneficial signaling pathways, decrease the benefit of engaging TrkB FL triggered pathways and

also inhibit the TrkB FL effects. Depending on the relative amounts of the TrkB receptors on the cells, BDNF-mimetic drugs could cause an over-all worsening of the conditions (Dorsey et al., 2002) by, for example, increasing the inflammation response. It will be important in the future to dissect the contributions of the TrkB isoforms to BDNF dependent and independent signaling pathways in the context of AD to better understand which ones are beneficial and which ones are not.

2.5 Figure Legends:

Figure 2.1: TrkB isoforms structure and functional domains. The extra-cellular portion of the receptor is conserved. The intracellular domain differs among the isoforms. TrkB FL contains a tyrosine kinase domain a SHC binding domain and a PLC-γ binding domain. TrkB SHC contains a SHC binding domain and TrkB T does not have any functional domain. The colored boxed areas represent the domains that have been mutated. The amino acid in red is the wilt type residue and the one in blue the mutation inserted.

Figure 2.2: AICD-mediated Luciferase activity in SH-SY5Y-APP-Gal4 cells with TrkB knock-down or TrkB over-expression. (A) ShRNA mediated NTRK2 knock-down: luciferase activity is decreased by *NTRK2* knock-down compared to non-silencing control ($p=0.03$). There is no significant difference between luciferase activity in *NTRK2* knock-down and *APP* knock-down cells. ShRNA constructs:

negative control, non -silencing, scrambled shRNA sequence; positive control, APP, shRNA targeting APP; experimental shRNA, NTRK2.1-3, shRNA constructs targeting all TrkB isoforms. The experiment was repeated four times independently and each time with six replicates. (B) TrkB isoform over-expression. Luciferase activity was not altered by TrkB T compared to GFP control. Luciferase activity was increased by TrkB FL ($p=0.01$) and decreased by TrkB SHC ($p=0.01$) compared to a GFP-control vector. The experiment was repeated 3 to 6 times independently with 6 replicates each.

The y-axis represents the arbitrary light units of firefly luciferase normalized to cell number (A) or transfection efficiency (B). Error bars=SEM. Statistical analysis: ANOVA and student t-test with Bonferroni correction where applicable.

Figure 2.3: TrkB FL mutants on the intracellular functional domains change the effect on AICD-mediated luciferase activity. (A) The TrkB SHC isoform significantly decreases luciferase activity when compared to TrkB T ($p=0.001$). The TrkB SHC Y515F mutant, unable to bind SHC, increases luciferase activity compared to TrkB SHC ($p<0.001$) and does not significantly alter luciferase activity compared to TrkB T ($p=0.1$). (B) TrkB Y515F (no SHC binding) does not significantly alter luciferase activity compared to TrkB FL. TrkB K571M with an inactive tyrosine kinase domain significantly decreases luciferase activity compared to TrkB FL ($p=0.0006$); TrkB Y515F/K571M significantly decreases luciferase activity compared to TrkB FL ($p=0.002$) but not to a greater extent of TrkB K571M. TrkB Y816F that does not bind PLC-γ also significantly decreases luciferase activity

compared to TrkB FL (p=0.0002). 4-8 independent experiments with 6 replicates each, error bars SEM. 4-6 independent experiments with 6 replicates each, error bars SEM. Statistical analysis: student t-test with Bonferroni correction.

Figure 2.4: *NTRK2* knock-down decreases APP full-length levels. (A) Representative Western Blot of APP full length and actin as a loading control in lysates from cells transfected with shRNA constructs targeting *APP*, *luciferase* (LUC), *NTRK2* and a negative, non silencing (CTRL), control. (B) Average quantification of APP full-length levels from four independent Western Blot analyses conducted on independent transfections. Within each transfection APP FL densitometry was normalized to the APP FL densitometry of the cell lysate obtained from cells transfected with CTRL shRNA. A statistically significant difference was observed between CTRL and the APP or NTRK2 shRNA targeting constructs ($p<0.05$). The experiment was repeated four times independently with three replicates each, error bars indicate SEM. Statistical analysis: ANOVA.

Figure 2.5: TrkB isoform over-expression affects APP FL levels and APP processing. (A) TrkB FL increases APP FL levels compared to TrkB T (p=0.03) and TrkB SHC isoforms (p=0.008). 5 independent experiments in duplicate, error bars indicate SEM. (B) TrkB FL increases AICD levels compared to TrkB T and TrkB SHC decreases AICD levels. (C) TrkB FL, TrkB T and TrkB SHC do not alter C83 levels. (D) sAPP levels in conditioned media. TrkB FL significantly decreases sAPP levels compared to TrkB T (p=0.003). (E) APPm/im ratio is increased by TrkB FL

(p=0.03) but not altered by TrkB SHC compared to TrkB T. The Western blot shows a representation of APP full length in its fully glycosylated form (APPm= mature) and partially glycosylated form (APPim= immature). Below the blot, the quantification of the APPm/im ratio (1, 1.4 and 0.9) is reported to facilitate the identification of the differences that are not readily apparent in the picture. The bar graph represents quantification of 4 independent experiments normalized to APPm/im values obtained with TrkB T transfection. The experiment was repeated four times independently in duplicate. Error bars indicate SEM. Statistical analysis: student t-test with Bonferroni correction.

Figure 2.6: γ-secretase inhibition increases sAPP levels independent of TrkB isoforms. (A) Representative Western blot showing sAPP levels in conditioned media of SH-SY5Y APP-Gal4 cells transfected with the different TrkB isoforms and treated with vehicle (DMSO) or L-685. (B) Quantification of 4 to five independent experiments. sAPP levels are normalized to the protein concentration of the corresponding cell lysates to normalize for cell number. The sAPP levels are then divided for the corresponding sAPP levels in TrkB T transfected cells within independent experiments and within treatments. TrkB T then has value 1 and all the statistical comparisons are made to TrkB T. TrkB FL induces a decrease in sAPP levels in both DMSO and L-685 treatments while TrkB SHC does not affect sAPP levels. (C) sAPP levels were normalized to the sAPP levels obtained in TrkB T transfected cells within independent experiments. This normalization method allows identifying differences induced by γ-secretase cleavage impairment (L-685 treatment)

in cells transfected with TrkB isoforms. L-685 treatment induced a significant increase in sAPP levels independently of TrkB isoforms (p=0.0002). Statistical analysis and comparisons performed are reported to the left of the bar graphs.

Figure 2.7: Short term or long term BDNF treatment of TrkB isoforms transfected cells. APP full length (FL) levels are increased by a 10 minutes BDNF treatment of TrkB FL transfected cells while a 24 hours treatment shows lower APP FL levels. TrkB T and TrkB SHC transfected cells are not significantly affected by short term or long term BDNF treatment.

Figure 2.8: **Co-transfection of TrkB FL with isoforms and mutants alters the TrkB FL effects on APP full length**. TrkB FL transfection increases APP FL levels compared to when TrkB FL is co-transfected with TrkB T, TrkB SHC or TrkB K571M. APP FL levels did not significantly differ between TrkB FL transfected cells and TrkB FL co-transfection with TrkB Y515F or TrkB Y816F. 24 hours BDNF treatment of co-transfected cells did not significantly alter APP FL levels compared to co-transfected but non-treated cells.

Figure 2.1

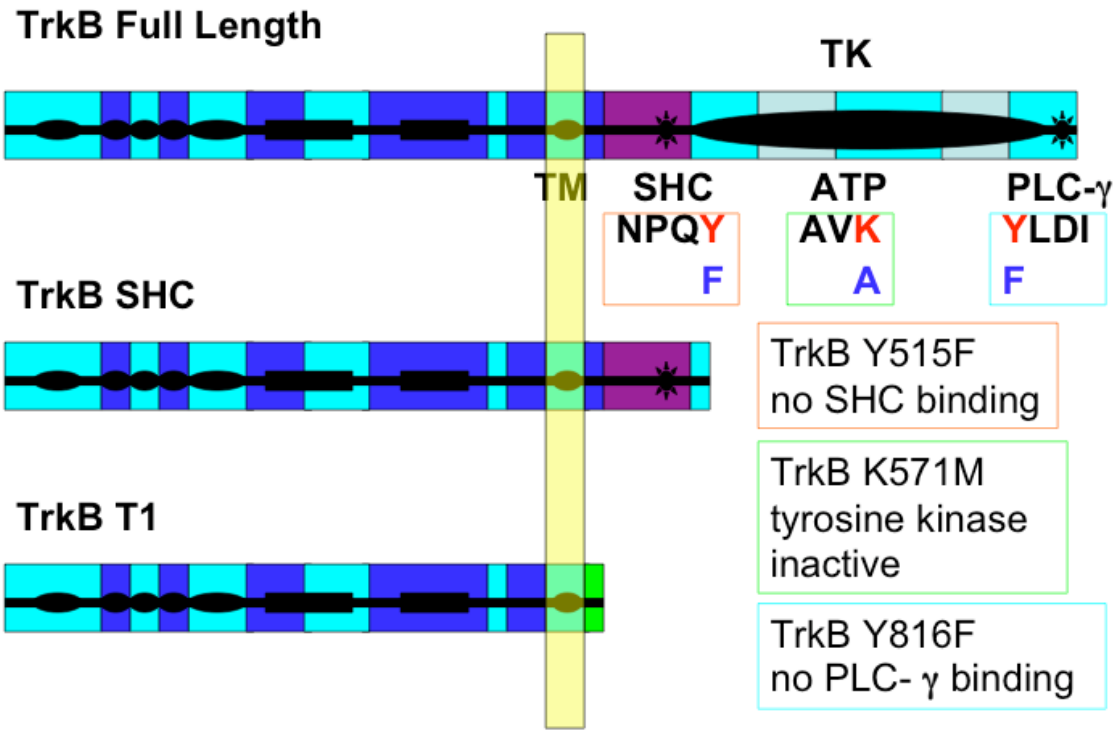

TrkB Full Length
TK
TM
SHC
NPQY
F
ATP
AVK
A
PLC-γ
YLDI
F
TrkB SHC
TrkB T1
TrkB Y515F
no SHC binding
TrkB K571M
tyrosine kinase
inactive
TrkB Y816F
no PLC- γ binding

Figure 2.2

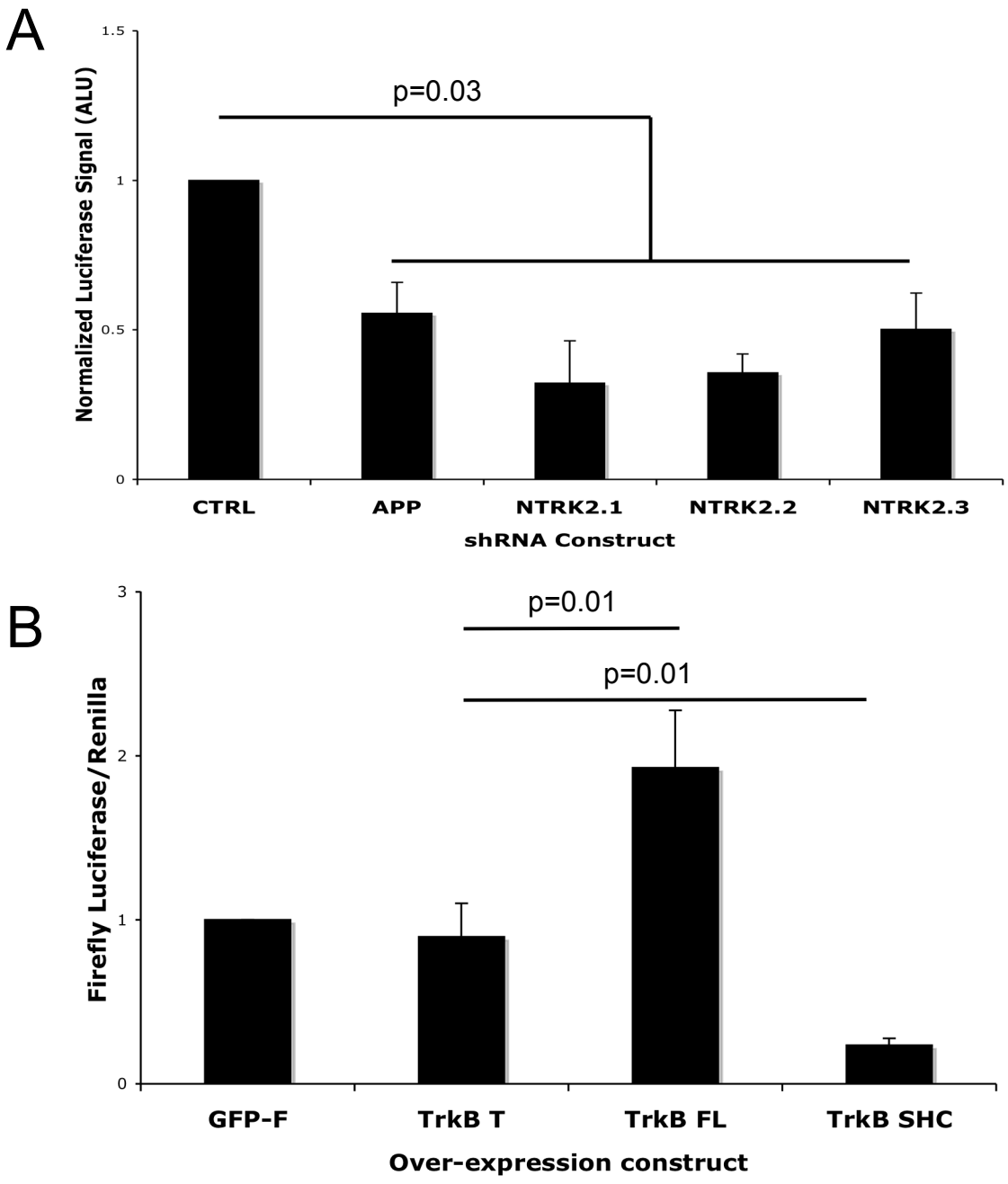

A
Normalized Luciferase Signal (ALU)
1.5
1
0.5
0
p=0.03
CTRL
APP
NTRK2.1
NTRK2.2
NTRK2.3
shRNA Construct
B
Firefly Luciferase/Renilla
3
2
1
0
p=0.01
p=0.01
GFP-F
TrkB T
TrkB FL
TrkB SHC
Over-expression construct

Figure 2.3

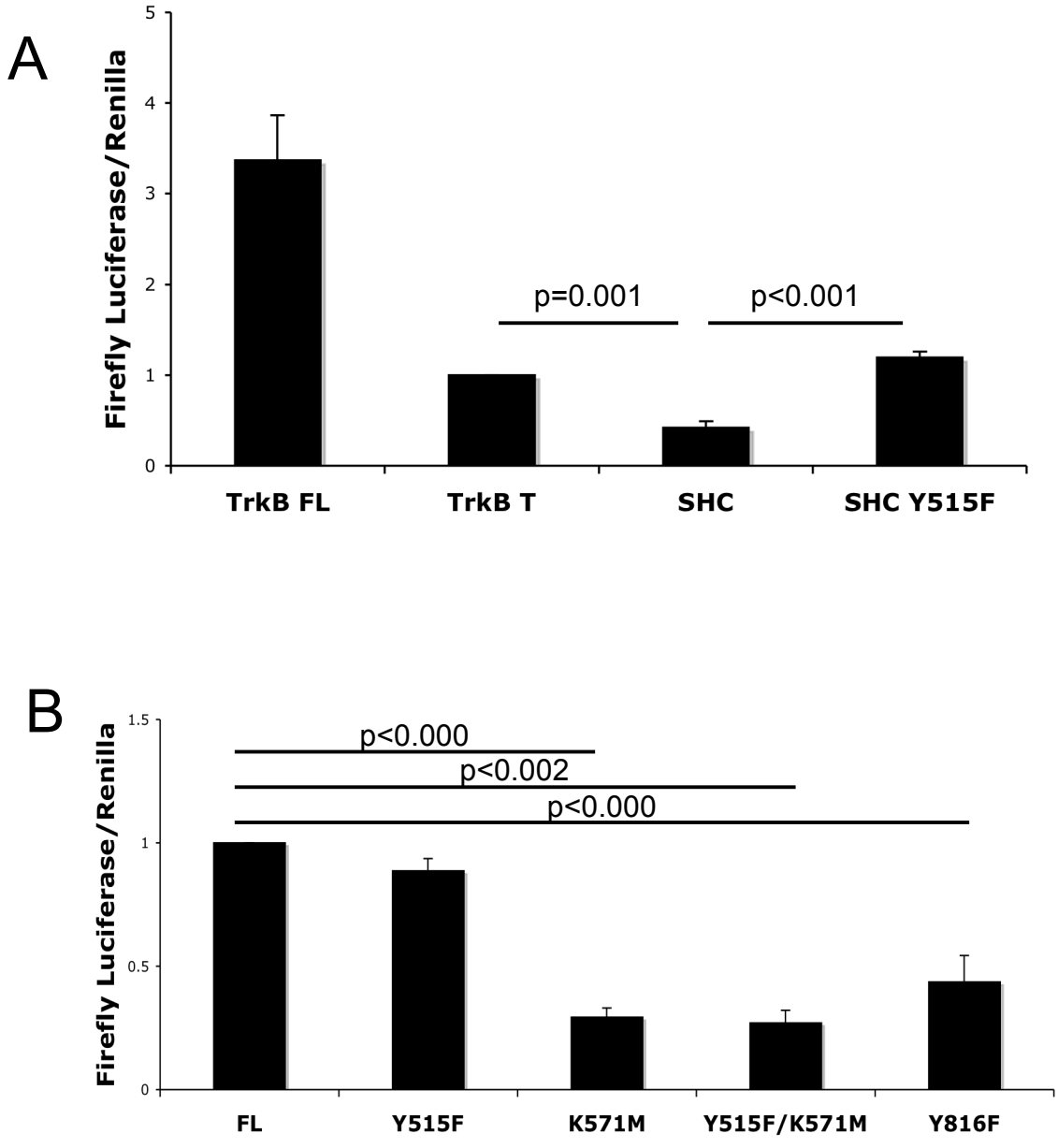

A
Firefly Luciferase/Renilla
5
4
3
2
1
0
p=0.001
p<0.001
TrkB FL
TrkB T
SHC
SHC Y515F
B
Firefly Luciferase/Renilla
1.5
1
0.5
0
p<0.000
p<0.002
p<0.000
FL
Y515F
K571M
Y515F/K571M
Y816F

Figure 2.4

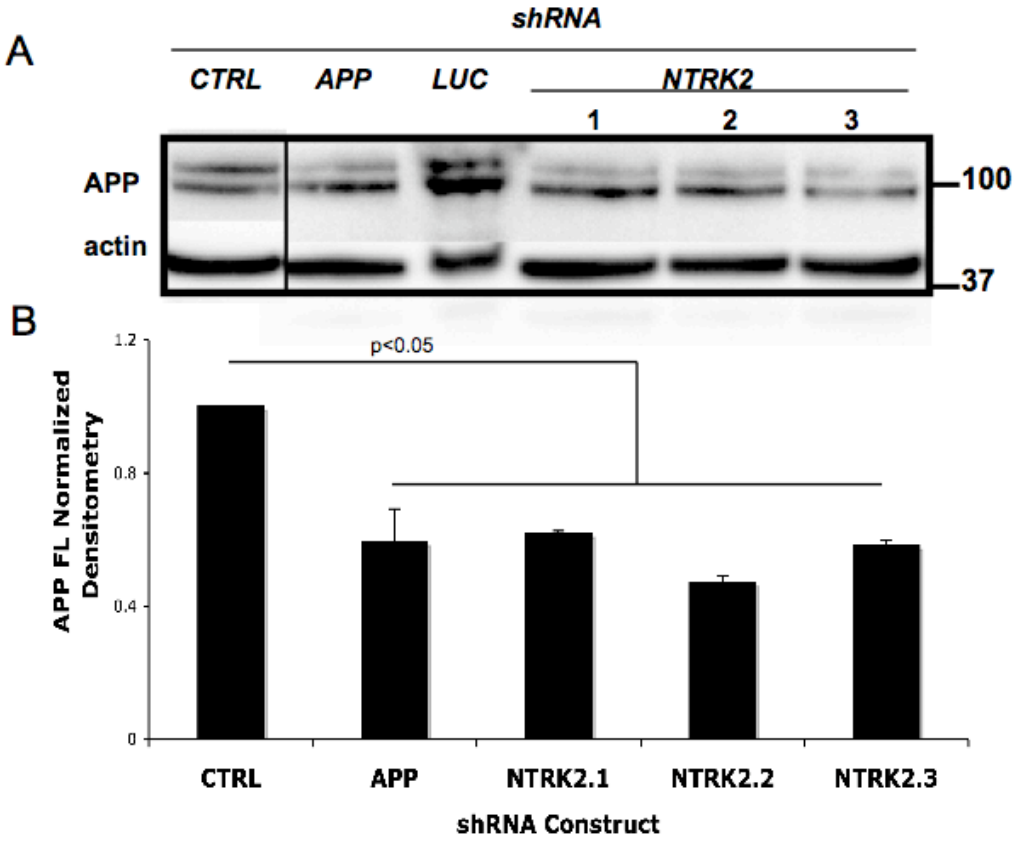
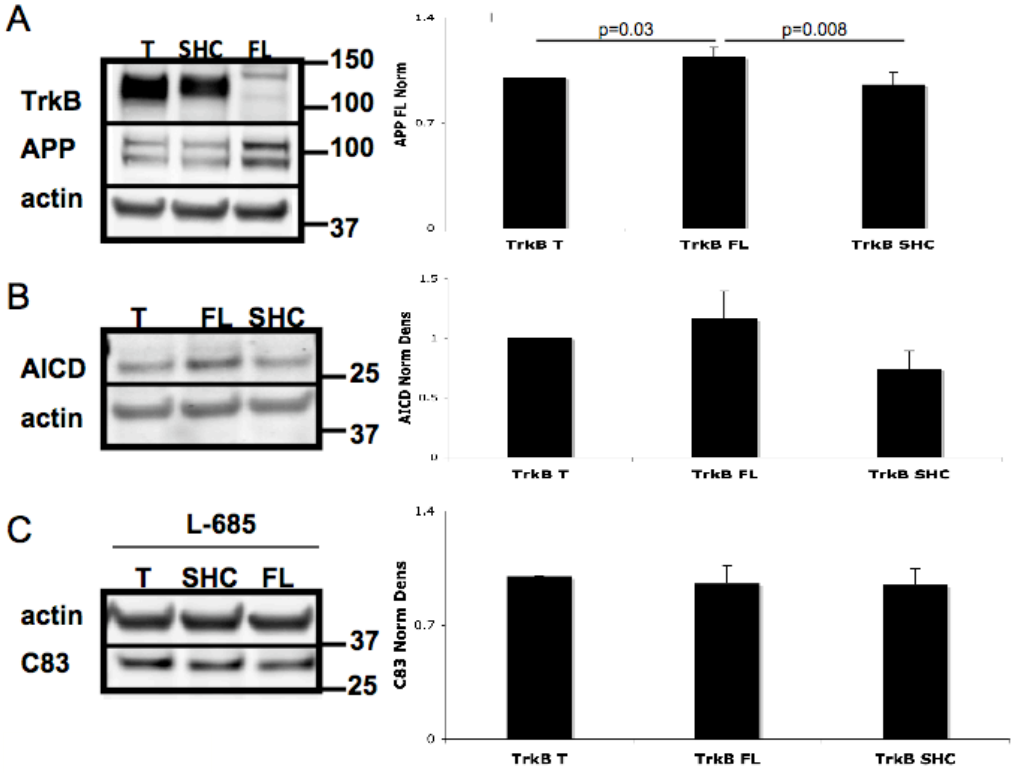

A
shRNA
CTRL
APP
LUC
NTRK2
1
2
3
APP
actin
100
37
B
p<0.05
APP FL Normalized Densitometry
1.2
0.8
0.4
0
CTRL
APP
NTRK2.1
NTRK2.2
NTRK2.3
shRNA Construct

Figure 2.5:


A
T SHC FL
TrkB
APP
actin
150
100
100
37
APP FL Norm
p=0.03
p=0.008
TrkB T
TrkB FL
TrkB SHC
B
T FL SHC
AICD
actin
25
37
AICD Norm Dens
TrkB T
TrkB FL
TrkB SHC
C
L-685
T SHC FL
actin
C83
37
25
C83 Norm Dens
TrkB T
TrkB FL
TrkB SHC

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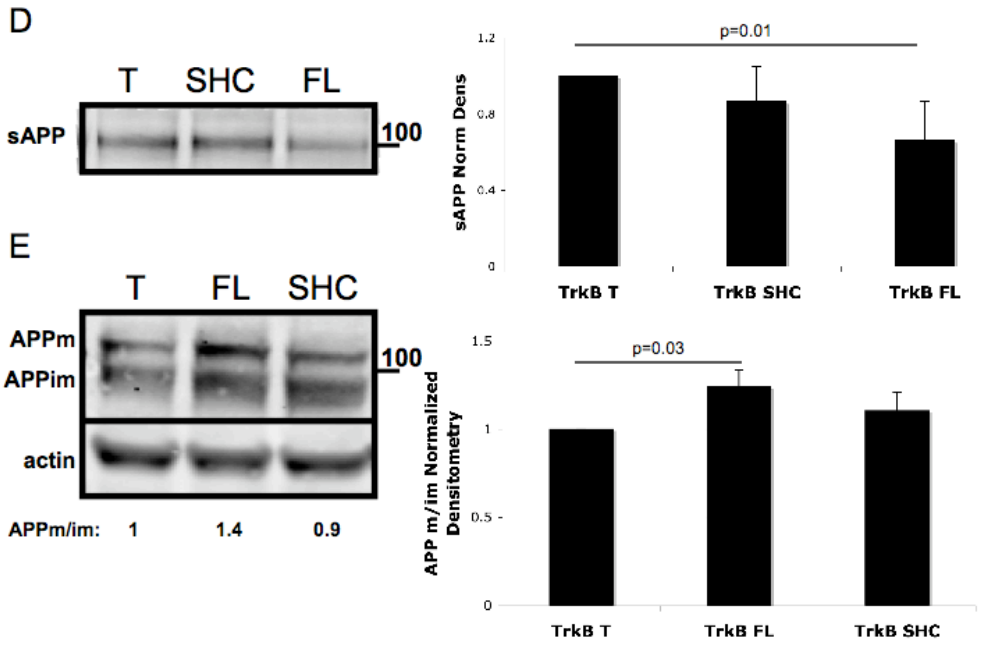


Figure 2.6

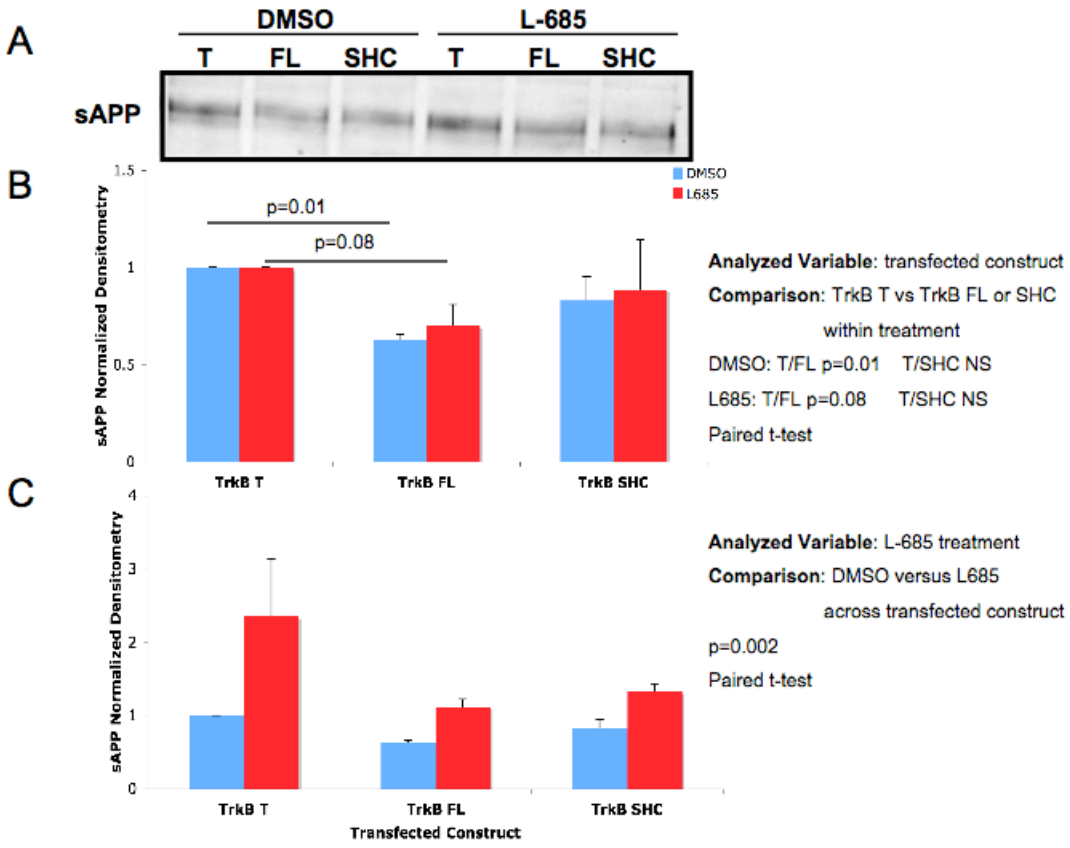

A
DMSO
L-685
T
FL
SHC
T
FL
SHC
sAPP
B
sAPP Normalized Densitometry
1.5
1
0.5
0
DMSO
L685
p=0.01
p=0.08
TrkB T
TrkB FL
TrkB SHC
Analyzed Variable: transfected construct
Comparison: TrkB T vs TrkB FL or SHC
within treatment
DMSO: T/FL p=0.01 T/SHC NS
L685: T/FL p=0.08 T/SHC NS
Paired t-test
C
sAPP Normalized Densitometry
4
3
2
1
0
TrkB T
TrkB FL
TrkB SHC
Transfected Construct
Analyzed Variable: L-685 treatment
Comparison: DMSO versus L685
across transfected construct
p=0.002
Paired t-test

Figure 2.7

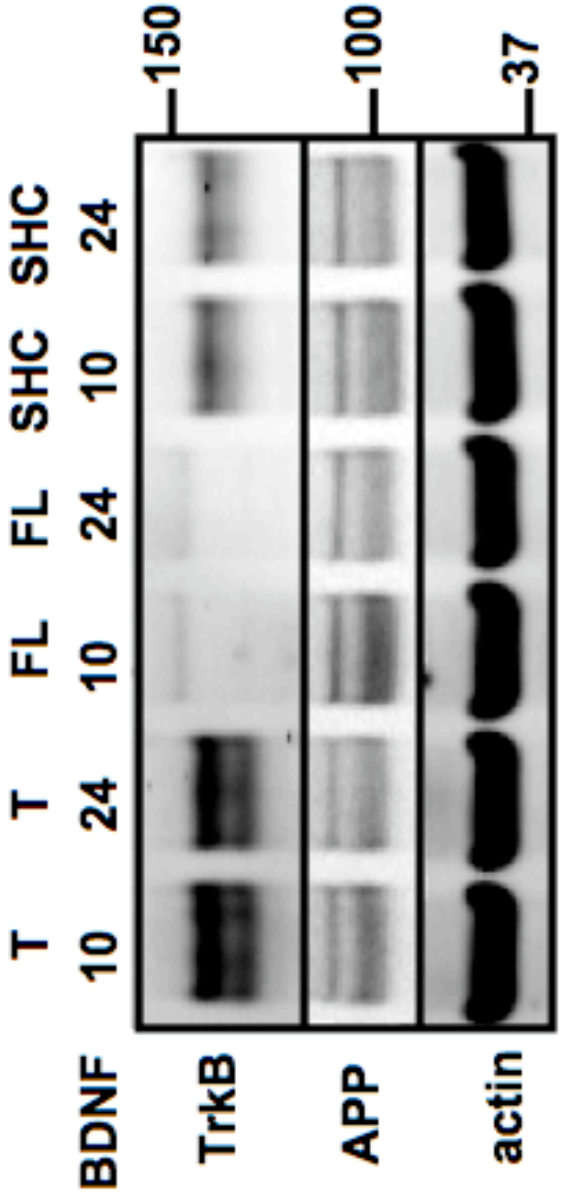
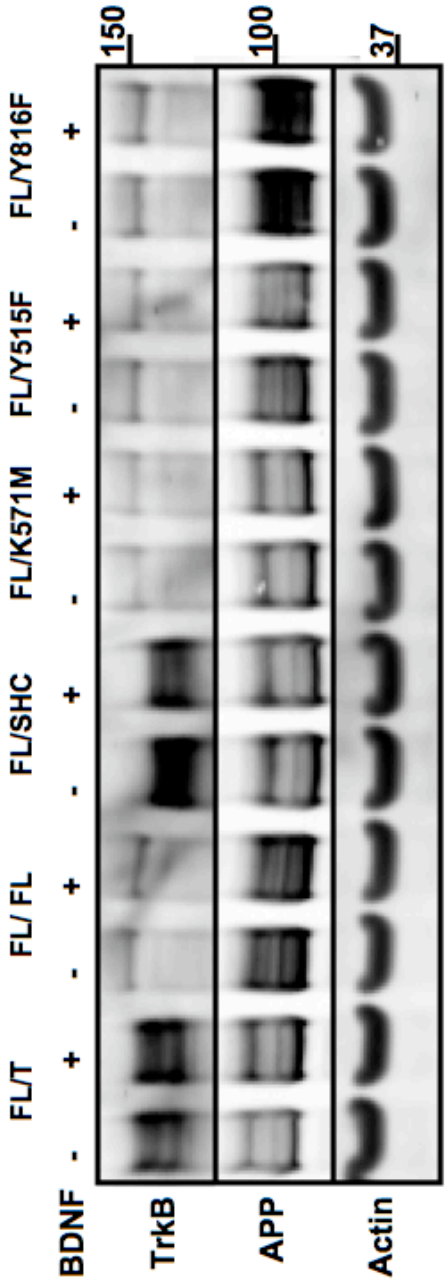


Figure 2.8


150
100
37
BDNF
TrkB
APP
Actin
FL/T
FL/ FL
FL/SHC
FL/K571M
FL/Y515F
FL/Y816F
-
+
-
+
-
+
-
+
-
+
-
+

3. CHAPTER 3: TrkB isoforms differentially regulate amyloid precursor protein Swedish mutant glycosylation and processing

Abstract

Alzheimer's Disease (AD) is a neurodegenerative disease that causes progressive memory loss which mainly affects people older than 65. At the molecular level, the main pathological mechanism of AD is the accumulation of Aβ, a cleavage product of Amyloid Precursor Protein (APP), which causes synaptic dysfunction and contributes to neuronal death. Age, environmental factors and genetic factors all contribute to determine susceptibility to AD. Polymorphisms in the *NTRK2* gene are among the genetic factors associated with increased AD risk. *NTRK2* encodes the neurotrophin receptor TrkB. Alternative splicing of the *NTRK2* mRNA results in at least three major receptor isoforms, TrkB FL, TrkB SHC and TrkB T. All of these bind the same ligand, Brain Derived Neurotrophic Factor (BDNF). While TrkB FL has been shown to regulate APP expression levels and processing, the exact role of TrkB FL in an AD context has not been defined. In addition, the effects of the TrkB SHC and TrkB T isoforms on APP metabolism have not been investigated. We tested the hypothesis that the TrkB isoforms differentially regulate APP metabolism. We found that TrkB FL increased APP FL levels, decreased APP glycosylation and decreased C99/C83 levels compared to the truncated isoforms. These effects were BDNF independent but dependent on the tyrosine kinase activity of the FL receptor. We also found that co-

expression of the TrkB FL receptor with the truncated isoforms can modulate its effect on APP. In particular TrkB T completely eliminated the effects of TrkB FL while TrkB SHC did not. These results demonstrate that TrkB isoforms differentially regulate APP metabolism. All these isoforms are co-expressed in neurons, it is important to understand the combinatorial and isoform-specific signaling in the context of AD. Especially, since TrkB FL/BDNF signaling has been shown to be beneficial in ameliorating cognitive deficits.

3. 1 Introduction

Late onset Alzheimer's Disease (LOAD) is the most common cause of dementia in Europe and the USA (Wancata et al., 2003). Since there is no current cure for AD and the number of cases is projected to triple in the next ten years (Wancata et al., 2003), the caregivers, the national health systems and the society are challenged to find an efficient, cost effective therapy (Maslow, 2010{Sloane, 2002 #143)}.

A major challenge in developing effective AD therapies is the lack of understanding of the causative pathological mechanism. The dominant mutations causing early onset AD (EOAD) are well characterized and located in three genes: amyloid precursor protein (APP) and presenilin 1 and 2 that are involved in APP proteolytic cleavage. APP cleavage is the most important pathologic mechanism underlying AD: α-secretase cleavage products (C83, p3 and sAPP-α) are non-toxic and generally protective while one of the β-secretase products (C99, Aβ and sAPP-β) Aβ, leads to synaptic dysfunction and eventually neuro-degeneration (De Strooper

and Annaert, 2000; Kamenetz et al., 2003; Oddo et al., 2003). Mutations in *APP* that increase the β-secretase over α-secretase processing cause EO heritable AD (Citron et al., 1992; Mullan et al., 1992). Studies on twins suggest that the genotype of individuals is a more important risk factor than environment even in the development of LOAD (Pedersen et al., 2004; Gatz et al., 2006). Therefore to further understanding the causes of LOAD is fundamental to focus the research on the genes that might affect APP metabolism.

To find putative APP metabolism regulators, we performed a cell-based functional screen (Zhang et al., 2007) of genes located between 80-120 cM of chromosome 9 since this region displays significant linkage to AD in multiple samples (Blacker et al., 2003; Bertram et al., 2007a; Perry et al., 2007). We identified genes that affect APP metabolism as possible genetic risk factors for AD and potential therapeutic targets (Ansaloni, Zhang, Khandelwal and Saunders, unpublished observations). One of these genes was *NTRK2,* the gene encoding for TrkB (Tropomyosin Related Kinase B) which is a receptor for Brain Derived Neurotrophic Factor (BDNF) (Soppet et al., 1991; Ansaloni et al., 2010).

Our data implicating TrkB in AD pathogenesis is supported by a number of other observations. Single nucleotide polymorphisms (SNPs) within the *NTRK2* gene have been significantly associated with AD in some genetic association studies (Chen et al., 2008; Cozza et al., 2008) but not in others (Vepsalainen et al., 2005; Bertram et al., 2007a). TrkB signaling via PI3K increases APP expression levels (Ruiz-Leon and Pascual, 2001, 2004). Moreover sAPP-α has been shown to increase when full-length TrkB is up-regulated by retinoic acid treatment in cell lines and when it is stimulated

by Brain Derived Neurotrophic Factor (BDNF) (Holback et al., 2005). The pathways that increase the production of BDNF and TrkB full length receptor, like an healthy diet and exercise, are generally protective in a AD setting (Ding et al., 2008; Komulainen et al., 2008; Schobel et al., 2008; Bousquet et al., 2009; Katsuki et al., 2009; Nichol et al., 2009). Conversely Aβ has been found to reduce TrkB FL/BDNF levels and to impair TrkB mediated signaling (Olivieri et al., 2003; Tong et al., 2004; Poon et al., 2009). Decreased levels of TrkB FL and BDNF are observed in AD brain tissue when compared to controls (Ginsberg et al., 2006). Interestingly, the lowest TrkB FL levels are found in brain samples of AD patients displaying the lowest global cognitive score (GCS) and mini-mental state examination score (MMSE) while higher TrkB FL levels are found in samples from patients displaying higher scores (Ginsberg et al., 2006).

TrkB is expressed in the human brain as three major splice variants. Each of these can bind BDNF in the conserved extra-cellular domain but differ in the cytoplasmic domain (Figure 3.1). The longest isoforms, full-length TrkB (TrkB FL; 822-838 amino acids in length) contain tyrosine kinase, SHC binding, and PLC-γ binding domains in the cytoplasmic portion. The intermediate length isoform (TrkB SHC; 537-553 amino acids in length) contains only a SHC binding domain while the truncated TrkB isoform (TrkB T; 477 amino acids in length) does not contain any known functional domains in the cytoplasmic domain (Klein et al., 1990a; Middlemas et al., 1991; Stoilov et al., 2002). Upon ligand binding, the TrkB FL receptors dimerize and auto-phosphorylate triggering intracellular signaling cascades such as MAK/ERK signaling, IP3K and IP3/DAG signaling (Eide et al., 1996; Reichardt,

2006). The intracellular domain of TrkB T is highly conserved and is important for mediating the receptor intra-cellular signaling (Armanini et al., 1995; Baxter et al., 1997; Rose et al., 2003). TrkB T seems to have a dominant negative effect on TrkB FL in neurons (Eide et al., 1996; Ninkina et al., 1996; Haapasalo et al., 2001). The antagonistic effect of TrkB T on TrkB FL signaling is evident since knock-down of TrkB T in mice with three copies of APP, which develop Alzheimer's like symptoms, improves cognitive decline (Dorsey et al., 2006). The role of the TrkB SHC isoform on TrkB FL regulation has not been characterized.

These isoforms are expressed at different levels in glia and neurons. TrkB T is expressed at higher levels in glial cells than neurons but its expression levels in neurons can change as a consequence of TrkB FL signaling (Armanini et al., 1995). During development and aging there is a decrease in the expression levels of TrkB FL receptors and an increase in the expression of TrkB T and SHC isoforms (Allendoerfer et al., 1994; Luberg et al., 2010). This suggests that TrkB truncated receptors might play an important role in Alzheimer's disease if we consider AD as a developmental disorder. The higher levels of expression of these isoforms on glial cells (Rose et al., 2003) compared to neurons also point to a possible role of TrkB truncated receptors in mediating inflammatory response which is central in AD.

Signaling of auto-activated TrkB receptor has been demonstrated in absence of BDNF (Rajagopal et al., 2004; Mojsilovic-Petrovic et al., 2006; Wiese et al., 2007; Jeanneteau et al., 2008; Schecterson et al., 2010). Since BDNF levels are decreased in AD patients (Peng et al., 2009), while the levels of TrkB truncated isoforms are

increased or unaltered (Allendoerfer et al., 1994; Luberg et al., 2010) BDNF independent activation of the TrkB signaling might have an important role in AD.

Our study aimed at characterizing the BDNF independent effects of the different TrkB receptor isoforms on the metabolism of APP. We used HEK293 cells over-expressing APP containing the Swedish mutation (APP^{Swe}; K670N and M671L) that causes EOAD in humans. We found that TrkB FL over-expression decreased the levels of N-O glycosylated APP and altered APP proteolysis leading to decreased ratio of C99/C83 fragments. These effects were dependent on the tyrosine kinase activity of the receptor. The TrkB FL mediated effects were altered when TrkB FL was co-expressed with the other isoforms. TrkB T or the TrkB FL tyrosine kinase activity deficient receptors eliminated the effect on APP maturation and processing while TrkB SHC did not. This demonstrates that truncated TrkB isoforms differentially regulate TrkB FL signaling. Moreover, BDNF application did not alter the observed effects suggesting that TrkB receptors can regulate APP metabolism through a BDNF independent mechanism.

3.2 Materials and Methods

3.2.1 Constructs and site directed mutagenesis

The TrkB full length and truncated GFP fusion constructs were a generous gift of Dr. Eero Castren (University of Helsinki, Finland). Site-directed mutagenesis (Stratagene Quikchange mutagenesis kit) of the TrkB full-length receptor was utilized to generate point mutants on the main functional domains. Here we refer to the mutants according to the TrkB sequence: TrkB Y515F, does not bind SHC, (TrkB FL Y515F); TrkB K571M, tyrosine kinase inactive (TrkB FL K571M) and TrkB Y816F, does not bind PLC-γ (TrkB FL Y816F). While in the literature the numbering of the mutant amino acid often refers to the analogous position in the TrkA receptor that shares the same functional domains of TrkB. Therefore TrkB FL K560M indicates the tyrosine kinase dead receptor since it is mutated on the ATP binding site; TrkB FL Y490F indicates the receptor mutated on the SHC binding site; TrkB FL Y785F indicates the receptor mutated on the PLC-γ binding site (Middlemas et al., 1991; Atwal et al., 2000). TrkB SHC indicates the other major human truncated isoform (isoform d and e, NCBI Gene NM_001018064$_{+}$2 and NM_001018066$_{+}$2). TrkB SHC was obtained by insertion of the exon 19 after the SHC binding site on the original TrkB FL construct followed by a stop codon. Mutagenesis was carried out according to manufacturer's instructions and the primers employed are reported in the table below (the bolded sequences represent the mutations/insertion):

Primers	Sequence 5' to 3'
TrkB.rat.Y515F.F	GTCATTGAAAACCCCCAGT**T**CTTCGGTATCACCAACAG
TrkB.rat.Y515F.R	CTGTTGGTGATACCGAAG**A**ACTGGGGGTTTTCAATGAC
TrkB.rat.Y816F.F	GCGTCGCCCGTCT**T**CCTGGACATCCTAG
TrkB.rat.Y816F.R	CGCAGCGGGCAGA**A**GGACCTGTAGGATC
TrkB.rat.Shc.F	CTCAAGCCGGACACA**TGGCCCAGAGGTTCCCCCAAGACCGCCTGATAGTAA**TTTGTTCAGCACATC
TrkB.rat.Shc.R	GATGTGCTGAACAAA**TTACTATCAGGCGGTCTTGGGGGAACCTCTGGGCCA**TGTGTCCGGCTTGAG
TrkB_Rat_K571M.F	GGTGGCCGTGA**T**GACGCTGAAGG
TrkB_Rat_K571M.R	CCTTCAGCGTC**A**TCACGGCCACC

Table 3.1: Primer sequences used to obtain the point mutations and the sequence insertion on the TrkB FL receptor. The mutated bases are represented in bold.

Successful mutations were identified by sequencing and one clone per construct was transformed in *E.coli* (DH5-α competent cells, InVitrogen). Transformed bacteria were selected on 100 μg/ml ampicillin LB-agar plates and liquid cultures were grown overnight at 37 °C. Bacterial cultures were miniprepped (MiniPrep Kit, Quiagen) and used for transfection after DNA quantification.

3.2.2 Cell culture, transfections and drug treatments

293HEK stably expressing human APP^{Swe} were cultured in DMEM media (Gibco) supplemented with 10% FBS (Gibco) with penicillin/streptomycin and 200 μg/ml of G418 as selection antibiotic. Cells were maintained at 37 °C in 5% CO_2. Twenty-four hours before transfection cells were split at approximately 60% confluency in 6-wells

plates or 12-wells plates. Cells were transfected for 6 hours in serum free media with Arrest-In transfection reagent (Open Biosystems) according to manufacturer's instructions. Plasmids were transfected in equimolar concentrations: 2 μg of the longest plasmid (TrkB FL and mutants) were used per well of a six-well plate and 1μg/well of a 12-well plate. Amounts in μg of the other plasmids were calculated accordingly based on base pair number. Two replicate wells per construct, per treatment were set up in each experiment and experiments were independently repeated at least three times. Six hours post-transfection, the media was changed to complete media (see above). The day after transfection, the cells were observed by fluorescence microscopy to assess transfection efficiency since all TrkB transfected constructs were expressed as GFP fusion proteins. Twelve to fourteen hours before collection the cells were treated with L-685,458 (Sigma) at 2.5 μg/ml final concentration in DMSO (0.5% concentration) or with 0.5% DMSO (Sigma) as a vehicle control. BDNF (Gemini, West Sacramento, CA) was prepared as a stock solution in sterile water at 0.1 mg/ml and stored at -20 °C. The final concentration in cell media was 50 ng/ml.

3.2.3 Western Blotting

To assess secreted APP products, sAPP and Aβ, conditioned media was collected in eppendorf tubes, centrifuged at 14,000 rpm for 10 minutes at 4 °C in a table-top centrifuge (Beckman Coulter, Microfuge 22R). The resulting supernatant was collected and 142 μl were mixed with 33 μl of 4X Reducing loading buffer

(InVitrogen) with 0.4% β-mercapto-ethanol (Sigma). These samples were mixed and heated at 70 °C for 10 minutes. The remaining conditioned media was stored frozen at -20 °C for later Western Blot or ELISA analysis. Whole cell lysates were prepared by lysing cells in the plate with ice cold radio immuno-precipitation buffer (150mM NaCl, 1% NP40, 0.5% DOC, 1% SDS, 50mM Tris, pH 8.0) supplemented with Halt cocktail of protease and phosphatase inhibitors (ThermoFisher). Cell lysates were sonicated in an ice-cold water bath sonicator for 6 minutes then centrifuged 20 minutes at 4 °C at 14,000 rpm. The resulting supernatants were collected and protein concentration measured with the BCA protein concentration kit (Pierce) according to manufacturer's instructions. Western blot samples were prepared at a final concentration of 1-2 μg/μl in 4X reducing loading buffer (InVitrogen) and heated at 70 °C for 10 minutes. 15-25 μg of total protein/well from the cell lysates were separated on 4-12% Tris-Glycine midi gels (InVitrogen) in MES-SDS running buffer (InVitrogen) and run at 190 mVolts for 45 minutes. The separated proteins were transferred to PVDF FL membranes (Millipore) using a Semi-Dry transfer apparatus (AA Hoefer TE77X) for 3 hours at 125 mAmp per gel. Membranes were blocked one hour at room temperature using Licor blocking buffer then probed overnight with primary antibodies diluted in Licor blocking buffer at 4-25 °C. Membranes were then washed for 5 minutes 4 times with 0.1% Tween (Sigma) in PBS. After washing, membranes were incubated in the dark with the appropriate secondary antibody IRDye (Licor) diluted in Licor blocking buffer for one hour. Again membranes were washed as above and finally rinsed with PBS. Membranes were scanned on an Odyssey InfraRed scanner (Licor) at appropriate intensities and images acquired at

159 μm resolution. Band intensities were quantified with the provided built-in software (Licor) and always normalized to the actin loading control. When conditioned media was analyzed the signals were normalized to the protein concentration of the corresponding lysates.

3.2.4 Antibodies

Detection of TrkB-GFP tagged constructs utilized mouse anti GFP antibody (1:1000, Living Colors, Clontech); detection of APP full length and C- terminal Fragments utilized A8717 rabbit antibody (1:2000, Rb, Sigma); detection of sAPP 22C11 utilized mouse antibody (1:1000, Millipore); detection of sAPP-α utilized 6E10 mouse antibody (1:1000, Covance); detection of actin A5441 utilized mouse antibody (1:15,000, Sigma); detection of TrkB phosphorylated on the SHC binding site utilized Phospho-TrkA (Tyr490) rabbit antibody (1:1000, Cell Signaling Technology Inc.). The secondary antibodies: IRDye700 anti mouse antibody (1:15,000) and IRDye800 anti rabbit antibody (1:15,000) were obtained from Licor.

3.2.5 Surface biotinylation

Forty-eight hours post-transfection, cells in a 6-well plate were washed in cold PBS twice then incubated 15 minutes in PBS at 4 °C with mild shaking. The PBS was removed and 700 μl/well 0.5 mg/ml **EZ-Link Sulfo-NHS-LC-LC-Biotin** solution (ThermoScientific) was slowly added to each well. Plates were incubated 30 minutes at 4 °C with mild shaking. The biotin solution was then aspirated and replaced with 2 ml/well of 0.1 M ice-cold glycine (EBM) solution to quench the remaining biotin reagent. Each well was then washed two more times with glycine. Finally the plates were incubated for 20 minutes at 4 °C in mild shaking with 2 ml/well of glycine solution. The glycine solution was then removed and cells were washed with PBS

three times. The PBS was aspirated and cells were lysed with RIPA as described above. Protein concentration was determined using a BCA assay (Pierce). Neutravidin agarose beads (High Capacity, #29202, Thermo Scientific) were washed according to manufacturer's instructions. 200 μg of total protein per sample were added to 50 μl of resuspended neutravidin agarose beads in 1.5 ml eppendorf tubes, the volume was adjusted to 200 μl with PBS. The tubes were then incubated in strong shaking for 1 hour at room temperature and centrifuged at 3000 rpm at 4 °C. The supernatant was removed and the beads washed three times in 200 μl cold PBS. The last wash was discarded. The pellet of beads was added with 40 μl of loading buffer (125μl 4X Dye InVitrogen, 25 μl β-mercapto-ethanol, 350 μl H_2O) and heated 10 minutes at 95 °C, then 12 μl of 4X Dye + 20% β-mercapto-ethanol were added to each sample. The samples were then spun for 5 minutes at 14,000 rpm and the supernatant recovered. All of the supernatant was loaded on the gel together with the corresponding lysates as a control.

3.2.6 Aβ 1-40 ELISA Assay

An Aβ 1-40 specific ELISA kit was purchased from Covance (Cat. No. SIG-38951-kit). Manufacture's instructions were followed to set up the assay. Conditioned media previously stored at -20 °C was thawed and 30 μl were diluted in 90 μl freshly made working buffer (provided with the kit). 100 μl of the diluted sample was loaded in each well of the plate. Aβ standards were run in duplicates. As a background control, conditioned media from a plate of HEK293APP_{Swe} cells that had been

transfected with TrkB T and treated with L-685, 458 for 12 hours was used. This treatment eliminates Aβ production. After overnight incubation at 4 °C, the plate was washed six times with 250 μl of washing buffer (provided with the kit). Every wash was performed in an automatic shaker at maximum speed for 20 seconds. The plate was read in a Top-Count Luminometer. Light emission was acquired for 1 second per well with no delay and the values recorded in an excel spreadsheet. The values in Arbitrary Luminescence Units (ALU) were divided by the protein concentration to normalize for cell number in the different wells (μg/ml).

3.2.7 Normalization and Statistical Analysis

Quantification of the Western Blot membranes was performed with the Odyssey System (Licor) analysis software. All the bands of interest were normalized to actin when whole cell lysate was used as a sample. When conditioned media was used, bands were normalized to protein concentration of the corresponding lysates. In each independent transfection involving the TrkB isoforms we normalized bands to the samples transfected with TrkB T because we have found no difference between the effect of TrkB T and a control GFP plasmid on APP (data not shown). Normalizing to TrkB T allowed us to take in account the variation among independent experiments and to run statistical analysis on all the data collected. When we co-transfected two TrkB constructs we normalized the values obtained within each experiment to the co-transfection of TrkB FL alone. The F-max test was used to test significant differences in variances and the Bonferroni correction for multiple comparisons was used to set

the appropriate α value (level of significance). ANOVA was used when the variances were equal among treatment groups followed by a post hoc t-test.

3.3 Results

3.3.1 TrkB FL alters full-length APP levels and APP glycosylation

Given our preliminary data that TrkB alters APP metabolism and the fact that increased APP levels can cause AD (Zigman and Lott, 2007; Ansaloni et al., 2010), we first tested whether TrkB isoforms modulate full-length APP levels. Over-expression of TrkB FL significantly increased APP levels, by ~ 50%, compared to TrkB T and TrkB SHC over-expression (Figure 3.2A and 3.2B). We also observed that TrkB FL over-expression altered APP glycosylation. Specifically, TrkB FL caused the accumulation of an intermediate APP glycosylation form, decreasing the ratio of fully glycosylated, mature APP to the partially glycosylated, immature APP (APPm/APPim) (Figure 3.2A and 3.2C). Over-expression of TrkB T or TrkB SHC had no effect on this ratio (Figure 3.2C) compared to a GFP vector control (data not shown). TrkB T and TrkB SHC transfected cells had a ratio of APP mature to immature close to one, TrkB FL transfected cells had a ratio of ~ 0.6 (Figure 3.2C).

Since APP levels were altered in cells transfected with the TrkB isoforms, we investigated if any of the TrkB isoforms also alters the levels of APP cleavage products. First we quantified the APP products of α- and β-secretase activity in whole cell lysates after treating the cells with L-685,458, a γ-secretase inhibitor. This drug

treatment allows the accumulation of the two membrane associated fragments C83 and C99, products of the α-secretase and β-secretase activities respectively (Figure 3.2A). We observed only modest effects of the TrkB isoforms on the C99 and C83 individual production. In fact, neither C99 levels nor C83 levels are significantly affected by transfection of the TrkB isoforms (not shown). However, we found that TrkB FL significantly decreases the C99/C83 ratio compared to TrkB T while TrkB SHC does not significantly alter the C99/C83 ratio compared to TrkB T, even if it shows a decreased average value (Figure 3.2D).

To further investigate changes in APP proteolysis, we analyzed the conditioned media of TrkB transfected cells to measure secreted APP levels, (sAPP). sAPP is comprised of the products of α and β secretases cleavage, sAPP-α and sAPP-β respectively. These N-terminal fragments of APP are released into the cell culture media. sAPP-α is mainly generated at the cell surface (Lichtenthaler, 2006; Schobel et al., 2008). We found no difference in sAPP levels in the conditioned media of cells transfected with the different TrkB isoforms (Figure 3.3A and 3.3B). We then compared the levels of sAPP-α and observed a trend in increase of sAPP-α levels as a result of TrkB FL over-expression (Figure 3.3A and 3.3C). Interestingly, TrkB FL is also the construct for which we find a significant decrease in C99/C83 ratio.

Secreted Aβ levels were then determined using ELISA. We observed a trend towards decreased $A\beta_{40}$ levels comparing TrkB SHC over-expression to TrkB T over-expression (Figure 3.3D; $p=0.07$). Consistent with the increase in sAPP-α levels, we observed a decrease, albeit non statistically significant, in $A\beta_{40}$ levels in TrkB FL over-expressing cells.

In conclusion, TrkB FL increased APP FL levels and decreased the APPm/im ratio compared to the truncated isoforms. There were no detectable differences between APP FL levels or APPm/im ratio in cells transfected with TrkB T compared to TrkB SHC. TrkB FL also decreased the ratio of C99/C83 while TrkB T and SHC did not differ from each other. There is a trend in increased sAPP-α levels of TrkB FL compared to TrkB T. Surprisingly TrkB SHC induced a significant decrease in Aβ40 levels compared to TrkB T while the $A\beta_{40}$ levels decrease induced by TrkB FL was not significant.

3.3.2 TrkB FL effects on APP metabolism are mainly mediated by the tyrosine kinase domain

To determine the mechanism by which TrkB FL modulates APP levels, glycosylation and processing we investigated the roles of the tyrosine kinase and SHC domains of TrkB. We over-expressed two TrkB FL mutants, TrkB K571M and TrkB Y515F, that eliminate tyrosine kinase activity and SHC binding respectively (Atwal et al., 2000). Compared to TrkB FL, the tyrosine kinase inactive mutant K571M significantly decreased APP levels ($p=0.0002$) while the SHC binding site mutant Y515F had no effect compared to TrkB FL (Figure 3.4A, 3.4B and 3.4C). The levels of APP FL in TrkB K571M over-expressing cells were comparable to the ones observed in TrkB T or TrkB SHC over-expressing cells (Figure 3.4B).

We observed that over-expression of the K571M mutant resulted in a significantly increased APPm/im ratio compared to TrkB FL ($p<0.0001$) while the Y515F mutant did not alter this ratio (Figure 3.4A, 3.4B & 3.4D). The APPm/im ratio

of cells transfected with the K571M mutant was comparable to the one of cells transfected with TrkB T or SHC isoforms.

We measured and calculated the C99/C83 ratio in cells transfected with these mutants (Figure 3.4A, 3.4B, and 3.4E). Again, the K571M mutant had different effects compared to TrkB FL over-expression, displaying a trend towards increased C99/C83 ratio ($p = 0.07$). The APPm/im ratio in TrkB K571M over-expressing cells is comparable to those observed with TrkB T and TrkB SHC over-expression. The TrkB Y515F mutant displayed no difference compared to TrkB FL over-expression.

We measured sAPP and sAPP-α levels to understand the role of the functional domains on sAPP secretion (Figure 3.5A). Interestingly, we observed that the TrkB Y515F mutant increased sAPP levels ($p=0.02$) compared to TrkB FL while K571M had no effect (Figure 3.5A and 3.5B). On the other hand, sAPP-α levels were decreased in K571M over-expressing cells but not Y515F over-expressing cells, compared to TrkB FL (Figure 3.5A and 3.5C). The sAPP-α levels observed in K571M over-expressing cells were comparable to levels observed in TrkB T and TrkB SHC over-expressing cells.

Finally, we measured secreted $A\beta_{40}$ from cells transfected with these mutants (Figure 3.5D). We observed a significant decrease in $A\beta_{40}$ levels upon TrkB Y515F over-expression ($p=0.04$) while TrkB K571M over-expression displayed a trend towards increased levels ($p=0.10$) compared to TrkB FL.

3.3.3 Impairment of γ-secretase activity increases sAPP and sAPP-α levels independently of TrkB isoform or mutant

Trafficking and cleavage of APP are intimately related because APP cleavage depends on it localization to the same cell compartments where the secretases are located. It has been shown that γ-secretase cleavage impairment also affects APP localization, increasing its cell surface retention (Kaether et al., 2002). APP retention at the cell surface increases sAPP-α production (Lichtenthaler, 2006). Our data suggest that TrkB FL and TrkB Y515F might be involved in increasing production of sAPP-α. Therefore we tested the hypothesis that both γ-secretase and TrkB FL/Y515F mediated increase in sAPP-α might occur through overlapping pathways. We then treated TrkB transfected cells with vehicle (DMSO) or L-685 (a γ-secretase inhibitor). We collected the conditioned media of these cells and quantified sAPP-α levels (Figure 3.6A and 3.6B). First, we checked if TrkB isoforms/mutants had an effect on sAPP-α levels in both treatments (Figure 3.6B). We found that both TrkB FL and TrkB Y515F showed a tendency to increase sAPP-α levels in DMSO treated cells compared to TrkB T (Figure 3.6B). Interestingly, TrkB FL did not show the same trend in cells treated with L-685 compared to TrkB T while TrkB Y515F displayed the same trend for both treatments (Figure 3.6B). Secondly, we wanted to assess if γ-secretase inhibition was effective in increasing sAPP-α levels independently of the TrkB isoform or mutant transfected in the cells. Therefore we normalized all sAPP-α values to the TrkB T DMSO treatment control and we compared the TrkB transfected DMSO treated cells with their corresponding L-685

transfections (Figure 3.6C). We found that in all cases γ-secretase inhibition increased sAPP-α levels compared to the DMSO treatment (p=0.00001, Figure 3.6C).

TrkB Y515F also showed a significant increase of sAPP total (sAPP-α+sAPP-β) levels compared to TrkB FL. We then assessed if the combination of TrkB isoforms and γ-secretase inhibition had different effects on sAPP total (Figure 3.7). In fact, γ-secretase impairment increases APP surface retention of APP allowing for more α-secretase cleavage. The remaining sAPP signal is attributable to sAPP-β. We compared TrkB isoforms and mutants within DMSO and L-685 treatments. We found that only TrkB Y515F showed a significant increase in sAPP compared to TrkB FL in DMSO (p=0.05, Figure 3.7A and 3/7B) and L-685 treatment (p=0.04, Figure 3.7B). The other TrkB isoforms did not alter sAPP total levels in either treatment. When we compared DMSO and L-685 treatment across the TrkB constructs we found that L-685 treatment increased sAPP levels in all cases, including TrkB Y515F transfection (p=0.003, Figure 3.7C). This result suggests that the increase in sAPP observed with L-685 treatment is due only to the treatment and is probably attributable to sAPP-α. Instead, since TrkB Y515F has an additive effect to sAPP, the additional sAPP signal might be due to an unknown interaction of TrkB Y515F affecting APP cleavage.

3.3.4 TrkB FL increases APP cell surface levels

The increased sAPP-α levels upon TrkB FL transfection compared to the truncated isoforms and the fact that the Y515F and K571M mutants have opposite effects on sAPP-α levels, suggest differential roles for the SHC and tyrosine kinase

domains in modulating APP cleavage at the cell surface. Since sAPP-α is mainly generated at the cell surface (Lichtenthaler, 2006; Schobel et al., 2008), we tested whether the TrkB isoforms and the TrkB FL mutants modulated cell surface APP levels. Higher surface APP levels would explain the increased sAPP-α levels we observe upon TrkB FL and TrkB Y515F over-expression. Indeed, compared to TrkB T, over-expression of TrkB FL results in a modest increase in cell surface APP levels ($p=0.06$; Figure 3.8A and 3.8B), while over-expression of TrkB SHC does not alter these levels. These TrkB FL effects are due to the tyrosine kinase domain since, compared to TrkB FL over-expression, the K571M mutant significantly reduced cell surface APP levels ($p=0.02$) while the Y515F mutant did not alter these levels. We also noticed the appearance of a faint band of immature APP at the cell surface that was more prominent in when full length TrkB constructs were transfected (Figure 3.8A).

3.3.5 BDNF stimulation does not change TrkB FL effects on APP metabolism

Since the experiments described so far were conducted using a cell line that does not produce BDNF and in absence of exogenous BDNF in HEK293 (Daniel et al., 2007) we wanted to determine if the presence of BDNF would alter the effects resulting from TrkB isoform over-expression. The tyrosine kinase activity seems essential to trigger TrkB FL effects on APP FL levels, APP glycosylation, sAPP levels and C99/C83 ratio, in fact these effects were not recapitulated by the tyrosine kinase inactive mutant TrkB K571M. TrkB FL tyrosine kinase activity is necessary to auto-

phosphorylate the TrkB receptor after BDNF mediated dimerization in physiologic conditions (Reichardt, 2006). We hypothesized that addition of exogenous BDNF might trigger the activation of different pathways resulting in a different effect on APP metabolism. To test this hypothesis we transfected cells with the TrkB isoforms and TrkB FL mutants and then we exposed the cells to either 50 ng/ml BDNF or H_2O for 24 hours (Figure 3.9A). We observed that the addition of BDNF did not change the effects that we had previously seen in its absence and that there was still a specific effect of the TrkB isoforms; that is TrkB T, SHC and the TrkB K571M mutant had no effect on APP full length levels and APP m/im ratio while TrkB FL and TrkB Y515F triggered almost exactly the same effects on APP in presence or absence of BDNF.

3.3.6 TrkB FL is phosphorylated in absence of BDNF stimulation

Since the tyrosine kinase activity of TrkB FL receptor was essential for mediating the observed effects on APP and BDNF did not change them, we hypothesized that in our experimental setting TrkB was able to auto-phosphorylate independently of BDNF exposure. There are in fact previous reports of BDNF independent phosphorylation of TrkB receptors (Rajagopal et al., 2004; Schecterson et al., 2010). We tested this hypothesis by using an antibody that recognizes the phosphorylated SHC binding site (tyrosine 515) of TrkB FL and TrkB SHC. We looked specifically at the phosphorylation state of the SHC binding site of the TrkB receptor. Over-expressing the TrkB isoforms in absence of BDNF, we observed that TrkB FL was phosphorylated while TrkB T and TrkB SHC were not phosphorylated

(Figure 3.9B). While TrkB SHC does not contain the tyrosine kinase domain, it does contain the Tyr 515 residue that is phosphorylated in the TrkB FL isoform. As expected, mutation of the Tyr 515 in the TrkB FL isoform abolished phosphorylation (Figure 3.9B). Also, inactivation of tyrosine kinase activity in the full-length isoform (TrkB K571M) abolished Tyr 515 phosphorylation (data not shown). The absence of phosphorylation on the SHC isoform and on the TrkB K571M mutant suggests that the tyrosine kinase activity of the receptor is necessary to transfer the phosphate group and that phosphorylation by other kinases does not take place, at least in this system.

Since we found that TrkB FL was phosphorylated independently of BDNF we concluded that BDNF is not necessary to trigger TrkB receptor phosphorylation in this experimental setting. Moreover, TrkB FL and TrkB Y515F both had the same effects on APP m/im ratio and APP full-length levels suggesting that the phosphorylation of the SHC binding site is not crucial in determining these effects.

3.3.7 Co-transfection of TrkB FL and truncated isoforms changes TrkB FL effects on APP metabolism

In our experimental setting BDNF application did not change the effects on APP metabolism suggesting that spontaneous dimerization occurs and triggers auto-phosphorylation of the receptors. We hypothesized that TrkB phosphorylation was mediated by the forced dimerization of the receptors that are over-expressed and aggregate spontaneously at the cell surface. Since we only transfected one isoform or mutant at a time the observed APP effects can only be a consequence of homo-

dimerization. We reasoned that co-transfection of the TrkB FL with the dominant negative isoform, TrkB T (Haapasalo et al., 2001), should decrease TrkB FL activation and therefore reduce the extent of its downstream effects on APP metabolism. We tested this hypothesis by co-transfecting equimolar amounts of TrkB FL and TrkB T over-expression plasmids. To further investigate the regulatory role of TrkB SHC on TrkB FL, which is not very clear, we also co-expressed these two receptors. Finally, to elucidate the role of the intracellular functional domains on the TrkB FL receptor, we also co-expressed the wild type TrkB FL with its mutant TrkB K571M and TrkB Y515F.

Since we co-transfected the TrkB receptors we used as a reference the transfection of TrkB FL only and we normalized the Western Blot quantifications to that value within each experiment. This way we can relate the results to the previous experiments where single isoforms/variants were over-expressed. After normalization, co-transfection of TrkB FL/T was compared to all the other co-transfections.

We observed that TrkB FL/TrkB T co-transfection (TrkB FL/T) resulted in lower APP FL levels than TrkB FL/FL (Figure 3.10A and 3.10B; $p=0.001$). Co-transfection of TrkB FL/SHC increased APP FL levels compared to TrkB FL/T (Figure 3.10B; $p=0.008$). This result is the first that differentiates the TrkB T and TrkB SHC isoforms effects on APP levels when measured in whole cell lysates and suggests a different regulatory role of the two truncated isoforms on TrkB FL. Co-transfection of TrkB FL/Y515F significantly increased APP FL levels similarly to TrkB FL/FL co-transfection compared to TrkB FL/T co-transfection ($p=0.006$). While co-transfection

of TrkB FL with the tyrosine kinase inactive K571M mutant resulted in APP FL levels comparable to TrkB FL/T.

The ratios of mature APP to immature APP were also altered by co-transfection of the TrkB constructs with TrkB FL (Figure 3.10C). As expected from single transfection results, TrkB FL/FL transfection lowered the APP m/im the most, all the values were normalized to these ratios. TrkB FL/T significantly increased the APP m/im ratio compared to TrkB FL/FL ($p=0.05$). TrkB FL/SHC decreased the APPm/im ratio when compared to TrkB FL/T ($p=0.009$). No significant difference was found between the TrkB FL/K571M co-transfection compared to TrkB FL/T. Surprisingly, no significant difference was detected between TrkB FL/Y515F co-transfection compared to TrkB FL/T.

We then measured the C99/C83 ratios to see what effect the co-transfection of TrkB FL with the other isoforms and mutants would have on β versus α-secretase cleavage of APP (Figure 3.10A and 3.10D). Again, all values were normalized to the C99/C83 ratio observed in the transfection of TrkB FL only. TrkB FL/T co-transfection compared to TrkB FL/FL co-transfection yielded a significantly higher C99/C83 ratio ($p=0.036$). TrkB FL/SHC co-transfection decreased the C99/C83 ratio compared to TrkB FL/T. Finally there was a trend towards decreased C99/C83 when either TrkB FL/Y515F ($p=0.071$) or TrkB FL/K571M ($p=0.069$) were co-expressed compared to TrkB FL/T.

sAPP levels were determined in conditioned media of 293Swe cells co-transfected with TrkB FL and other TrkB isoforms/variants (Figure 3.10E). As in the single transfection experiments, there were no statistically significant differences between

sAPP levels from co-transfections of the TrkB FL with each of the isoforms (Figure 3.10F). We also found no significant difference when we co-transfected TrkB FL with the TrkB FL mutants Y515F and K571M (Figure 3.10E and 3.10F). This result suggests that TrkB FL eliminates the TrkB FL Y515F mediated increase in sAPP that is detectable when TrkB Y515F is transfected by itself.

Since co-transfecting TrkB FL and the isoforms/mutants had similar effects on APP metabolism compared to the single TrkB transfections we tested the hypothesis that in this more physiologic context, BDNF treatment might alter the effects. We then treated the co-transfected cells with 50 ng/ml BDNF or vehicle (Figure 3.11A). Again, BDNF treatment did not alter the effects observed on APP levels, suggesting that the APP effects observed are dependent on the TrkB over-expression and do not depend on BDNF stimulation. Therefore the effects observed in the co-expression experiments are due to the presence of the truncated isoforms or mutant.

Since we have observed that auto-phosphorylation of the TrkB FL receptor is essential for triggering the effects on APP metabolism and that BDNF does not change the observed effects on APP, we hypothesized that co-transfection of TrkB FL with the isoforms lacking tyrosine kinase activity, or mutants in which this activity is disrupted, can reduce or eliminate TrkB FL phosphorylation thereby inhibiting intracellular signaling. Therefore we assessed the phosphorylation state of the TrkB FL receptor when co-transfected with the other TrkB isoforms/variants (Figure 3.11B). We observed TrkB FL phosphorylation when TrkB FL was transfected alone or co-transfected with TrkB SHC. TrkB FL phosphorylation was greatly reduced when TrkB FL was co-transfected with TrkB T, TrkB Y515F or the TrkB K571M

mutant (Figure 3.11B). These results demonstrate that TrkB FL phosphorylation can be prevented by TrkB T but not by the TrkB SHC isoform.

3.4 Discussion

A variety of experimental evidence suggests that TrkB/BDNF signaling has an important role in AD pathogenesis. Genetic linkage and association studies (Blacker et al., 2003; Bertram et al., 2007a) as well as TrkB expression analysis in post-mortem brain tissue (Ginsberg et al., 2006) implicate TrkB in AD. TrkB FL/BDNF signaling can activate several signaling pathways through its intracellular domains: a SHC binding domain, a tyrosine kinase domain and a PLC-γ binding domain (Middlemas et al., 1991). Some of these pathways have been shown to directly affect APP metabolism. *In vitro* studies show a direct effect of TrkB FL on APP transcription and proteolytic processing (Ruiz-Leon and Pascual, 2001, 2004; Holback et al., 2005). TrkB FL can activate PLC-γ, which is upstream of PKC, an activator of ADAM10 (Yamada et al., 2002; Musumeci et al., 2009); and SHC, that binds to TrkB, has been shown to modulate BACE expression levels (Xie et al., 2007). In addition, $A\beta_{42}$ has been shown to down-regulate TrkB /BDNF signaling in primary neurons (Tong et al., 2004; Poon et al., 2009), while activation of TrkB signaling reduces Aβ production (Rohe et al., 2009). All these results point to the existence of a dynamic relationship between APP metabolism and TrkB.

3.4.1 TrkB isoforms differentially affect APP metabolism

The role of the truncated TrkB isoforms, TrkB T and TrkB SHC, on APP metabolism has not been investigated. All TrkB isoforms are co-expressed in neurons and glia (Armanini et al., 1995; Fryer et al., 1996; Fryer et al., 1997; Ohira et al., 2005; Cheng et al., 2007; Ohira et al., 2007; Carim-Todd et al., 2009). TrkB T was initially thought to be the inactive version of TrkB FL (Haapasalo et al., 2001), however it can activate signaling pathways independently of TrkB FL (Rose et al., 2003). Therefore it is important to determine if and how these truncated isoforms affect APP metabolism.

To determine these effects we transfected HEK293 cells stably over-expressing APP^{Swe} with over-expression plasmids for TrkB isoforms or mutants. HEK293 cells do not express endogeneous TrkB, unlike neuronal cell lines, allowing the study of single TrkB isoforms. These cells also do not express BDNF which activates TrkB signaling and differentially affects TrkB isoforms turnover (Daniel et al., 2007; Colley et al., 2009). In fact is known that TrkB FL is rapidly transported to the surface after BDNF stimulation and then degraded (Sommerfeld et al., 2000) while TrkB T expression remains constant (Rohe et al., 2009). We initially transiently over-expressed individual TrkB isoforms under the hypothesis that each isoform differentially affects APP metabolism. We found that TrkB FL was able to modify APP levels, glycosylation and C99/C83 ratio. TrkB T and SHC did not differ from each other in the effects on APP metabolism.

The two major effects that we observed were altered full-length APP (APP FL) levels and altered APP glycosylation, specifically the ratio of fully (mature, N +

O glycosylation) to partially glycosylated APP (immature, N glycosylation). We believe that these two findings are related. Increased APP FL levels cannot be due to a transcriptional effect of TrkB FL on the APP promoter. Even though increased transactivation of the endogenous APP promoter by TrkB FL has been previously demonstrated (Ruiz-Leon and Pascual, 2001, 2003). In our experimental setting, APP is expressed under the control of the CMV promoter therefore it cannot be physiologically regulated. The altered APP m/im ratio we observe is consistent with the recent finding that auto-activated TrkB receptor can alter glycosylation of proteins (Schecterson et al., 2010). We believe that the observed increase in APP FL in our experimental setting is due to an accumulation of APP in the pre-Golgi compartment that leads to decreased APP turnover thereby increasing steady state APP levels. This hypothesis is supported by the decreased ratio of APP m/im. In fact the majority of APP that is transported to the cell surface is fully glycosylated. After shedding, APP is endocytosed and degraded (Lichtenthaler, 2006). Therefore impaired glycosylation of the protein might prevent its transport to degradation compartments.

APP glycosylation state can regulate APP proteolysis. In fact inhibition of O-glycosylation, which occurs in the Golgi compartment, inhibits APP α and β processing (Tomita et al., 1998). Over-expression of TMEM59, a trans-membrane protein that is resident in the Golgi, results in APP retention in the Golgi (even if no direct interaction with APP was observed) and impaired N- and O-glycosylation (Ullrich et al., 2010). TMEM59 seems to act as a general inhibitor of glycosylation since it was also able to inhibit N-glycosylation of BACE1 and the prion protein. The authors do not describe any effects on C83/C99 production but observe a decrease in

α and β sAPP levels that was not due to loss of BACE1or ADAM10 activity (Ullrich et al., 2010).

Since we do not observe major changes in C83 and/or C99 levels we do not have a reason to hypothesize that ADAM10 and BACE1, the two enzymes responsible for α and β secretase APP cleavage, are strongly affected by TrkB isoform over-expression. Glycosylation defects would impair ADAM10 activity (Escrevente et al., 2008) and could affect BACE activity even if BACE activity is largely independent of its glycosylation status (Huse et al., 2000; Benjannet et al., 2001; Vanoni et al., 2008). We also do not observe a statistically significant difference in sAPP-α levels when TrkB isoforms and/or mutants are over-expressed. We only observed a slight increase in sAPP-α caused by TrkB FL over-expression, and a TrkB FL induced glycosylation defect is unlikely to increase ADAM10 activity. Since the secretase activity is not likely affected, we hypothesize that the significant decrease in C99/C83 ratio that we observe is due to altered APP trafficking. In fact, the production of C83 and C99 is influenced by the availability of APP to the secretases. That is why we did not normalize the levels of C99 and C83 (CTFs) to the total amounts of APP full-length. In fact, even if TrkB FL increases total APP FL levels, it might not necessarily increase the pool of APP that is available for cleavage. Therefore increases in APP levels do not necessarily correlate to a proportional increase in APP proteolytic products. Since the main pathologic mechanism in AD is the increased β-secretase cleavage of APP compared to α-secretase cleavage, we analyzed the ratio of C99 to C83 to assess if any of the TrkB isoforms displays preferential generation of the amyloidogenic fragment (C99) over the non-

amyloidogenic fragment (C83). Calculating the C99/C83 ratio normalizes the effects of altered APP FL levels and also addresses the question of the APP cleavage route that might be favored by the different TrkB isoforms. We found that tyrosine kinase active receptors significantly decreased the C99/C83 ratio compared to the truncated/TK inactive receptors. This suggests that the TrkB FL beneficial effects might be partially mediated by decreased β-secretase cleavage of APP. In agreement with this hypothesis is the reduced Aβ40 levels found in the media of cells transfected with TrkB FL and TrkB Y515F. The magnitude of the decrease of Aβ40 and C99/C83 ratio was modest. C83 and C99 can be generated in different intracellular compartments and at the cell surface (Muresan et al., 2009). Some compartments might be more subjected than others to TrkB mediated effects. When we measure protein levels in the whole cell lysate, we only monitor an average effect that might be less obvious that the net effect in specific compartments. This is most likely the reason we do not see a robust difference in C99/C83 ratios. The same consideration must be applied to secreted Aβ40 levels.

A general experimental observation we made in the course of these experiments was TrkB FL expression levels were lower than the expression levels of the truncated counterparts. This is despite using equimolar amounts of DNA used for transfection and the identical vector containing the TrkB isoform cDNAs. This suggests that there is a regulation mechanism aimed at maintaining TrkB FL levels low. Tight regulation of TrkB FL levels after BDNF stimulation has been previously demonstrated (Sommerfeld et al., 2000) further underlining the importance of this receptor in the homeostasis of the cells.

3.4.2 The TrkB FL effects on APP metabolism depend on the tyrosine kinase domain but are BDNF independent

Since over-expression of TrkB FL altered APP metabolism most significantly, we hypothesized that either the SHC binding domain, tyrosine kinase (TK) domain, or both must be responsible for these effects. We therefore mutated the functional residues within these domains to determine the relative contribution of these domains to the observed effects.

Only full length TrkB variants with tyrosine kinase activity, FL and Y515F, were able to increase APP FL levels and decrease the APPm/im ratio suggesting that this is a tyrosine kinase dependent effect. The importance of an active tyrosine kinase domain on glycosylation of proteins has been documented by another study that extended the finding to all the tyrosine kinase family members: TrkB A, B and C. In this study the authors demonstrated how tyrosine kinase inactive receptors were unable to alter glycosylation of other proteins (Schecterson et al., 2010). While this might seem a rather unspecific effect of intracellular receptor auto-activation, the same authors found that TrkB does not have the same effect as the others Trks on NOTCH1 (Schecterson et al., 2010). While TrkA and C increase NOTCH1 levels and alter its glycosylation, compared to the control cells, TrkB does not. On the contrary, TrkB, TrkC and TrkA similarly affect other proteins such as NHR2 and TNFR2 (Schecterson et al., 2010). NOTCH1 and APP are generally compared since they have a similar metabolism and it is interesting to observe that while TrkB FL over-expression had little effect on NOTCH1 glycosylation it has a considerable effect on

APP. This suggests that TrkB effects on APP might be specifically related to this neurotrophin receptor and therefore merit through investigation.

We found similar effects of TrkB over-expression on the glycosylation of the TrkB receptor itself: in fact we observed two distinct bands (Figure 3.4A and 3.4B) when we over-expressed the tyrosine kinase active receptors (TrkB FL or Y515F, Figure 3.4A) and not when we expressed the kinase dead (K571M) receptor (Figure 3.4B). These two bands represent glycosylated receptor and non-glycosylated or partially glycosylated receptor as previously described (Schecterson et al., 2010). Similarly we consistently observed the formation of intermediate glycosylation forms of APP that migrate between the immature APP and the fully glycosylated APP (Figure 3.4A/B, 3.2A, 3.8A, 3.9A, 3.10A, 3.11A). These glycosylation effects have been related to modifications of Golgi trafficking mediated by proteins such as Trks or TMEM59 (Schecterson et al., 2010; Ullrich et al., 2010).

sAPP levels were unchanged among all the TrkB isoforms and the TrkB FL K571M variant while TrkB Y515F significantly increased sAPP levels. There was no significant difference in sAPP-α levels among TrkB isoforms even if both TrkB FL and TrkB Y515F displayed a tendency to increase them. The tendency to increase sAPP-α of both TrkB FL and TrkB Y515F mutant is consistent with the decreased C99/C83 ratio that these constructs yield in whole cell lysates compared to TrkB truncated isoforms. It is also consistent with the observed decrease in Aβ 1-40 levels detected in conditioned media.

The TrkB Y515F variant displayed significantly higher sAPP levels compared to the other isoforms. This effect is likely not due to an increased sAPP-α level since

both TrkB FL and TrkB Y515F showed similar sAPP-α levels. β-secretase cleavage also seems unlikely to be increased by TrkB Y515F transfection given the decreased C99/C83 ratio and decreased Aβ40 levels. It is possible, though, that mutation of the SHC binding site affects the secretion of sAPP-β and that the stability of this fragment in the extra-cellular media allows for detection of differences that are not evident with other TrkB isoforms and mutants.

TrkB FL and Y515F both showed a tendency to increase sAPP-α levels. Since sAPP-α levels are thought to be directly proportional to the amount of cell surface APP (Lichtenthaler, 2006), we investigated TrkB effects on APP transport to the cell surface. We found that tyrosine kinase active TrkB (FL receptor and Y515F) increased APP cell surface levels while decreasing TrkB surface levels. TrkB FL effects were not significantly altered upon mutation of the SHC binding site (Y515F) despite SHC binding has been shown to be important for trafficking of proteins (Colley et al., 2009). Since we attribute the observed APP effects to trafficking alteration, we expected that SHC binding would play some important role. Especially considering that SHC binds to both TrkB and APP (Tarr et al., 2002). EndophilinA3 was found to strongly up-regulate α-secretase APP cleavage by inhibiting its endocytosis (Schobel et al., 2008). Endophilin has a SH3 domain that can bind the SHC adaptor protein like the TrkB receptor. It is hypothesized that interaction of APP with proteins favoring its endocytosis reduces α-secretase cleavage and favors β-secretase cleavage (Lichtenthaler, 2006). Despite this, SHC binding does not seem to be the main factor contributing to sAPP-α generation in our experimental setting.

We also observed different surface levels of the receptors: TrkB FL and Y515F surface levels were lower than the truncated isoforms and the TrkB K571M mutant. The altered transport of tyrosine kinase active TrkB receptors might be due to the effect that auto-activated TrkB receptors have on the Golgi compartment. This would also explain why we did not observe reduced cell surface levels of TrkB K571M (Schecterson et al., 2010). Tight regulation of the TrkB receptor surface expression has been previously described and the findings agree with what we observed: TrkB receptors that have tyrosine kinase activity also display lower surface expression levels than TrkB receptors that are tyrosine kinase inactive (Haapasalo et al., 2002).

Interestingly we also observed that TrkB FL and TrkB Y515F (both TK active receptors) had a similar effect on the C99/C83 ratio. This effect, even if more modest, seemed to be related to the effects described earlier (APP FL and APP m/im ratio). We initially hypothesized that TK active receptors would increase C83 production through PLC-γ and PKC/ADAM10 activation. In fact it has been previously demonstrated that BDNF mediated activation of TrkB FL increases α-secretase cleavage of APP (Holback et al., 2005). Surprisingly we did not observe a significant increase in C83 suggesting that α-secretase processing is not significantly affected in our experimental setting. There are many ways in which SHC binding to TrkB could modulate APP trafficking/intracellular processing leading to altered C99/C83 ratios. First, TrkB FL binds SHC adaptor proteins and engages ERKs which can decrease APP β-secretase processing by increasing transcription of the gene sortin protein mediated receptor A (*SORLA1*)(Rohe et al., 2009). SORLA mediates transport of

APP in the trans-Golgi network and early endosomes (Rohe et al., 2009). Secondly, siRNA mediated knock-down of Fe65 was able to reduce Aβ production without affecting BACE1 levels but altering APP trafficking (Xie et al., 2007). Similarly, siRNA mediated SHC knock-down reduces Aβ 40/42 levels but also reduces BACE levels. The authors conclude that both Fe65 and SHC might affect APP trafficking (Xie et al., 2007).We could then speculate that the decrease in C83/C99 ratio that we observe when over-expressing TrkB FL, might result from the increased *SORL1* transcription while in the case of TrkB Y515F by a mechanism mimicking SHC knock-down. It is possible that C99/C83 ratio reduction is achieved through different but parallel pathways: reduced endocytosis from the cell surface, *SORLA1* gene expression and maybe other unknown mechanisms that are also TrkB mediated. The existence of these multiple pathways complicates the attribution of effects to a specific mechanism. Consistently, with the decreased C99/C83 ratio we observed reduced Aβ levels when TrkB FL and TrkB Y515F are over-expressed. This Aβ effect can also be partially explained with the SHC mediated signaling described above. TrkB FL binding SHC can mediate decreased Aβ production via the SORLA protein while the TrkB Y515F mutant can act on APP trafficking.

Finally the SHC binding might play some other role in APP metabolism regulation since TrkB Y515F seems to increase APP FL levels less than TrkB FL and also to cause less accumulation of immature APP (Figure 3.4A).

Importantly all these isoform-specific changes in APP levels, glycosylation and processing are independent of BDNF binding. Over-expression of TrkB FL alone is sufficient to cause phosphorylation of the intracellular domain in the absence of

BDNF. BDNF binding does not change the effects on APP metabolism of any of the isoforms suggesting that the intracellular signaling triggered by BDNF independent TrkB activation is the same. These results suggest that BDNF just mediates dimerization of the receptors and this event is what causes the auto-phosphorylation (activation) of the receptors and the downstream signaling pathways. When the receptors are over-expressed they spontaneously aggregate and can trigger intracellular signaling.

3.4.3 Truncated TrkB isoforms suppress TrkB FL mediated effects on APP metabolism.

TrkB signaling is achieved by BDNF induced dimerization of the receptors that triggers auto-phosphorylation of the intracellular domain on TrkB FL. This initiates intracelluar signaling by the binding of the adaptor protein SHC and of PLC-γ to the TrkB phosphorylated residues (Reichardt, 2006; Minichiello, 2009). The three TrkB isoforms, studied here, are co-expressed on the surface of neurons and form hetero-dimers (Ohira et al., 2001). Co-expression of TrkB T with TrkB FL inhibits TrkB FL mediated intracellular signaling (Ninkina et al., 1996; Haapasalo et al., 2001). It has been shown that, in a mouse model of Down Syndrome (where there are three copies of the APP gene) down-regulation of TrkB T reduces neuronal death re-establishing TrkB FL signaling (Dorsey et al., 2006). The effect of TrkB SHC co-expression on TrkB FL signaling has not, until now, been investigated.

Co-transfection of TrkB FL with TrkB T or TrkB K571M (the tyrosine kinase inactive variant) decreases APP FL, increases APPm/im ratio and increases the C99/C83 ratio compared to TrkB FL transfection. These two receptors (TrkB T and TrkB K571M) act in a dominant negative fashion on TrkB FL. Co-transfection of TrkB SHC with TrkB FL, instead, significantly increases APP FL and also significantly decreases APPm/im ratio and the C99/C83 ratio compared to TrkB FL/T co-transfection. TrkB FL/Y515F co-transfection had the same effects as TrkB FL transfection compared to TrkB FL/T co-transfection, suggesting, once again, that the tyrosine kinase domain has a pivotal role in determining the effects on APP while the SHC binding site on the receptor is a minor contributor.

Importantly co-transfection of TrkB FL and SHC also increases sAPP-α underlining the importance of further investigating the role of this isoform. In fact, while stimulation of TrkB FL signaling for therapeutic purposes might be too aggressive since TrkB FL releases Ca^{2+} from intracellular storage via PLC-γ activation. This is not advisable since increased Ca^{2+} signaling has many adverse effects in AD context (Mattson and Chan, 2003). TrkB SHC, instead, lacking the PLC-γ binding site, might attenuate the Ca^{2+} response but still promote α-secretase processing of APP by promoting its transport to the cell surface.

An intriguing open question of TrkB signaling regulation by dimerization is if there is a preference of the receptors for homo-dimerization or hetero-dimerization and in this second case if one isoform is preferred over the other. In our experimental setting we co-transfected equimolar quantities of TrkB receptors and we cannot assess whether homo or hetero dimerization is preferred. Our results suggest that

hetero-dimerization is probably the cause of the elimination of the TrkB FL mediated effects on APP. Supporting this hypothesis, co-transfection of TrkB FL and TrkB T at different ratios induced a dose dependent decrease in TrkB FL mediated survival of primary neurons when the amount of TrkB T plasmid was increased (Ninkina et al., 1996). This work would suggest that the hetero-dimerization is mainly driven by relative amounts of the receptors at the cell surface. Another intriguing possibility is that homo-dimerization of TrkB FL and TrkB T occurs at the same time and activates signaling pathways that are interfering with each other. TrkB T has been shown before to be able to generate Ca^{2+} intracellular currents in glial cells independently of TrkB FL (Rose et al., 2003). It is known that the domains regulating dimerization of Trk receptors are located in the extra-cellular region. There are both domains that favor dimerization and domains that inhibit it (Arevalo et al., 2000; Arevalo et al., 2001). It has been shown that in macaque brains the extent of formation of homo or hetero-dimers is directly proportional to BDNF concentration and the composition of the dimers changes during development (Ohira et al., 2001). These authors elegantly describe the formation of the TrkB dimers by cross-linking the receptors but do not show the levels of the TrkB FL receptor versus the TrkB T receptor in the tissues under investigation (Ohira et al., 2001). Therefore it remains to be determined if the composition of the dimers is solely dependent on the relative concentration of the TrkB isoforms or there are other mechanisms involved. This question is particularly important since the composition of the dimers can have such different effects on APP metabolism. It would be crucial to be able to modulate TrkB signaling with small molecules that can be effectively used as drugs in the central nervous system. It has

already been shown that engineered BDNF can induce Trk receptors dimerization (Ibanez et al., 1993) and that small molecules can be easily synthesized and used as agonists to promote TrkB receptor dimerization and activation (O'Leary and Hughes, 2003; Jang et al., 2009). This drug development approach will become more effective once we gain a better molecular understanding of how these different TrkB isoforms interaction with each other and modulate signaling.

In conclusion, TrkB truncated isoforms and TrkB FL have different effects on APP metabolism. These specific differences are important in AD and have to be considered when designing BDNF based therapies. Our results suggest that BDNF treatment might be ineffective in AD patients that do not express TrkB FL at high levels. In fact BDNF treatment could cause more damage. We also show that co-expression of the TrkB FL receptor with the truncated isoforms could modulate its effect on APP. This result is very important since the three isoforms are co-expressed on both neuronal and non-neuronal cells. The relative expression levels of the three isoforms in AD are determined by alternative splicing that can be altered by polymorphisms in the gene. Studying the relationship between the splicing regulation and the TrkB isoforms protein levels might give insight in one more genetic risk factor for LOAD.

3.5 Figure Legends:

Figure 3.1: Schematic representation of the TrkB human isoforms. Modified from Stoilov et al.

Figure 3.2: TrkB isoforms differentially affect APP full-length levels, glycosylation (APPm/im) and C99/C83 ratio. (A) Representative western blot of whole cell lysate showing TrkB signal and the corresponding APP signal with actin as a loading control. (B) APP full-length levels are increased by TrkB FL transfection and not by TrkB T or SHC transfection ($p=0.01$). (C) The ratio of mature to immature APP is decreased by TrkB FL transfection but not by TrkB T or SHC transfection ($p=0.002$). (D) TrkB FL significantly decreases the C99/C83 ratio compared to TrkB T ($p=0.010$). (n=6-7).

Figure 3.3: TrkB isoforms effects on total sAPP and sAPP-α levels. (A) Representative Western blot showing total sAPP and sAPP-α levels in conditioned media from HEK239-APP^{swe} cells transfected with TrkB isoforms. (B) TrkB isoforms do not alter total sAPP levels. (C) TrkB FL displays a non-significant increase in sAPP-α compared to TrkB T and TrkB SHC. (D) TrkB SHC displays a trend in decreased Aβ40 levels compared to TrkB T ($p=0.077$). Values represent arbitrary luminescence units (ALU) and are divided by the protein concentration of the corresponding cell lysate. (n=4-5).

Figure 3.4: TrkB FL mutants effect on APP full-length, glycosylation (APPm/im) and C99/C83 ratio. (A) and (B) representative Western blots showing whole cell lysate probed for TrkB, APP, C99, C83 and actin as a loading control. The transfected TrkB isoforms/mutants are indicated above each lane. Note that TrkB FL and TrkB Y515F show two bands corresponding to different glycosylated receptors while the TrkB T, the TrkB SHC isoforms and TrkB K571M migrate as a single band. (C) TrkB K571M causes a significant decrease in APP levels when compared to TrkB FL wild type (n=3; p=0.0002); there is no significant difference in APP levels between TrkB FL and TrkB Y515F mutant (n=7, p=0.5). (D) TrkB K571M significantly increases the APP m/im ratio when compared to TrkB FL (n= 4, p=0.006); the TrkB Y515F mutant does not alter the APPm/im ratio compared to TrkB FL (n=5). (E) TrkB K571M shows a trend in increasing the C99/C83 ratio compared to TrkB FL (p=0.07, n=3), TrkB Y515F does not affect the C99/C83 ratio compared to TrkB FL.

Figure 3.5: Total sAPP, sAPP-α, Aβ40 levels in conditioned media of HEK293-APP^{swe} cells transfected with TrkB isoforms or TrkB FL mutants. (A) Representative Western blot of conditioned media probed for sAPP total and sAPP-α. The transfected TrkB constructs are indicated above each lane. (B) Total sAPP levels are significantly increased by transfection of TrkB Y515F compared to TrkB FL (p=0.02) but not by transfection of TrkB FL K571M. (C) TrkB Y515F does not significantly affect sAPP-α levels and TrkB K571M decreases non significantly sAPP-α compared to TrkB FL. (D) TrkB Y515F significantly decreased Aβ1-40 levels compared to TrkB FL (p=0.05) while TrkB K571M showed a non significant increase.

Figure 3.6: Impairment of γ-secretase cleavage increases sAPP-α levels independently of TrkB transfected construct. (A) Representative Western blot showing sAPP-α levels in cells transfected with TrkB isoforms and mutants and treated with DMSO or L-685. (B) sAPP-α levels were normalized to the values detected in TrkB T transfected cells within treatments. TrkB FL and TrkB Y515F show a tendency to increase sAPP-α in DMSO treated cells compared to TrkB T but this increase is not significant. Only TrkB Y515F displays the tendency to further increase sAPP-α levels compared to TrkB T in L-685 treated cells. TrkB FL Transfected cells treated with L-685 display the same sAPP-α levels of TrkB T transfected cells and L-685 treated. (C) L-685 treatment significantly increases sAPP-α levels independently of TrkB construct transfected ($p=0.0001$).

Figure 3.7: Impairment of γ-secretase cleavage increases sAPP levels independently of TrkB transfected construct but does not alter the effects of the TrkB isoforms. (A) Representative Western blot showing sAPP levels in cells transfected with TrkB isoforms and mutants and treated with DMSO or L-685. (B) sAPP levels were normalized to the values detected in TrkB T transfected cells within treatments. TrkB Y515F shows increased sAPP in DMSO treated cells compared to TrkB T ($p=0.05$) and in L-685 treated cells ($p=0.04$). (C) L-685 treatment significantly increases sAPP- levels independently of TrkB construct transfected ($p=0.0001$) even if the TrkB Y515F levels are higher compared to other TrkB constructs.

Figure 3.8: Cell surface APP levels in cells transfected with TrkB isoforms and TrkB FL mutants. (A) Representative Western blot of whole cell lysates from cells transfected with different TrkB construct and the corresponding biotinylated surface protein. Note that the APP detected at the cell surface is mainly fully glycosylated APP. A low level of immature and intermediately glycosylated APP is more prominent in the TrkB FL and TrkB Y515F transfected cells. (B) APP glycosylated surface levels are higher in TrkB FL and TrkB Y515F transfected cells, there is no statistically significant difference between TrkB FL and TrkB T. There is a statistically significant difference between TrkB FL and TrkB K571M transfected cells (n=3; p=0.02).

Figure 3.9: TrkB mediated effects on APP metabolism are BDNF independent and TrkB FL is phosphorylated in absence of BDNF stimulation. (A) Treatment of TrkB transfected cells with BDNF (50 ng/ml) does not alter the isoform/mutant specific effects on APP levels and/or glycosylation. (B) TrkB FL auto-phosphorylates when over-expressed in HEK293-APP^{swe} without BDNF treatment. A Y515 phospho-specific antibody was used to detect TrkB FL phosphorylated at tyrosine 515. Note that TrkB T, TrkB SHC and the full-length Y515F mutant are not phosphorylated.

Figure 3.10: TrkB FL co-transfection with TrkB isoforms and TrkB FL mutants.
(A) Representative Western blot of whole cell lysates from HEK293-APP^{swe} co-transfected with TrkB FL and truncated isoforms or mutants. Note the several bands corresponding to the molecular weight of the different isoforms transfected, when

only TrkB FL is transfected the bands are reduced to two or one. (B) APP full-length levels are increased in cells co-transfected with TrkB FL/SHC compared to TrkB FL/T (p= 0.008); TrkB FL/Y515F co-transfection also increases APP full-length levels compared to TrkB FL/T (p= 0.006); TrkB FL/K571M APP full-length levels are not statistically significantly different from TrkB FL/T. (C) APPm/im ratios are decreased by co-transfection of TrkB FL/SHC compared to TrkB FL/T (p=0.009); surprisingly no significant difference was found between TrkB FL/Y515F compared to TrkB FL/T. As expected there was no significant difference between co-transfection of TrkB FL/K571M compared to TrkB FL/T (n=6-8). (D) TrkB FL/SHC co-transfection decreases the C99/C83 ratio compared to TrkB FL/T (p=0.036); TrkB FL/Y515F co-transfection shows a trend in decreased C99/C83 ratio compared to TrkB FL/T (p=0.07). There is no significant difference between TrkB FL/K571M co-transfection and TrkB FL/T co-transfection. (E) Representative Western blot of total sAPP in conditioned media of TrkB co-transfected cells. (F) There is no difference in sAPP total levels in conditioned media from cells co-transfected with TrkB FL and the other TrkB isoforms or mutants.

Figure 3.11: BDNF treatment of cells co-transfected with TrkB FL and TrkB isoforms/mutants. (A) Treatment with BDNF does not change the effects of TrkB FL co-transfection with isoforms/mutants on APP full-length or APP glycosylation. (B) Phosphorylation on the SHC binding site of the TrkB FL receptor is detectable when TrkB FL is co-transfected with TrkB FL or TrkB SHC. No detectable

phosphorylation is evident in TrkB FL/T, TrkB FL/Y515F and TrkB FL/K571M co-transfections.

Figure 3.1

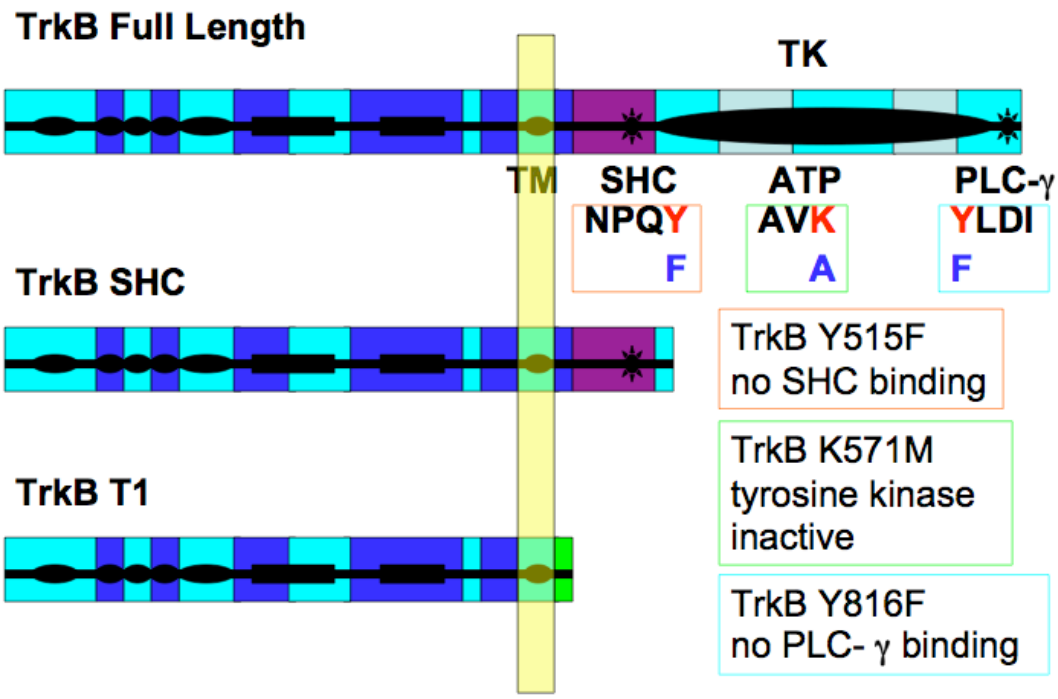
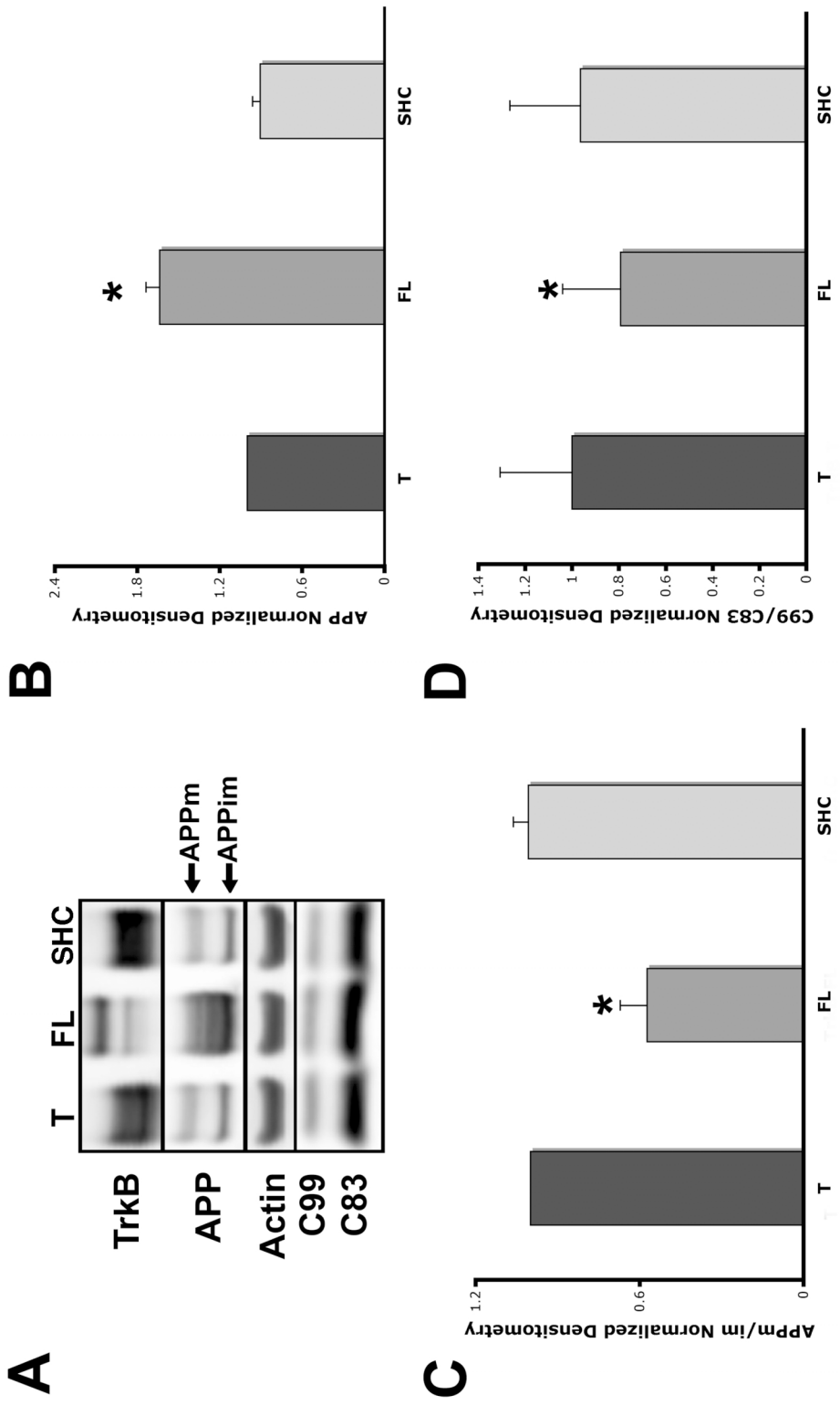

TrkB Full Length
TK
TM
SHC
NPQY
F
ATP
AVK
A
PLC-γ
YLDI
F
TrkB SHC
TrkB T1
TrkB Y515F
no SHC binding
TrkB K571M
tyrosine kinase
inactive
TrkB Y816F
no PLC- γ binding

Figure 3.2


A
T
FL
SHC
APPm
APPim
TrkB
APP
Actin
C99
C83
B
APP Normalized Densitometry
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1.8
1.2
0.6
0
*
T
FL
SHC
C
APPm/im Normalized Densitometry
1.2
0.6
0
*
T
FL
SHC
D
C99/C83 Normalized Densitometry
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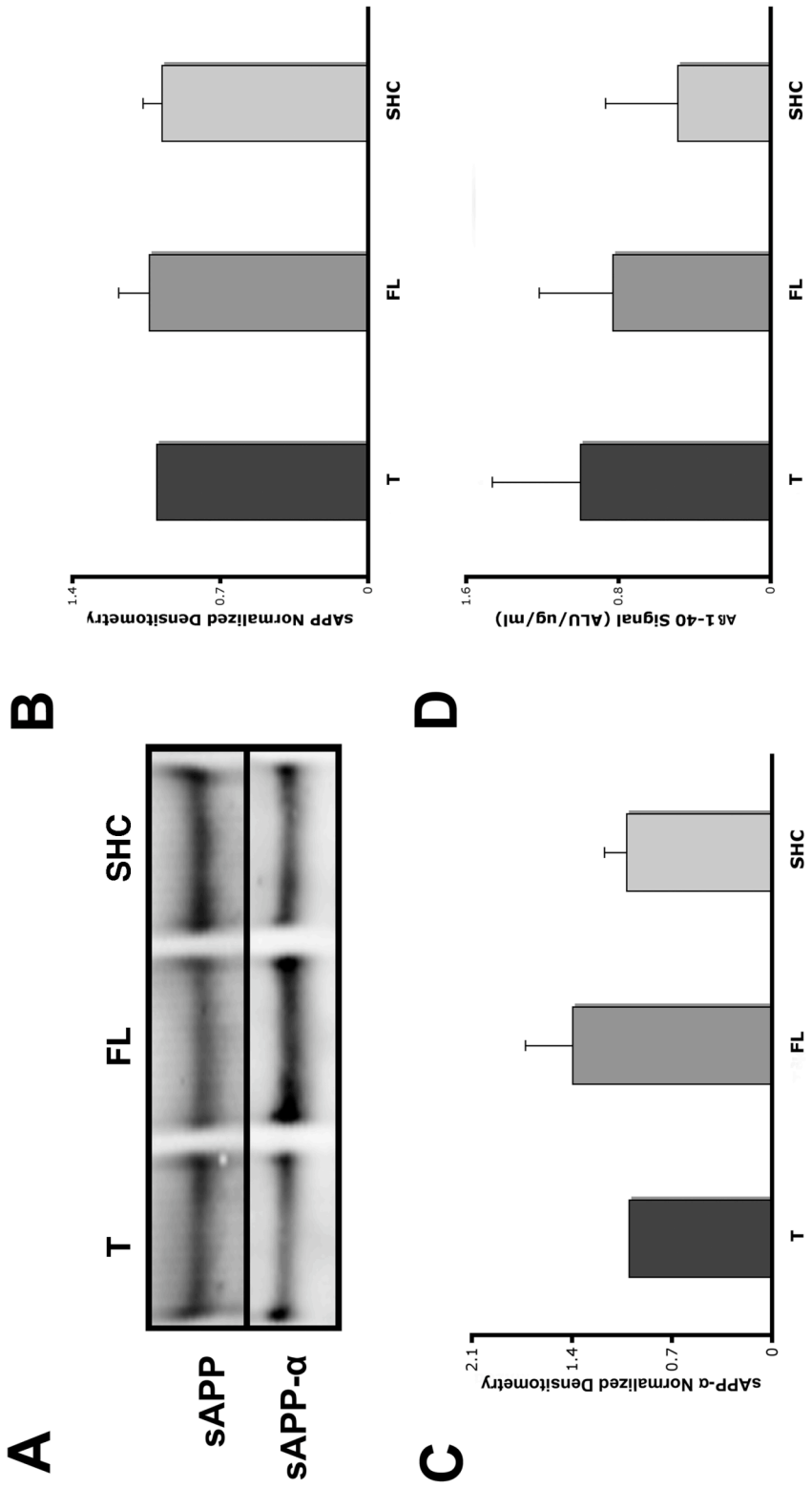
1.2
1
0.8
0.6
0.4
0.2
0
*
T
FL
SHC

Figure 3.3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FL
SHC
sAPP
sAPP-α
B
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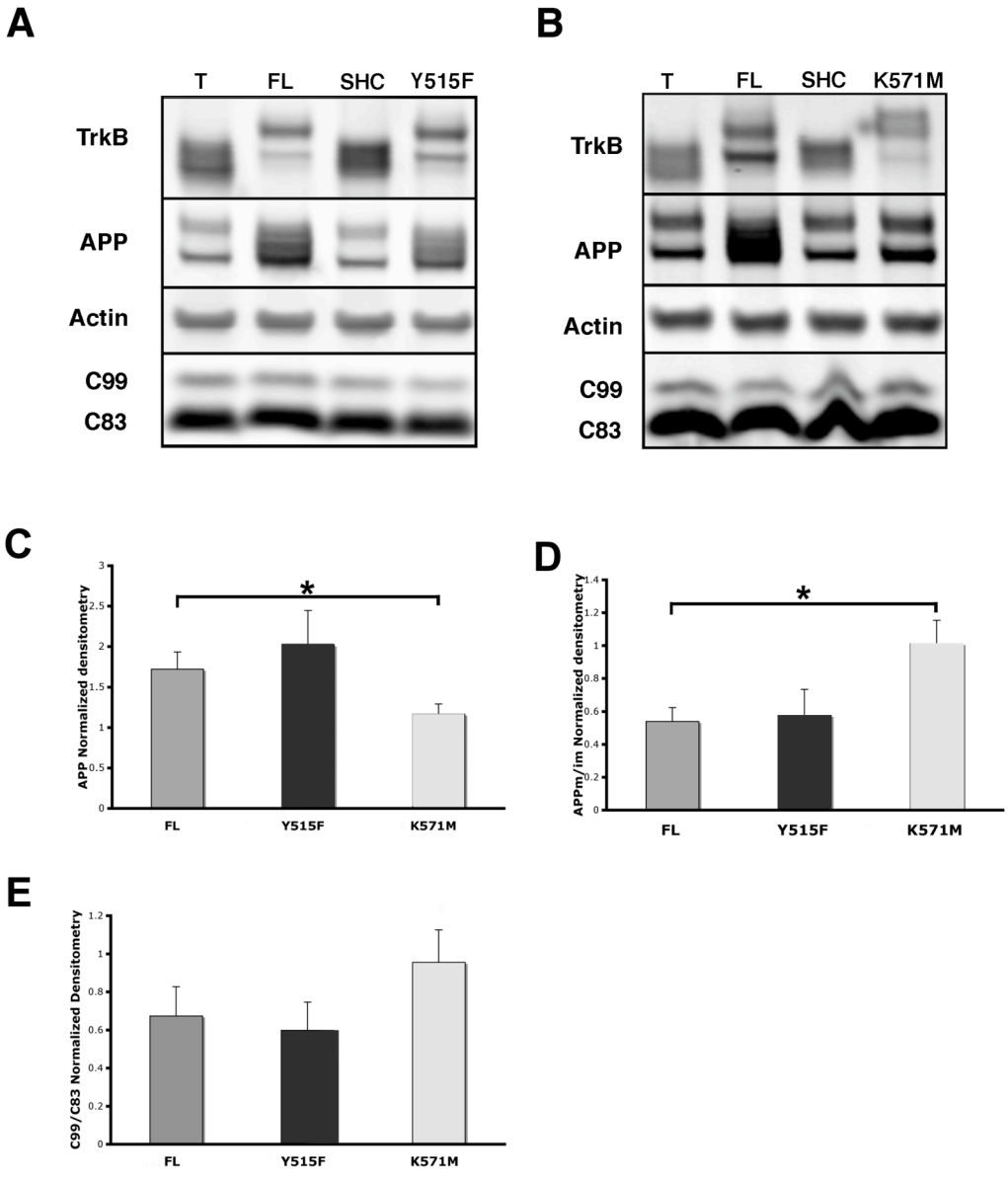
0.7
0
T
FL
SHC
C
sAPP-α Normalized Densitometry
2.1
1.4
0.7
0
T
FL
SHC
D
Aß 1-40 Signal (ALU/ug/ml)
1.6
0.8
0
T
FL
SHC

Figure 3.4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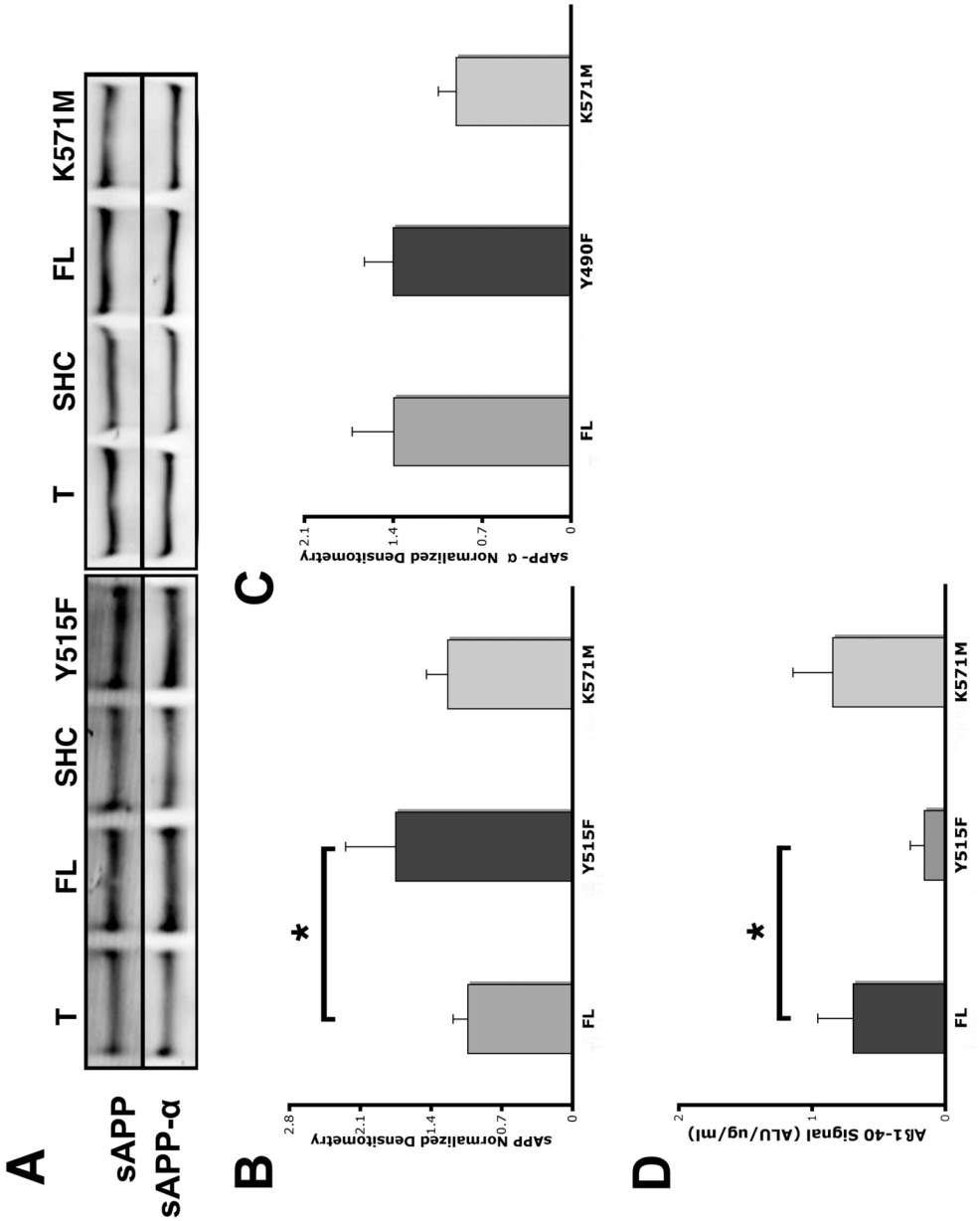
FL
SHC
Y515F
TrkB
APP
Actin
C99
C83
B
T
FL
SHC
K571M
TrkB
APP
Actin
C99
C83
C
APP Normalized densitometry
*
FL
Y515F
K571M
D
APPm/im Normalized densitometry
*
FL
Y515F
K571M
E
C99/C83 Normalized Densitometry
FL
Y515F
K571M

Figure 3.5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sAPP-α
T
FL
SHC
Y515F
T
SHC
FL
K571M
B
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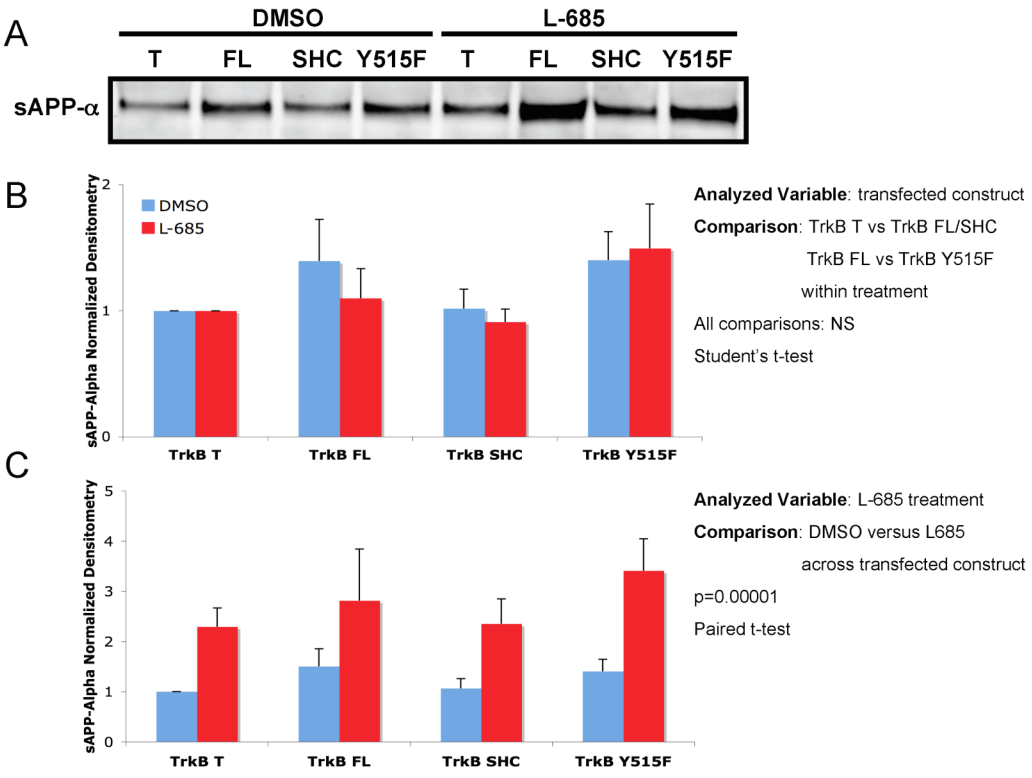
2.1
1.4
0.7
0
*
FL
Y515F
K571M
C
sAPP- α Normalized Densitometry
2.1
1.4
0.7
0
FL
Y490F
K571M
D
Aβ1-40 Signal (ALU/ug/ml)
2
1
0
*
FL
Y515F
K571M

Figure 3.6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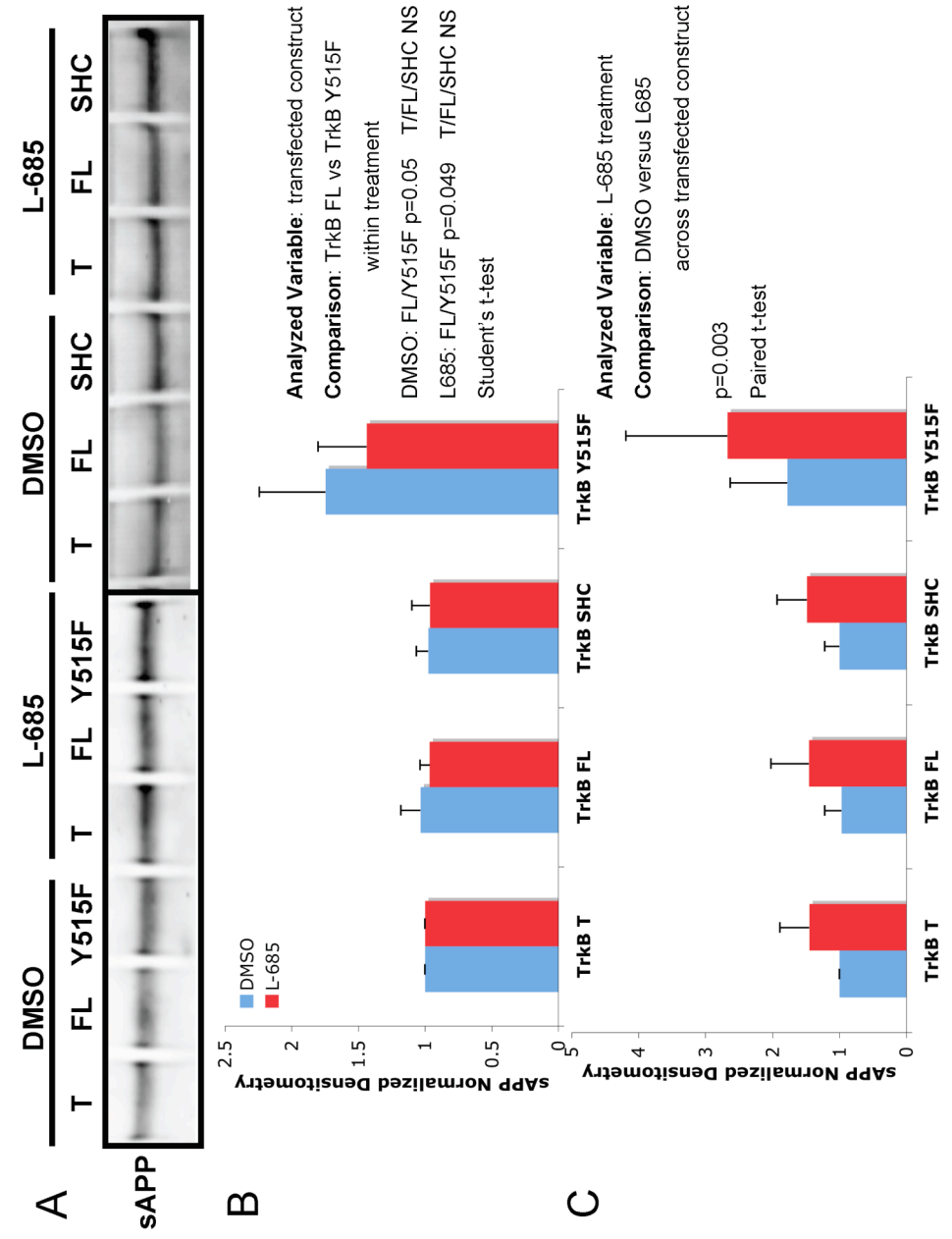
L-685
T
FL
SHC
Y515F
T
FL
SHC
Y515F
sAPP-α
B
sAPP-Alpha Normalized Densitometry
DMSO
L-685
TrkB T
TrkB FL
TrkB SHC
TrkB Y515F
Analyzed Variable: transfected construct
Comparison: TrkB T vs TrkB FL/SHC
TrkB FL vs TrkB Y515F
within treatment
All comparisons: NS
Student's t-test
C
sAPP-Alpha Normalized Densitometry
TrkB T
TrkB FL
TrkB SHC
TrkB Y515F
Analyzed Variable: L-685 treatment
Comparison: DMSO versus L685
across transfected construct
p=0.00001
Paired t-test

Figure 3.7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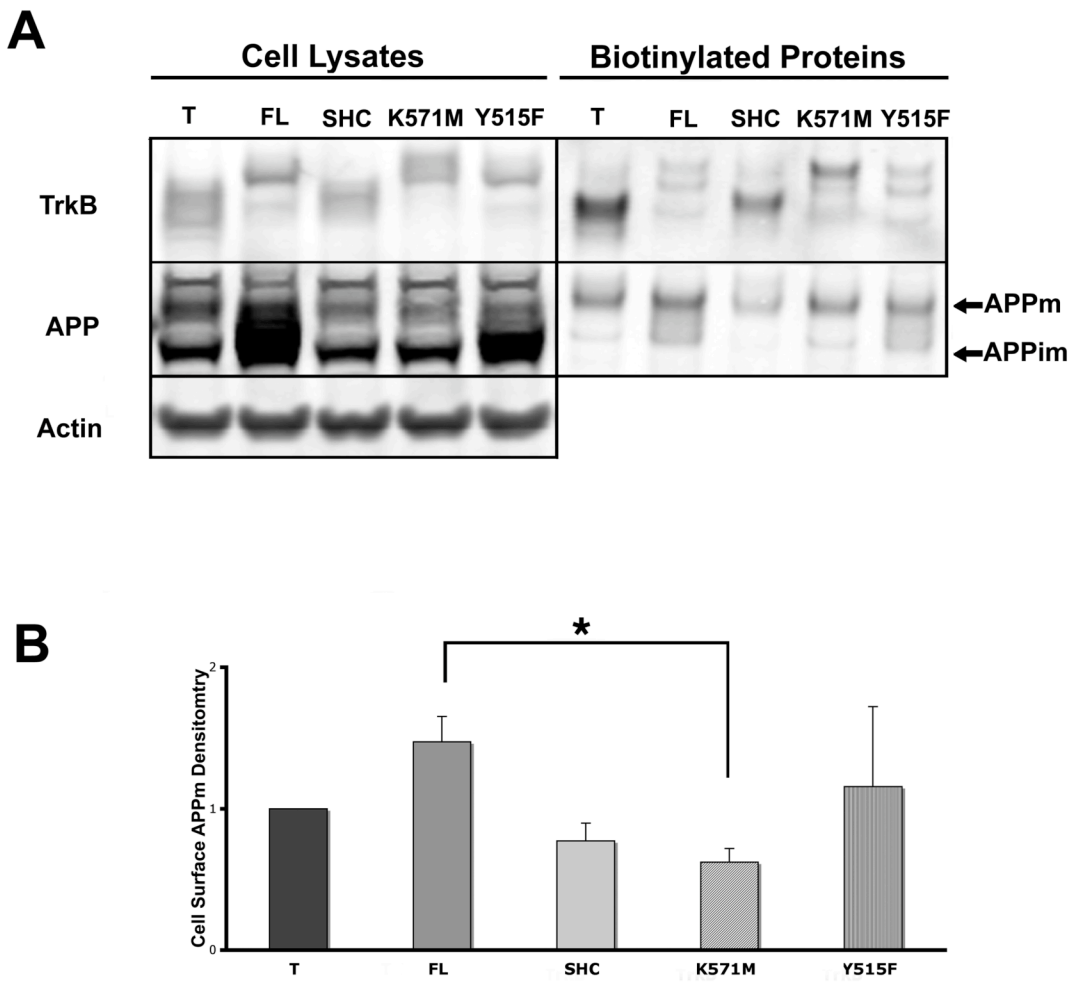
DMSO
L-685
DMSO
L-685
T
FL
Y515F
T
FL
Y515F
T
FL
SHC
T
FL
SHC
sAPP
B
DMSO
L-685
sAPP Normalized Densitometry
2.5
2
1.5
1
0.5
0
TrkB T
TrkB FL
TrkB SHC
TrkB Y515F
Analyzed Variable: transfected construct
Comparison: TrkB FL vs TrkB Y515F
within treatment
DMSO: FL/Y515F p=0.05 T/FL/SHC NS
L685: FL/Y515F p=0.049 T/FL/SHC NS
Student's t-test
C
sAPP Normalized Densitometry
5
4
3
2
1
0
TrkB T
TrkB FL
TrkB SHC
TrkB Y515F
Analyzed Variable: L-685 treatment
Comparison: DMSO versus L685
across transfected construct
p=0.003
Paired t-test

Figure 3.8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Cell Lysates
Biotinylated Proteins
T
FL
SHC
K571M
Y515F
T
FL
SHC
K571M
Y515F
TrkB
APP
Actin
APPm
APPim
B
*
Cell Surface APPm Densitomtry
2
1
0
T
FL
SHC
K571M
Y515F

Figure 3.9

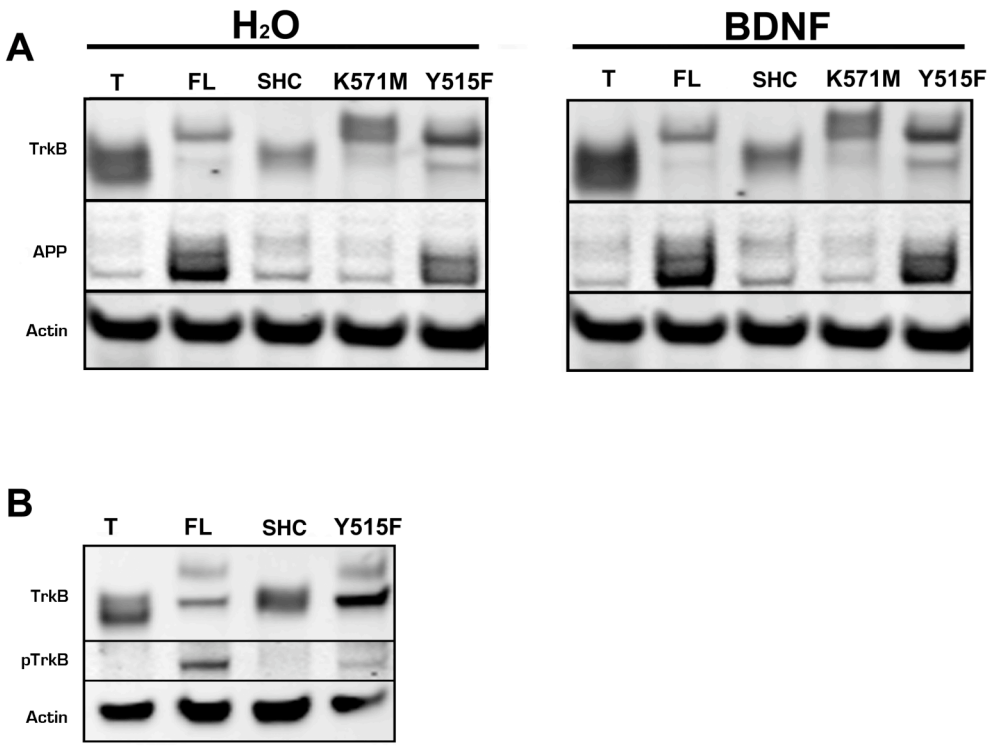
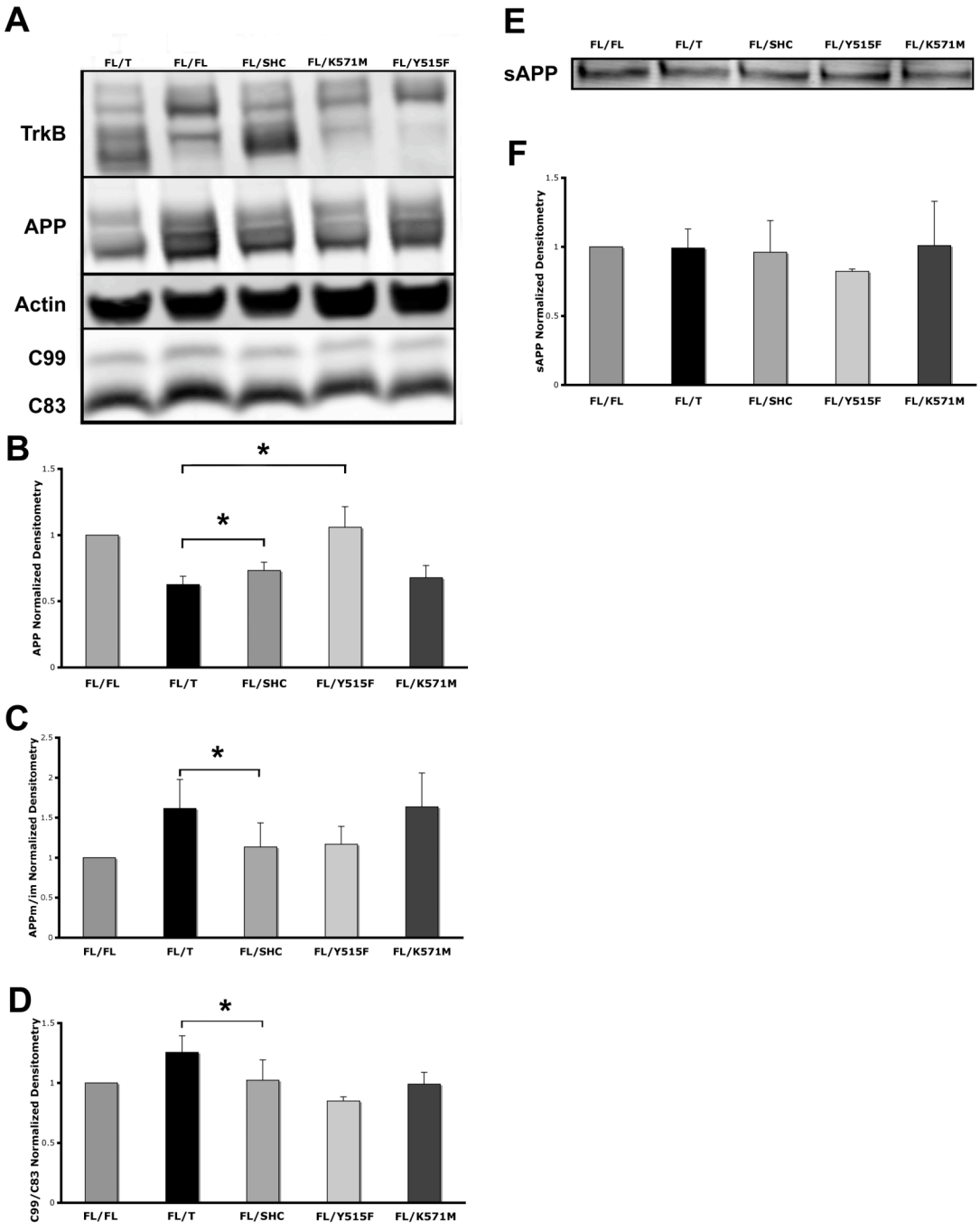


Figure 3.10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FL/T
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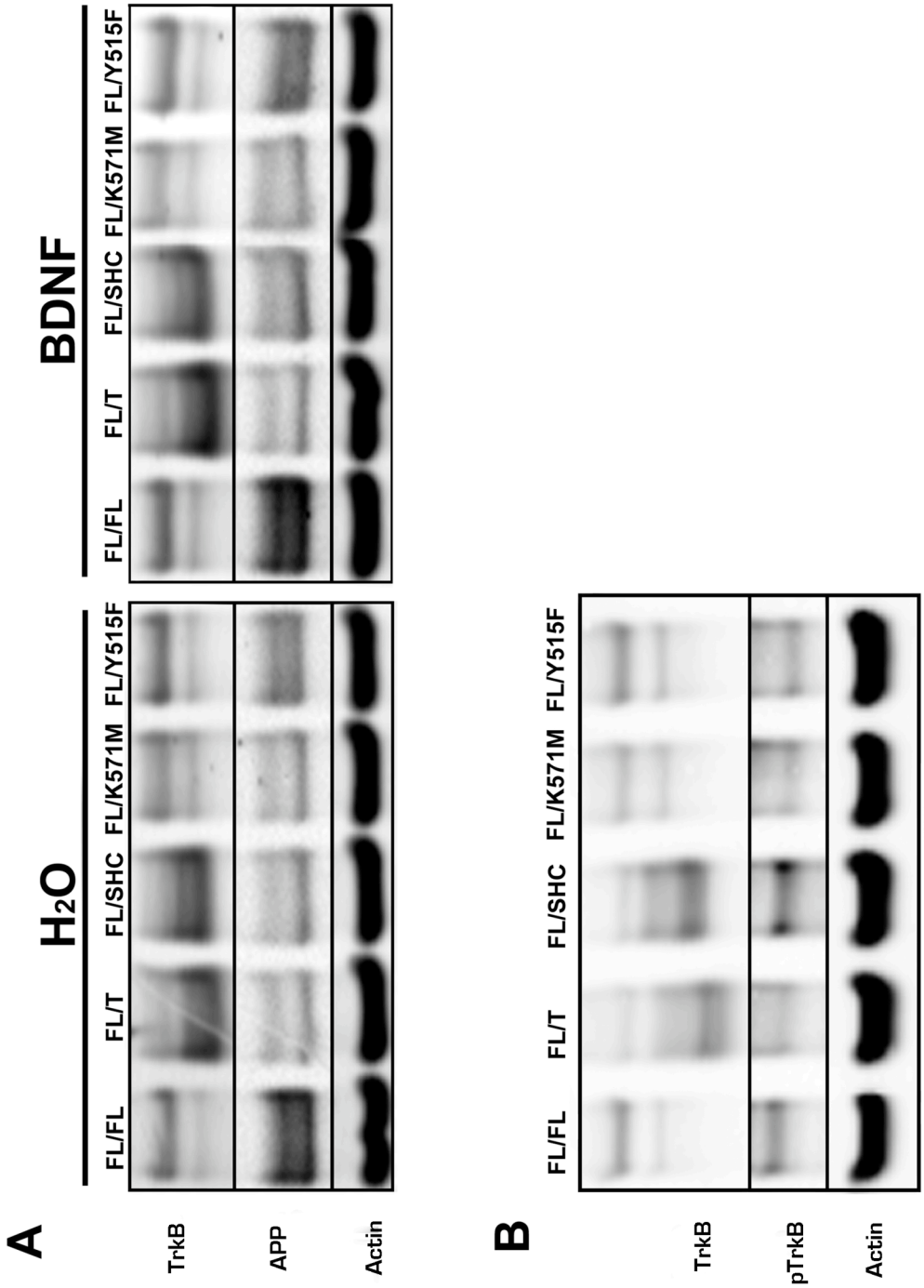
FL/SHC
FL/K571M
FL/Y515F
TrkB
APP
Actin
C99
C83
B
APP Normalized Densitometry
C
APPm/im Normalized Densitometry
D
C99/C83 Normalized Densitometry
E
sAPP
F
sAPP Normalized Densitometry

Figure 3.11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H2O
BDNF
FL/FL
FL/T
FL/SHC
FL/K571M
FL/Y515F
TrkB
APP
Actin
B
FL/FL
FL/T
FL/SHC
FL/K571M
FL/Y515F
TrkB
pTrkB
Actin

4. CHAPTER 4: TrkB mediates endogenous APP metabolism, BACE endogenous levels and NFAT mediated transcriptional activity

Abstract

TrkB isoforms differentially affect APP levels and processing in SH-SY5Y APP Gal4 cells and HEK293 APP^{Swe} cells. These two cell lines over-express APP and are used as a model for several reasons. SH-SY5Y APP Gal4 cells are a neuroblastoma cell line that had been modified to allow AICD levels detection through a luciferase reporter system. HEK293 APP^{Swe} cells over-express an APP mutant that causes early onset Alzheimer's disease in patients. These cell lines cannot be used to investigate the effects of TrkB isoforms on endogenous APP.

We therefore investigated the effects of TrkB FL and TrkB T to test the hypothesis that TrkB might have different effects in the two corresponding naive cell lines. Because we have observed TrkB isoforms effects on APP processing, we also tested the hypothesis that TrkB FL might affect endogenous ADAM10 activity and BACE1 levels in HEK293 cells.

We find that TrkB FL increases APP endogenous levels in both HEK293 and SH-SY5Y naïve cells. We do not find an increase in ADAM10 activity in HEK293 cells but an increase in BACE1 levels. We also find that TrkB FL is able to increase NFAT mediated transcriptional activity and we suggest that this might be the mechanism of transcriptional activation of the BACE1 promoter that causes increased BACE1 levels.

4. 1 Introduction

We have found that TrkB mediated signaling can affect the processing and the expression levels of over-expressed APP in two different cell lines: SH-SY5Y and HEK293 cells. Interestingly, we found that in SH-SY5Y cells APP full-length levels were increased and the TrkB FL receptor increased APP mature levels. In HEK293 cells APP full-length levels were also increased but APP mature levels were decreased (the glycosylation of APP was impaired). This result suggests that there are specific regulation mechanisms dependent on the cell line used. We therefore wanted to test if TrkB FL and TrkB T had different effects on endogenously expressed APP in these two different cell lines.

TrkB FL also had an effect on APP processing that was dependent on the tyrosine kinase activity of the receptor. This was evident in both cell lines. In SH-SY5Y APP Gal4 cells, TrkB FL increased AICD mediated transcriptional activity and AICD-Gal4 levels. Previous experimental evidence suggested increased ADAM activity in SH-SY5Y cells stimulated by RA that induces up-regulation of TrkB (Holback et al., 2005). These studies did not investigate if RA was able to increase ADAM activity or levels but only measured the products of APP α-secretase cleavage, sAPP-α as indirect evidence of increased ADAM activity. First we demonstrated that RA treatment of SH-SY5Y cells specifically increases ADAM activity and levels. We observed a slight increase in sAPP-α levels upon transfection of TrkB FL in HEK293 APP^{Swe} cells. We then tested the hypothesis that TrkB FL can

directly affect ADAM activity in HEK293 APP^{Swe} cells in a BDNF independent manner.

In HEK293 APP^{Swe} cells, TrkB FL decreased the C99/C83 levels but did not alter C83 levels, suggesting a specific effect on β-secretase cleavage of APP. BACE1 is responsible for β-secretase cleavage of APP and its levels are influenced by SHC levels and by NFAT mediated transcription (Groth and Mermelstein, 2003; Xie et al., 2007). Since both SHC and NFAT are mediators of TrkB FL signaling pathways, we tested the hypothesis that TrkB FL auto-activation in HEK293 cells might modulate BACE1 levels via NFAT mediated transcriptional regulation.

We found that, in both SH-SY5Y and HEK293 cells, TrkB FL over-expression increases endogenous levels of APP. Moreover, TrkB FL specifically increases NFAT mediated transcription in both cell lines. We also found that in HEK293 APP^{Swe} cells endogenous BACE levels were increased by TrkB FL over-expression.

4.2 Materials and Methods

4.2.1 Cell lines and stable populations

SH-SY5Y Naïve and 293HEK cells were maintained in DMEM medium (Gibco) with 10% FBS (Gibco) with penicillin streptomycin. SH-SY5Y APP-Gal-4 cells were maintained in the same media but added with 200 μg/ml of G418 (Gibco).

We also obtained stable cell populations expressing farnesylated GFP, TrkB T and TrkB FL both GFP tagged. The transfected cells were selected with 400 μg/ml G418 from 48 hours after transfection up to one month. Liquid nitrogen stocks were obtained. Expression of the constructs was verified by fluorescence microscopy.

For ADAM activity assays cells treated with 10μM in 0.5% DMSO of the ADAM10 agonist PMA (phorbol 12-myristate 13-acetate, Sigma) for 10 hours or DMSO control.

We also treated cells with 20 μM of all trans retinoic acid (ATRA or RA, Sigma) in 0.5% DMSO for 48 hours before collection, when this treatment was performed, control cells were treated 48 hours with 0.5% DMSO.

4.2.2 ADAM10 Assay

Cell lysates were collected in extraction buffer provided with the R&D Systems ADAM activity kit and centrifuged at 4 °C. The supernatant was saved and combined with reaction buffer and substrate in a 96-well plate. The plate was incubated at 37 °C

in the dark for 2 hours and then placed in a Tecan Infinite 200 plate reader controlled by the commercial software Magellan. The plate reader was set at 355nm excitation wavelength and absorption was read at 510nm, the gain was automatically optimized and the integration time was set at 80 μS. Each well was read only once. As a positive control we used lysates from PMA treated cells. Each lysate was run in duplicates and readings were averaged. Background readings from wells containing reagents and substrate were divided by the experimental values and positive controls so that the ADAM10 activity is expressed in fold increase over background. The experiment was repeated twice independently for the cell lines and three times independently for the stable population of HEK293 cells transfected with TrkB constructs or GFP-F controls. Statistical analysis was conducted using the student t-test.

4.2.3 Western blotting procedure and antibodies

Cells were collected 48 hours after all transfection procedures Whole cell lysates were prepared by lysing cells in the plate with ice cold radio immuno-precipitation buffer (150mM NaCl**,** 1% NP40**,** 0.5% DOC**,** 1% SDS**,** 50mM Tris, pH 8.0) supplemented with Halt cocktail of protease and phosphatase inhibitors (ThermoFisher). Cell lysates were sonicated in an ice-cold water bath sonicator for 6 minutes then centrifuged 20 minutes at 4 °C at 14,000 rpm. The resulting supernatants were collected and protein concentration measured with the BCA protein concentration kit (Pierce) according to manufacturer's instructions. Western blot samples were prepared at a final concentration of 1-2 μg/μl in 4X reducing loading

buffer (InVitrogen) and heated at 70 °C for 10 minutes. 15-25 μg of total protein/well from the cell lysates were separated on 4-12% Tris-Glycine midi gels (InVitrogen) in MES-SDS running buffer (InVitrogen) and run at 190 mVolts for 45 minutes. The separated proteins were transferred to PVDF FL membranes (Millipore) using a Semi-Dry transfer apparatus (AA Hoefer TE77X) for 3 hours at 125 mAmp per gel. Membranes were blocked one hour at room temperature using Licor blocking buffer then probed overnight with primary antibodies diluted in Licor blocking buffer at 4-25 °C. Membranes were then washed for 5 minutes 4 times with 0.1% Tween (Sigma) in PBS. After washing, membranes were incubated in the dark with the appropriate secondary antibody IRDye (Licor) diluted in Licor blocking buffer for one hour. Again membranes were washed as above and finally rinsed with PBS. Membranes were scanned on an Odyssey InfraRed scanner (Licor) at appropriate intensities and images acquired at 159 μm resolution. Band intensities were quantified with the provided built-in software (Licor) and always normalized to the actin loading control.

APP was detected using a rabbit antibody A8717 (Sigma) at 1:2000 dilution; TrkB GFP tagged proteins were detected using a mouse GFP antibody (Clontech) at 1:500 dilution; TrkB was also detected with a pan-TrkB rabbit antibody (Santa Cruz Biotechnology); BACE1 was detected using a rabbit antibody ab2077 (Abcam) at 1:1000 dilution; ADAM10 was detected using a rabbit antibody (Chemicon); actin was detected using a mouse antibody (Sigma) at 1:15,000 dilution. Secondary IRDye antibodies (anti rabbit IR800 and anti mouse IR700) were purchased from Licor and used at 1:15,000 dilution.

4.2.4 Transfection and luciferase reporter assays

Cells were transfected at approximately 50-60% confluency in 96-well plates using Arrest-In transfection reagent (OpenBiosystems) according to manufacturer's protocol. For the reporter experiments TrkB plasmids, the GFP-F (negative control, Clontech) and the NFAT3-GFP plasmids (positive control, a gift of Dr. Chris Norris) were co-transfected with the NFAT luciferase reporter (Promega) or the pGL3 luciferase reporter (Promega). Experimental or control plasmids were transfected in 1:1 molar ratio with the luciferase reporter plasmids. In all transfections a renilla luciferase reporter (pRLSV40 or pRLTK, Promega) was used as a transfection normalization control at a molar ratio of 1:40 to the luciferase plasmid.

Cells were collected in Dual Glo Lysis Buffer (E2920, Promega) 48 hours post transfection and the assay was carried out as per manufacturer's instructions. Plates were read in a plate reader luciferase signal was normalized to renilla and data was analyzed using t-tests with Bonferroni correction for multiple comparisons.

4.3 Results

4.3.1 Effects of TrkB T and TrkB FL transfection on endogenous APP in HEK293 and SH-SY5Y cells

TrkB FL has been reported to increase transcription of the APP promoter via IP3K and Akt mediated pathways in SH-SY5Y cells (Ruiz-Leon and Pascual, 2004).

Our previously employed cell models utilized cells that over-express APP under the CMV promoter and therefore impede the assessment of the effects of TrkB on endogenous APP. We hypothesized that, in both HEK293 naive cells and SH-SY5Y naive cells, TrkB FL transfection increases APP transcription. In fact, the signaling pathways triggered by TrkB FL engage molecules that are ubiquitously expressed in all cell lines.

We transiently transfected GFP-F, TrkB T and TrkB FL in HEK293 cells and measured APP levels 48 hours post-transfection. APP detection was difficult in this naïve cell line. We found that TrkB FL transfection significantly increased APP levels compared to GFP-F or TrkB T transfection (Figure 4.1A and 4.1B). The extent of the increase, though, was modest. We then measured APP in a stable population of cells that had been selected for TrkB or GFP-F control expression with antibiotic treatment for at least one month. We found that cells expressing TrkB FL displayed higher levels of APP compared to GFP-F and TrkB T expressing cells (Figure 4.1C).

We transiently transfected TrkB T, TrkB FL and the GFP-F plasmid control in SH-SY5Y naïve cells and found similar results (Figure 4.2A and 4.2B).

We conclude that TrkB FL expression increases endogenous APP full-length levels in two different cell lines while TrkB T does not.

4.3.2 ADAM10 activity does not differ in 293HEK cells stably transfected with TrkB T or TrkB FL

TrkB FL has been shown to increase APP promoter transcription but also to increase sAPPα levels in the media of SH-SY5Y cells (Holback et al., 2005). We

hypothesized that this increase might be due to TrkB FL mediated PLC-γ activation that produces DAG, an ADAM activity stimulator. Therefore we employed an ADAM activity assay to measure ADAM activity in cells that had been transfected with TrkB T or FL.

We initially checked for the assay reliability and the baseline ADAM activity of three different cell lines. A neuroblastoma cell line SH-SY5Y and the same cell line stably expressing APP fused to Gal4. We also used HEK293 cells. We collected lysates of cells treated with vehicle (DMSO) or PMA and read the fluorescence signal emitted at 510 nm. PMA is a phorbol ester that mimics DAG activity.

SH-SY5Y cells showed a higher ADAM basal activity compared to 293HEK cells. PMA was able to increase the ADAM activity signal in all cell lines (Figure 4.3A). SH-SY5Y cells have been shown to display accumulation of sAPP-α when treated with retinoic acid (Holback et al., 2005). The ADAM10 promoter contains RARE, so we hypothesized that this increased sAPP-α release is mediated by an increased transcription and maybe activity of ADAM10 in this cell line. To verify this, we treated SH-SY5Y cells for 48 hours with 20 μM RA or DMSO and measured ADAM activity in the cell lysates. We also treated cells for 10 hours with PMA as a positive control. Both RA and PMA treatments induced a detectable increase in ADAM10 activity, the increase induced by PMA was higher (Figure 4.3B). To verify if the increase in ADAM activity observed after RA treatment, was due to an increase in ADAM levels, we performed a Western blot analysis on the same cell lysates used for the ADAM activity assay. We found that there was an increase in ADAM10

levels in the cells treated with RA compared to the cells treated with vehicle only (DMSO) (Figure 4.3C and 4.3D).

These experiments show that the ADAM10 assay is able to detect differences in ADAM activity and validate its use in our *in vitro* system.

We then applied this assay to test the hypothesis that TrkB FL activates ADAM when transfected in cells. We obtained populations of 293HEK cells stably transfected with a control farnesylated GFP plasmid, TrkB T or TrkB FL. We measured ADAM10 activity of both untreated cells or PMA treated cells and compared them (Figure 4.3). Only the GFP transfected cells showed a small, non significant, increase in ADAM activity when treated with PMA, while both TrkB T or TrkB FL transfected cells displayed the same ADAM activity levels with or without PMA treatment (Figure 4.3). There was no statistical difference between the ADAM activity levels detected in TrkB FL or TrkB T transfected cells (Figure 4.3).

4.3.3 BACE1 levels are increased by TrkB FL transfection in 293Swe cells but not by TrkB T or TrkB SHC

We did not observe altered ADAM activity when transfecting TrkB FL or TrkB T in HEK293 cells. ADAMs are responsible for C83 generation. We did observe alteration of C83/C99 ratios when transfecting TrkB FL, TrkB T, TrkB SHC or the TrkB FL mutants. Therefore we hypothesize that BACE1, responsible for C99 production, levels might be decreased upon TrkB transfection. To test this hypothesis we measured BACE1 levels in cells transfected with the TrkB isoforms and mutants (Figure 4.5A).

Surprisingly, we found that TrkB FL significantly increased BACE1 levels compared to TrkB T while TrkB SHC did not. Also TrkB FL Y515F did decreased BACE1 levels compared to TrkB FL, while the tyrosine kinase inactive mutant did not affect BACE1 levels compared to TrkB FL (Figure 4.5B).

4.3.4 TrkB FL activates NFAT3 mediated transcription

We observed an increase in BACE1 levels upon TrkB FL transfection. BACE1 levels have been shown to be modulated by the Ca^{2+} activated transcription factor NFAT1 but not by NFAT4 (Cho et al., 2008). Because TrkB FL can activate NFATc4, also referred to as NFAT3, the neuronal specific form of NFAT, we hypothesized that TrkB FL might mediate activation of NFATc4 and thus increase BACE1 transcription. To test whether TrkB FL transfection was able to activate NFATc4 we transfected a NFAT luciferase reporter construct together with TrkB FL or TrkB T. We used a GFP tagged NFAT3 over-expression construct as a positive control and a renilla luciferase reporter to normalize transfection efficiency. In both SH-SY5Y Naïve and HEK293 Naïve cells, we found that TrkB FL transfection increased NFAT mediated luciferase activity compared to TrkB T and GFP-F control but not compared to the NFAT3 over-expression construct (Figure 4.6A and 4.6B).

TrkB FL can activate signaling pathways that increase release of Ca^{2+} from intracellular storage, therefore the effects that we observe might be due to Ca^{2+} mediated unspecific increase in transcription. To rule out this possibility we co-transfected the TrkB constructs, the control constructs and the NFAT3 construct

together with a pGL3 luciferase reporter that does not respond to NFAT specific activation but would be subjected to general increases in transcription activity within the cells. We did not observe any differences in luciferase activity between TrkB T and TrkB FL when utilizing pGL3 as a reporter construct (Figure 4.6C and 4.6D) in both cell lines. This demonstrates that TrkB transcriptional effects are specific to NFAT. The increase in luciferase activity observed when transfecting NFAT3 and pGL3 luciferase is due to the fact that NFAT binds to the SV40 promoter enhancing its transcription (Manley et al., 2008).

4.4 Discussion

We have here investigated the TrkB mediated modulation of endogenous APP and of the APP processing enzymes ADAM10 and BACE1.

APP levels are increased by TrkB FL auto-activation and the increase is more dramatic in the HEK293 cells line than in the SH-SY5Y cell line. This might be due to the presence of all TrkB isoforms and BDNF in the latter cell line that might contribute to regulate levels of the TrkB FL/T exogenously expressed. APP promoter activation mediated by TrkB FL had been previously shown through reporter assays in SH-SY5Y cells, here we show that APP protein levels are increased by TrkB FL transfection compared to TrkB T and that this increase is apparent even in absence of exogenous BDNF. We observe the same increase in APP levels in HEK293 cells that do not express BDNF/TrkB endogenously. Therefore, over-expression of the TrkB FL receptor probably causes auto-activation and signaling through of the IP3K

pathway that has been shown to mediate APP transcription (Ruiz-Leon and Pascual, 2001).

The fact that we observe a TrkB FL mediated increase in APP levels in both cell lines suggests that the pathways engaged are common among the two. Interestingly, TrkB FL/BDNF signaling is associated with beneficial effects in the context of AD while any factor that increases APP levels is generally associated with worse degeneration. This apparent contradiction can be solved by several considerations. First, increased APP levels are not necessarily associated with more degeneration since APP is important in cell-matrix interaction and cell-cell interactions, is expressed on dendritic spines and plays important roles in synaptic regulation (Kamenetz et al., 2003; Hoe et al., 2009). Secondly, increased APP levels do not necessarily correlate to increased production of neurotoxic fragments. The outcome of APP proteolytic cleavage is central for the pathogenesis. If APP is cleaved by α-secretases, it generates fragments that have growth factor like characteristics, such as sAPP-α (Caille et al., 2004). Finally, TrkB activates many signaling pathways that are anti-apoptotic and favor synaptic transmission and LTP (Minichiello, 2009). In the broader context of all these interactions and balance, TrkB FL beneficial effects can be associated even with over-all increased APP levels.

The hypothesis that TrkB FL might favor α-secretase cleavage of APP has been marginally addressed before. In previously performed experiments RA treatment of cells had been showed to promote accumulation of sAPP-α. Even is the authors show that BDNF stimulation of the cells increases sAPP-α accumulation, it is difficult to unequivocally determine if this effect is only, or at least mainly, mediated

by TrkB. In fact RA can activate ADAM10 transcription TrkB independently. The authors also do not use the TrkB T isoform to check if the effect was specifically due to the tyrosine kinase activity of the full-length receptor. Here we show that ADAM10 levels are increased in SH-SY5Y cells after RA stimulation and that ADAM activity is also increased. We could not detect an ADAM activity increase in HEK293 cells transfected with TrkB T or TrkB FL. There could be several reasons for this. We found that the assay employed was sensitive to the GFP fluorescence emission. Our TrkB constructs are GFP positive. This might have altered the readings introducing an unknown factor in the quantification. A reading of the samples at 540nm, the optimal emission wavelength of GFP, did not highlight differences in signal among lysates obtained from different stable populations (transfected with different constructs). While this would suggest that the GFP signal, constant across samples, would not contribute to the reading of the experimental samples, we cannot exclude that, at 510nm, the optimal wavelength for ADAM activity readings, the emission spectra of GFP interferes with the quantification. It must also be noted that HEK293 cells show a lower baseline value of ADAM activity, lowering the sensitivity and dynamic range of the assay for this particular cell line. Experimental issues aside, we could also speculate that auto-activation of the TrkB FL receptor, shown to differ from the BDNF dependent activation, is not efficient at mediating PLC-γ binding and therefore at increasing ADAM activity. We performed the experiment in HEK293 cells because that was the cell line where we observed a decrease in C83/C99 ratio and this cell line lacks BDNF and endogenous TrkB expression, it might therefore lack specific mediators of TrkB signaling that are

necessary to engage ADAMs. Another explanation is that TrkB FL does not mediate ADAM activation. To investigate all these possibilities more experiments are needed. TrkB FL over-expression could be employed in SH-SY5Y cells in combination with its isoforms and mutants to determine if there is an ADAM activity effect and if the PLC-γ binding site is mediating this effect. Moreover BDNF stimulation of exogenously expressed TrkB FL could be employed to investigate whether an ADAM activity effect can be recapitulated even in the HEK293 cell line.

ADAMs are mediators of α-secretase cleavage of APP and we did not find any evidence for altered ADAM activity in HEK293 APP^{Swe} cells. BACE1 is responsible for the β-secretase cleavage of APP that generates the toxic fragment Aβ. We observed that TrkB FL was able to reduce the C83/C99 ratio in HEK293 APP^{Swe} cells, therefore we tested if BACE1 levels were decreased by TrkB FL or its isoforms. We found that BACE1 levels were increased by TrkB FL but not by its tyrosine kinase inactive mutants or truncated isoforms. SHC knock-down has been shown to decrease BACE1 levels and therefore Aβ production (Xie et al., 2007). SHC mediates downstream signaling of TrkB FL. Moreover, NFAT1 activation has been shown to increase BACE1 levels, while NFAT4 does not mediate the same effect (Cho et al., 2008). NFAT3 is activated by TrkB FL/BDNF signaling (Groth and Mermelstein, 2003). We then suggest a mechanism for BACE1 transcriptional regulation mediated by TrkB FL/SHC and NFAT.

In conclusion we find that TrkB FL over-expression increases APP levels even in absence of exogenous BDNF, suggesting that auto-activation of the receptor can mediate APP transcription. We also find that BACE1 levels are increased by

TrkB FL auto-activation in HEK293 APP^{Swe}. We speculate that altered trafficking of APP and/or BACE1 is responsible for decreased C83/C99 levels in HEK293 APP^{Swe}. We finally identify NFAT mediated transcriptional activation as a possible mechanism of BACE1 transcription up-regulation.

4.5 Figure Legends:

Figure 4.1: TrkB FL transfection increases endogenous APP full-length levels in HEK293 cells. (A) Transient transfection of TrkB FL induces an increase in APP full-length levels while TrkB T does not affect APP levels compared to GFP-F control. (B) Stable transfection of TrkB FL also induces an increase in APP full-length levels compared to both TrkB T and GFP-F stably transfected cells. (C) Quantification of APP FL levels in cells transfected with TrkB FL shows a statistically significant increase in APP levels over both TrkB T and GFP-F.

Error bars represent STDEV; *=$p<0.025$ after t-test, Bonferroni corrected for multiple comparisons.

Figure 4.2: TrkB FL transfection increases endogenous APP full-length levels in SH-SY5Y Naïve cells. (A) Representative Western blot of SH-Sy5Y Naïve cells lysates transiently transfected with TrkB T or FL. (B) TrkB FL transfection induces an increase in APP full-length levels compared to TrkB T transfection.

Figure 4.3: ADAM10 activity/expression in cell lines treated with PMA or stably transfected with TrkB. (A) PMA treatment of three different cell lines induces a significant increase in ADAM activity compared to the vehicle treated controls. (B) RA treatment of SH-SY5Y cells induces an increase in ADAM activity compared to vehicle control treated cells but not compared to PMA treated cells. (C) Representative Western blot for detection of ADAM10 levels in RA treated SH-SY5Y and SH-SY5Y-APP-Gal4 cells. (D) Quantification of the Western blot in (C) showing that ADAM10 levels are increased by RA treatment of the SH-SY5Y neuroblastoma cell line.

Figure 4.4: TrkB FL and TrkB T stable transfection does not increase ADAM activity compared to GFP-F transfected cells. PMA treatment also does not affect ADAM activity in these cell lines.

N=3; t-test with Bonferroni correction for multiple comparisons.

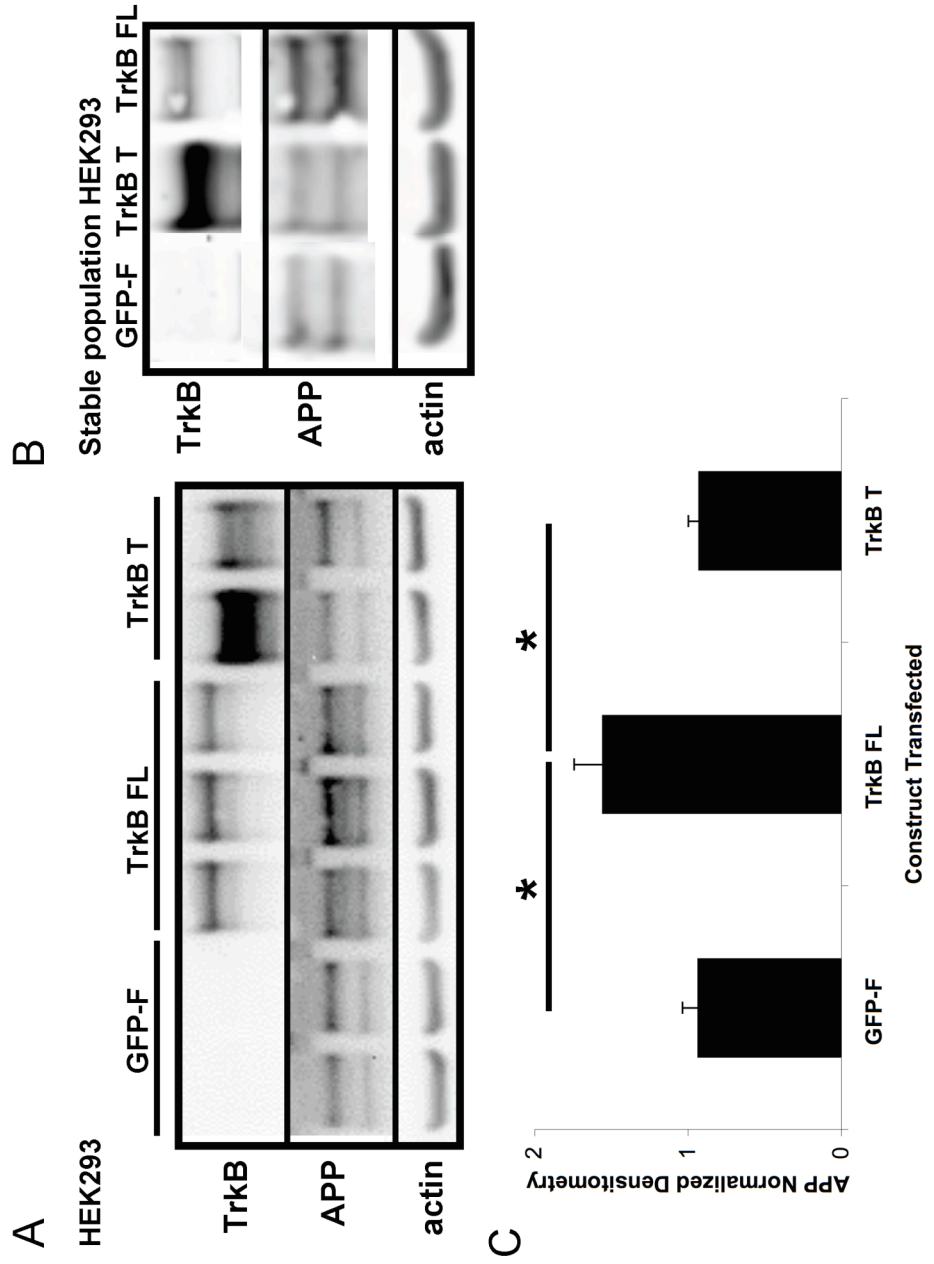
Figure 4.5: TrkB transfection affects BACE1 levels in HEK293 APP^{Swe} cells. (A) Representatives Western blots of BACE1 in cells transfected with TrkB isoforms and mutants. (B) Quantification of at least two independent experiments. TrkB FL significantly increases BACE1 levels compared to TrkB T ($p=0.01$) while TrkB SHC does not. Transfection of the mutant on the SHC binding site of TrkB FL, Y515F, decreases BACE1 levels compared to TrkB FL.

Error bars: SEM; n=6 for TrkB T, FL, SHC; n=4 for TrkB Y515F; n=2 for K571M. Statistical analysis: paired t-test with Bonferroni correction.

Figure 4.6: TrkB FL increases NFAT mediated transcription. (A) TrkB FL and TrkB T were transfected in SH-SY5Y Naïve cells with a NFAT luciferase reporter constructs. TrkB FL significantly increased NFAT mediated luciferase compared to TrkB T (p=0.02). GFP-F was used as a negative control while NFAT3 GFP tagged was used as a positive control. (B) TrkB FL and TrkB T were transfected in HEK293 Naïve cells with a NFAT luciferase reporter constructs. TrkB FL significantly increased NFAT mediated luciferase compared to TrkB T (p=0.0001). (C) TrkB FL did not increase transcription of a pGL3 reporter construct co-transfected in SH-SY5Y Naïve cells. NFAT3 increased transcription of pGL3 because it can bind to its SV40 viral promoter. (D) TrkB FL did not increase transcription of a pGL3 reporter construct co-transfected in HEK293 Naïve cells. NFAT3 increased transcription of pGL3 because it can bind to its SV40 viral promoter.

N=3 for SH-SY5Y cells with 6 replicates, n=2 for HEK293 cells with 6 replicates; error bars represent STDEV; statistical analysis: student t-test.

Figure 4.1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GFP-F
TrkB FL
TrkB T
TrkB
APP
actin
B
Stable population HEK293
GFP-F
TrkB T
TrkB FL
TrkB
APP
actin
C
APP Normalized Densitometry
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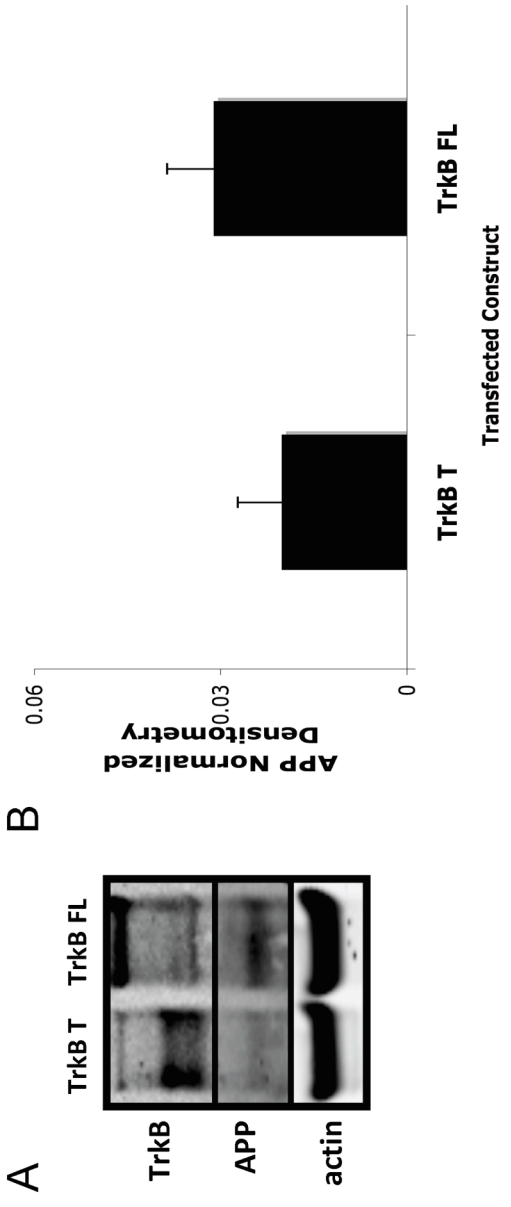
1
2
*
*
GFP-F
TrkB FL
TrkB T
Construct Transfected

Figure 4.2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TrkB FL
TrkB
APP
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B
APP Normalized Densitometry
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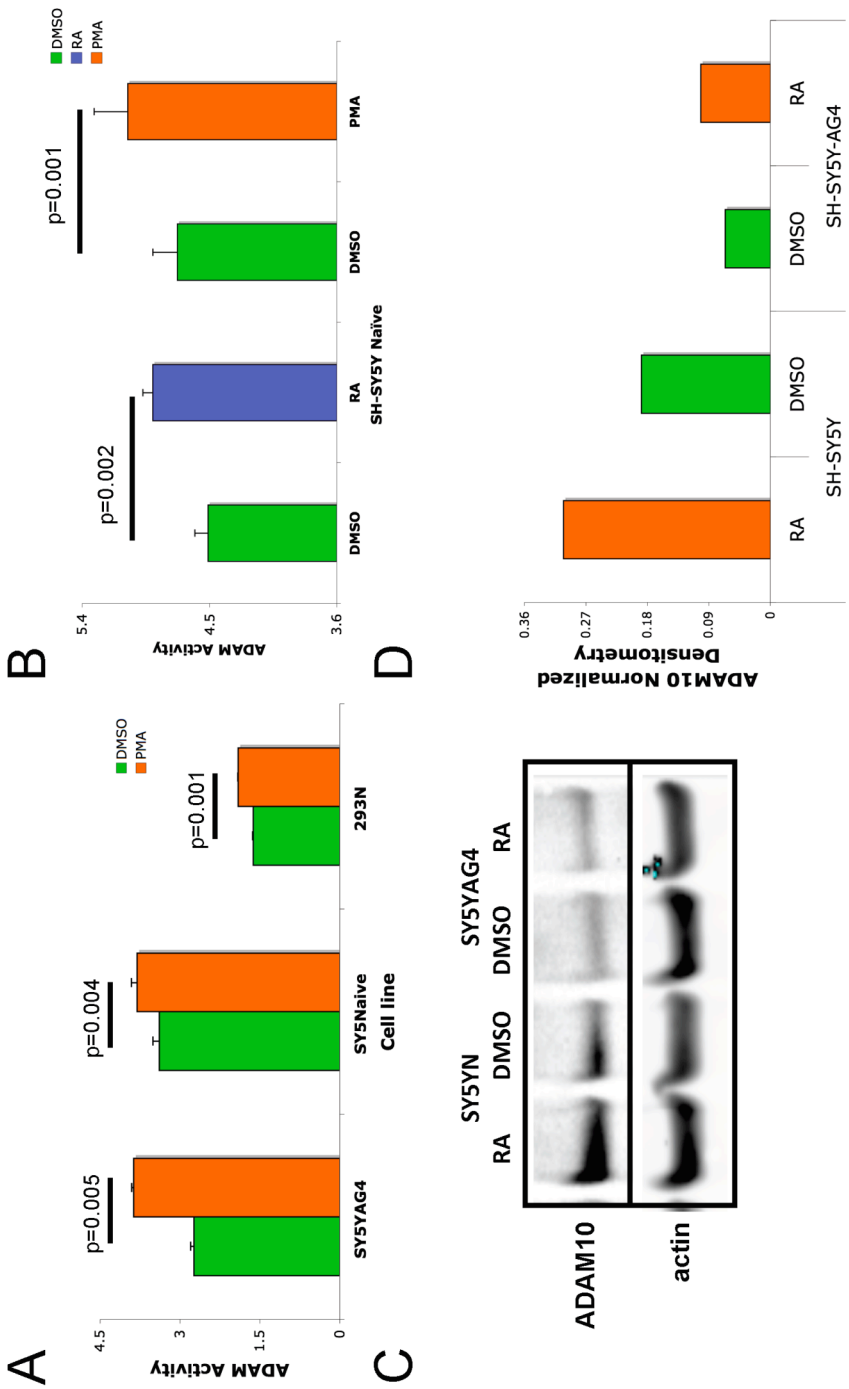
0.03
0
TrkB T
TrkB FL
Transfected Construct

Figure 4.3


A
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3
1.5
0
p=0.005
p=0.004
p=0.001
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PMA
SY5YAG4
SY5Naive
293N
Cell line
B
ADAM Activity
5.4
4.5
3.6
p=0.001
p=0.002
DMSO
RA
PMA
DMSO
RA
SH-SY5Y Naïve
C
SY5YN
SY5YAG4
RA
DMSO
DMSO
RA
ADAM10
actin
D
ADAM10 Normalized Densitometry
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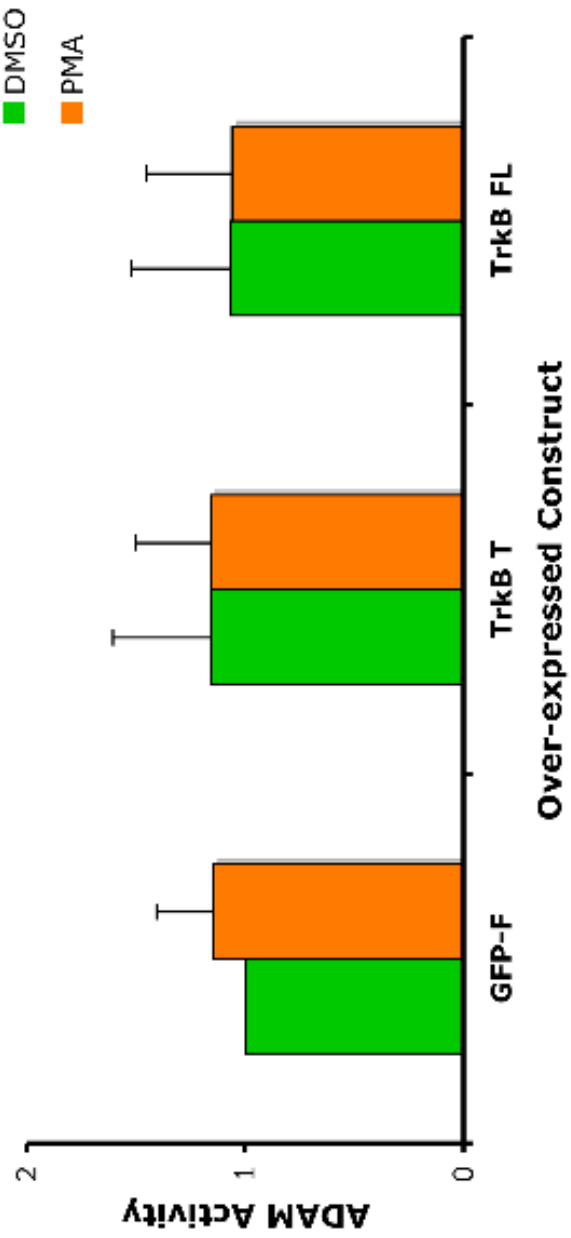
0.27
0.18
0.09
0
RA
DMSO
SH-SY5Y
DMSO
RA
SH-SY5Y-AG4

Figure 4.4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PMA
ADAM Activity
0
1
2
GFP-F
TrkB T
TrkB FL
Over-expressed Construct

Figure 4.5

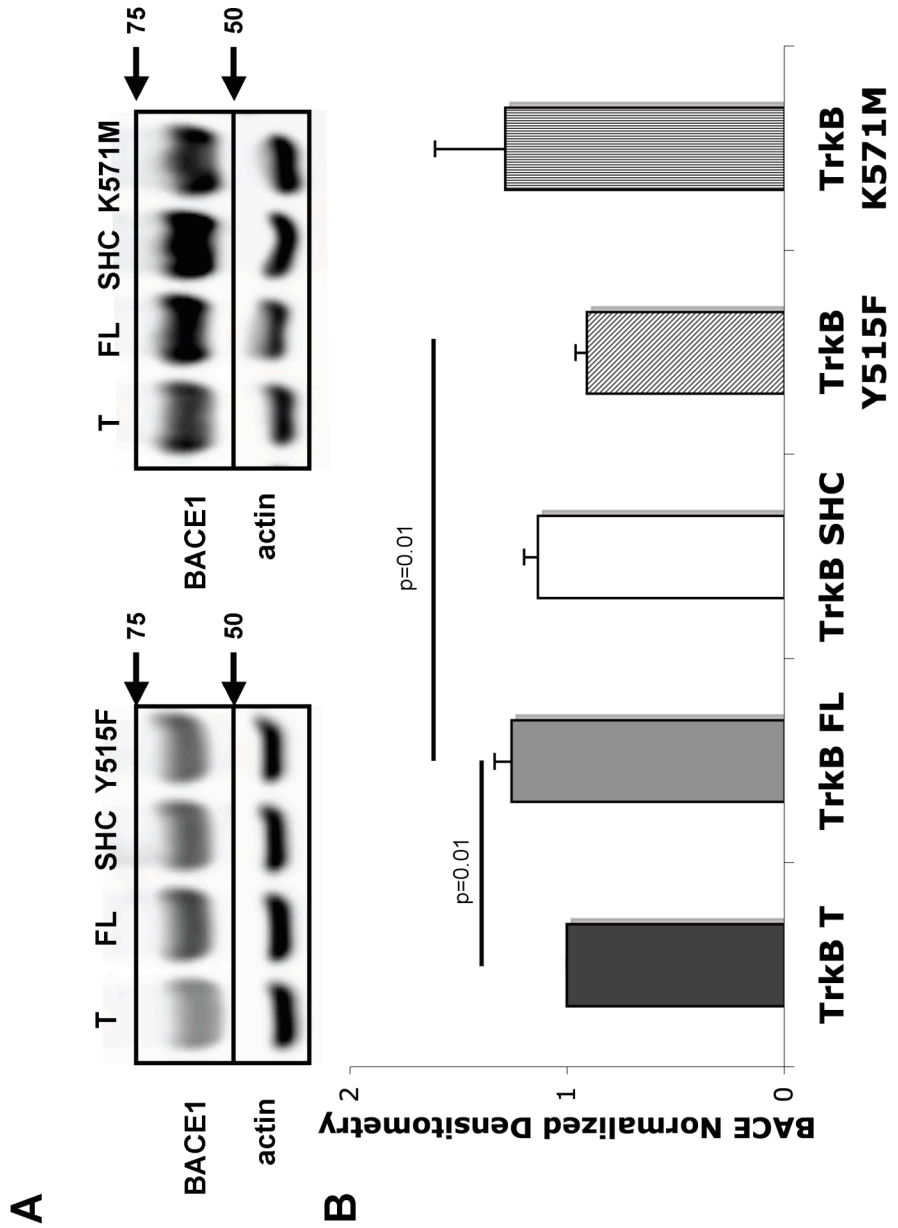
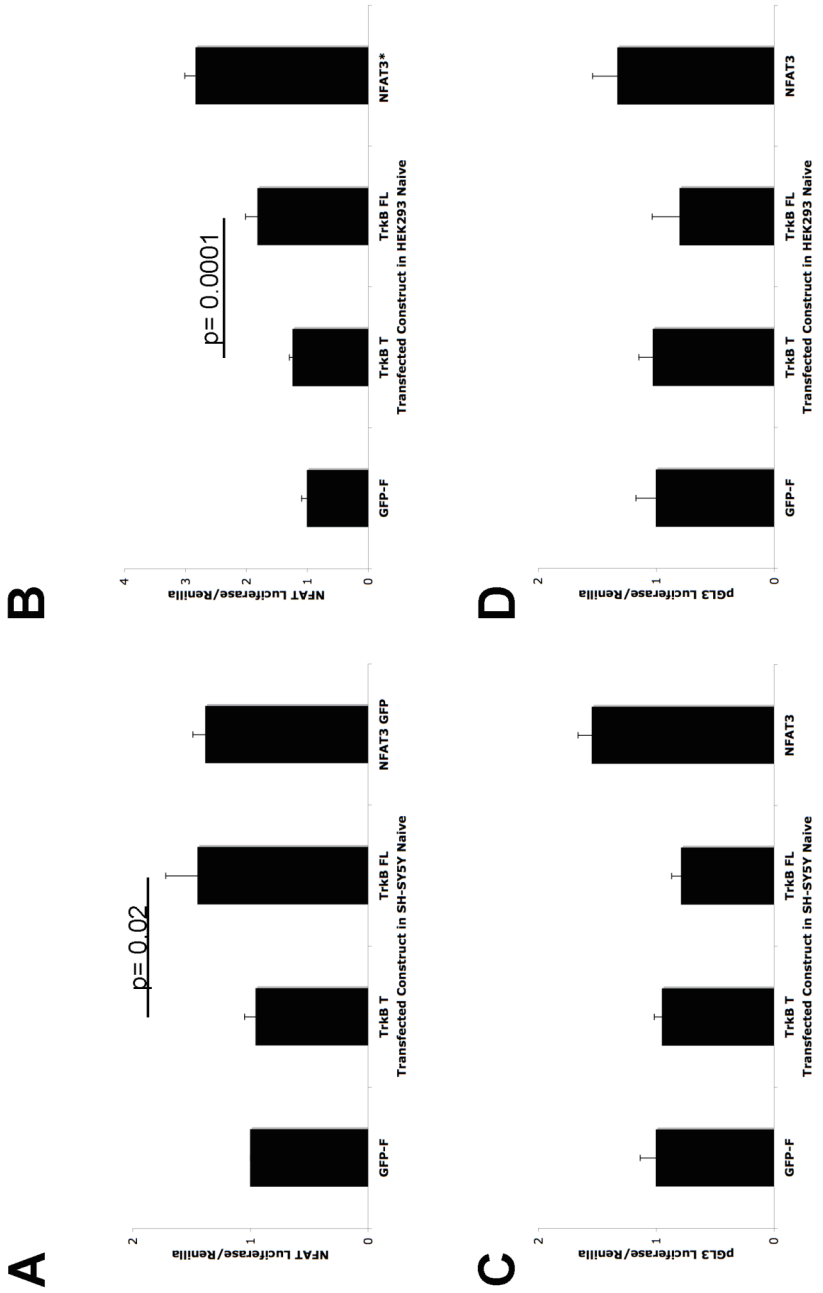

A
T
FL
SHC
Y515F
75
50
BACE1
actin
T
FL
SHC
K571M
75
50
BACE1
actin
B
BACE Normalized Densitometry
0
1
2
p=0.01
p=0.01
TrkB T
TrkB FL
TrkB SHC
TrkB Y515F
TrkB K571M

Figure 4.6


A
B
C
D
NFAT Luciferase/Renilla
pGL3 Luciferase/Renilla
p= 0.02
p= 0.0001
GFP-F
TrkB T
TrkB FL
NFAT3 GFP
NFAT3*
NFAT3
Transfected Construct in SH-SY5Y Naive
Transfected Construct in HEK293 Naive

5. SUMMARY AND FUTURE DIRECTIONS

5.1 Summary of findings

TrkB is an important neurotrophic receptor expressed on neurons and implicated in synapses formation, neuronal survival/differentiation and long-term potentiation. TrkB levels are developmentally regulated and are affected in Alzheimer's neurodegeneration and other conditions such as depression.

TrkB is expressed in three different splicing isoforms, TrkB full length (FL), TrkB truncated (T) and TrkB SHC, that share a common extracellular domain that binds brain derived neurotrophic factor (BDNF) and differ in their intracellular domain. The intracellular domain of TrkB can activate different signaling pathways and bind to the adaptor protein SHC and to the enzyme PLC-γ. TrkB FL has been shown to affect APP transcription and processing, moreover, some studies associated single nucleotide polymorphisms of *NTRK2*, the gene encoding for TrkB, to Alzheimer's disease. *NTRK2* is located on chromosome 9p22, a region with a high likelihood of disease score.

We hypothesized that TrkB isoforms differentially modulate APP metabolism and tested this hypothesis in different cell lines. Initially we employed the SH-SY5Y neuroblastoma cell line that expresses basal levels of TrkB and BDNF and has been used before to investigate TrkB mediated signaling pathways. Then we employed HEK293 APP^{Swe} cells that provide a TrkB/BDNF free model system that simplifies data interpretation. This cell line also over-expresses APP^{Swe} that causes early onset

Alzheimer's disease (AD) in humans and is a commonly used *in vitro* AD model. Finally we determined TrkB mediated effects on APP endogenous in both SH-SY5Y and HEK293 cells. We also investigated TrkB effects on endogenous ADAM10 and BACE1.

In SH-SY5Y APP Gal4 cells, we found that APP levels are increased by TrkB FL over-expression but are not affected by TrkB T and TrkB SHC. Knock-down of the TrkB receptors causes decreased levels of APP and knock-down of TrkB FL and TrkB SHC only causes cell death, suggesting that TrkB T might impair cell survival. We found that TrkB FL increases AICD mediated transcriptional activity and that this effect is mediated by the tyrosine kinase activity of the receptor and by the PLC-γ binding activity. AICD mediated transcriptional activity is not affected by TrkB T but is decreased by TrkB SHC through its SHC binding site. Therefore we demonstrate that TrkB isoforms can affect APP levels and processing.

We then investigated the role of the TrkB isoforms in APP^{Swe} metabolism. We hypothesized that BDNF independent signaling of TrkB can affect APP and we tested this hypothesis on APP^{Swe} that causes early onset AD in humans. We found that TrkB FL auto-activates in HEK293 APP^{Swe} cells and that it impairs APP^{Swe} glycosylation and decreases the C99/C83 ratio. TrkB T and TrkB SHC do not affect APP^{Swe} glycosylation or processing. These effects are mainly mediated by the tyrosine kinase activity of the receptor and not by its SHC or PLC-γ binding. We also found that co-transfection of the TrkB truncated isoforms regulates TrkB FL effects on APP. In particular, TrkB T acts as a dominant negative, while TrkB SHC shows the same effects as TrkB FL. In agreement with this finding, TrkB FL was not phosphorylated

when co-transfected with TrkB T but was phosphorylated when co-transfected with TrkB SHC. This demonstrates, for the first time, that TrkB truncated isoforms have a different regulatory role on TrkB FL.

While TrkB had different effects on the metabolism of exogenously expressed APP in the two cell lines employed, we found that it increases endogenous APP levels in both without affecting APP glycosylation. This demonstrates that TrkB affects APP levels through mechanisms that are common between the two cell lines. TrkB does not affect endogenous ADAM10 activity in HEK293 cells but increases BACE1 levels. We speculate that BACE1 levels increase is due to TrkB FL mediated increased NFAT transcriptional activity.

TrkB FL had different effects on APP over-expressed in two different cell lines. We speculate that this difference is due to the mechanism of activation of the receptor: BDNF dependent, in SH-SY5Y cells, or BDNF independent, in HEK293 cells. In SH-SY5Y cells, BDNF binding to the TrkB FL receptor causes internalization of the receptor in activated endocytic vesicles and its rapid degradation in endosomal compartments. This physiologic route of activation does not affect APP glycosylation but only its processing. In HEK293 cells, BDNF is not present therefore TrkB FL auto-activation occurs in intracellular vesicles in the trans-Golgi network impairing protein glycosylation and trafficking. The fact that TrkB FL activation can be prevented in both cell lines by co-transfection of TrkB T but not by TrkB SHC suggests that TrkB FL regulation through the truncated isoforms is conserved and probably occurs through similar mechanisms.

5.2 Future Directions

The aforementioned findings are relevant in AD context for several reasons. TrkB FL/BDNF signaling pathways are associated with beneficial effects and BDNF agonists are being investigated as potential therapies. The demonstration that TrkB isoforms have different effects on APP metabolism suggests that these therapeutic approaches might be effective only in patients that express TrkB FL at higher levels than the truncated isoforms. In patients where TrkB FL levels are reduced relative to TrkB T/SHC, BDNF, or its agonists, would be scavenged by these isoforms. Binding of TrkB truncated isoforms to BDNF would negatively regulate TrkB FL signaling and maybe activate pathways that increase neurodegeneration rather than ameliorating it. The fact that TrkB can modulate APP metabolism but also is subjected to APP mediated regulation suggests the presence of a self-sustaining loop of interactions between the two proteins in AD. If Aβ down-regulates TrkB FL/BDNF signaling, it is unlikely that BDNF agonists will be an effective therapy in patients that already have a high Aβ load. On the contrary, if TrkB FL/BDNF signaling pathways can promote α-secretase processing and neuronal survival, they might be valuable drug targets during the early stages of AD. Given the regulatory role of the truncated isoforms on TrkB FL, these can also be considered as drug targets. Instead of triggering TrkB FL signaling, an alternative approach could be TrkB T down-regulation or TrkB SHC up-regulation. Therefore, RNAi therapeutic strategies targeting TrkB T specifically or targeting the splicing mechanism of the TrkB gene to increase production of TrkB SHC/FL could also be investigated.

The design of effective therapies targeting TrkB will benefit from a more through investigation of its role in regulating APP metabolism and as a target of APP regulation. In particular, based on our findings, we think necessary to perform additional experiments to address important outstanding questions.

The experiments conducted on SH-SY5Y APP Gal4 and HEK293 APP^{Swe} cells suggest that BDNF dependent or independent TrkB activation might have different effects on over-expressed APP metabolism. Moreover we find that TrkB truncated isoforms can regulate TrkB FL effects in both cell lines. Further characterization of the TrkB mediated effects in these cell lines should address the questions:

1. What are the intracellular levels of Aβ in HEK293 APP^{Swe} cells after TrkB isoform transfection? Do levels of sAPP-β correlate with these? Based on the Aβ extracellular levels, the C83/C99 ratio and the sAPP-α quantifications, we hypothesize that TrkB FL would lower these levels. TrkB Y515F might also decrease Aβ and sAPP-β products.
2. Since BACE1 levels are increased by TrkB FL transfection, based on the results of Aβ quantification, it would be essential to investigate how APP cleavage is modified by TrkB isoforms. Co-localization and cellular fractionation experiments aimed at detecting the location of the secretases and APP in the cells upon transfection of the TrkB isoforms could be employed. We hypothesize that trafficking of these proteins is altered causing the altered processing.

3. Aβ, sAPP-β and BACE1 expression levels should also be checked in SH-SY5Y APP Gal4 cells and possibly in the same cell line expressing APP^{Swe}.
4. Characterization of the TrkB SHC Y515F mutant should be performed in both aforementioned cell lines since it might help uncover the mechanism of its lack of inhibition of AICD-mediated transcription.

It can be hypothesized that basal expression of TrkB FL followed by BDNF stimulation of the receptor in HEK293 APP^{Swe} cells, will be able to recapitulate the effects seen in SH-SY5Y APP Gal4. Specifically, lower levels of expression of TrkB FL should avoid intracellular auto-activation of the receptor and BDNF application would trigger a physiologic activation of the receptor at the cell surface.

1. Titration experiments to determine TrkB FL amounts that transfected avoid intracellular auto-activation.
2. Investigation of the mechanism of TrkB SHC mediated TrkB FL activation. We hypothesize that this isoform, co-localizing with TrkB FL, helps the crowding of this receptor intracellularly promoting its auto-activation.
3. Co-transfection experiments aimed at dissecting the optimal ratios of TrkB FL inhibition by TrkB T and exogenous BDNF application experiments to check for the role of BDNF in promoting TrkB dimerization. The question of isoform homo/hetero dimerization

preference can also be addressed in this system by performing chemical cross-linking of the receptors.

Finally, our experiments employed cell lines over-expressing APP as a model for this initial characterization of the TrkB effects on APP metabolism. Cell lines are valuable models for molecular and cellular biology investigations but have limited relevance to the physiologic situation. Therefore investigation of the same problems in primary neurons and animal models is needed.

1. Primary neurons express all the TrkB isoforms but nobody has performed an exact characterization of the relative levels of the TrkB isoforms. Primary neuronal cultures should be obtained and mRNA of the TrkB isoforms as well as protein levels should be measured. It would be interesting to see if cultures display different TrkB ratio at different days *in vitro*.
2. Investigation of the effects of a specific isoform at a time can be performed by using shRNA mediated knock-down of the other isoforms. This approach is easy to implement because the mRNA of the isoforms are different. Over-expression of the isoforms/mutants, using lower efficiency promoters can also be employed to this end.
3. Investigation of the TrkB SHC isoform will be difficult in mouse/rat neuronal cultures because these animals do not express this isoform. Alternatively, human neuronal stem cell lines could be employed after performing characterization experiments (see point 1).

After the work in primary neurons, if the findings are promising in light of future applicable therapeutic strategies, animal models can be used. These models could include TrkB or BDNF knock-outs and AD models.

6. APPENDIX I: Brefeldin A inhibits APP glycosylation and inhibits APP cleavage in SH-SY5Y APP Gal4 cells

We have observed that TrkB FL over-expression in HEK293 APP^{Swe} cells decreases APP glycosylation. We hypothesized that this effect is due to disruption of the Golgi apparatus caused by the auto-activated TrkB FL receptors as it has been shown previously (Schecterson et al., 2010). We also hypothesize that the impaired glycosylation of APP causes its decreased processing as measured by the decreased C83/C99 ratio that we observe. We do not observe the same effect in SH-SY5Y APP-Gal4 cells and we hypothesize that this is due to the different regulation of TrkB FL signaling in this cell line that expresses BDNF and TrkB endogenous. We speculate that in SH-SY5Y APP Gal4 cells, APP glycosylation is not affected because TrkB activation and regulation is different and therefore activation of the TrkB FL does not affect the Golgi apparatus/protein glycosylation. If APP impaired glycosylation is causing the decreased APP processing observed in HEK293 APP^{Swe} cells, we should be able to recapitulate the same effect in SH-SY5Y APP Gal4 cells. Therefore we hypothesize that inhibiting APP glycosylation in SH-SY5Y APP Gal4 cells will inhibit APP processing. To test this hypothesis we treated with Brefeldin A SH-SY5Y APP-Gal4. Brefeldin A (BFA) is a drug commonly used to impair protein glycosylation.

BFA is lactame antibiotic that affects the Golgi compartment. It impedes the trafficking of proteins from the ER to the Golgi thus inhibiting post-translational

modification of proteins. These trafficking changes do not alter transferrin and iron transport from the cell surface to the intracellular compartment and vice versa. Therefore normal membrane trafficking is not completely compromised (Klausner et al., 1992). BFA also inhibits the delivery of proteins from the early endosomal compartment to lysosomes (Klausner et al., 1992).

We initially demonstrated decreased APP processing through an AICD-mediated luciferase activity assay (Figure 6.1). AICD mediated luciferase activity decreased about 30% in BFA treated cells compared to vehicle control. Parallel L-685 treatment as a positive control showed a luciferase decrease of about 50% (Figure 6.1).

APP processing is mainly mediated by α-secretase activity. We therefore tested the hypothesis that ADAM10 levels could be decreased by BFA treatment. In particular, we thought that BFA would affect ADAM10 maturation and that we would find higher levels of immature pro-enzyme ADAM10. We quantified ADAM10 levels in lysates treated with BFA or controls and we did not detect a significant difference in total ADAM10 levels. Furthermore, we could not detect a shift in molecular weight of ADAM10 that would suggest an impaired ADAM10 maturation in treated cells (Figure 6.2).

APP glycosylation was impaired by BFA treatment as demonstrated by an accumulation of an intermediate glycosylated form of APP (Figure 6.3). We also observed an increase in APP total when treating cells with BFA for 4 hours (Figure 6.3 and 6.4A). This accumulation is probably due to BFA mediated inhibition of mature endosomes formation. APP does not get degraded and the time period of the

treatment is enough to cause APP accumulation. In fact APP half-life is approximately 1.5 hours. Even if total APP levels were increased by BA treatment, we found that BFA treatment decreases CTFs levels, hence APP processing (Figure 6.3).

C83 levels in lysates of SH-SY5Y APP Gal4 cells treated for 4 hours with BFA were very low, almost below Western blot detection limit (Figure 6.3 and 6.4B). Combined BFA and L-685 treatment showed accumulation of the CTFs to the same extent as non-treated cells (Figure 6.4B). This suggests that APP processing by both α and β secretases, but not γ-secretase, is inhibited by BFA. 3 hour treatment with BFA followed by one hour incubation with L-685 lead to significant accumulation of CTFs compared to the vehicle control. This suggests that BFA inhibition of APP α and β processing is quickly reversible.

We conclude that BFA mediated APP impaired glycosylation can inhibit APP processing in SH-SY5Y APP Gal4. BFA does not affect ADAM10 levels or maturation. Decreased APP processing is therefore probably due to the impaired trafficking of APP between the endoplasmic reticulum and the Golgi.

Figures:

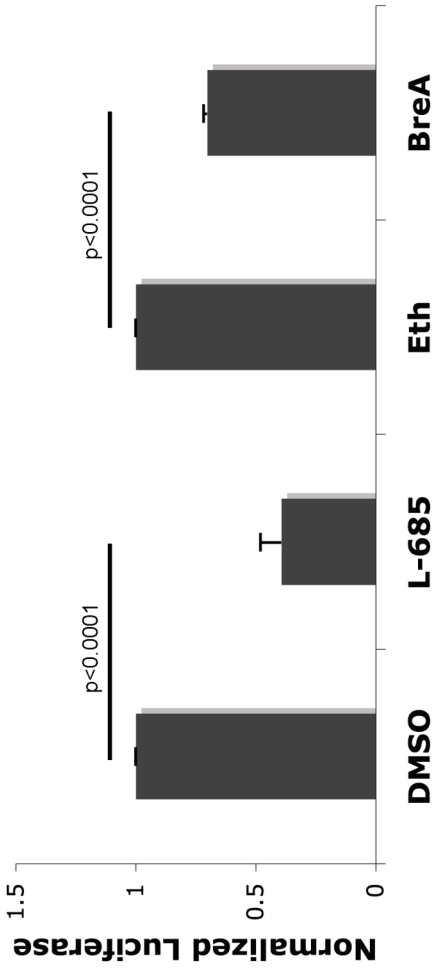
Figure 6.1: BFA treatment of cells decreases AICD-mediated luciferase activity in SH-SY5Y APP Gal4 cells. Cells were treated with L-685, a γ-secretase inhibitor that inhibits formation of AICD as a positive control. DMSO treated cells were the negative control for γ-secretase treated cells. Cells were treated with BFA for 4 hours and control cells were treated 4 hours with ethanol only (BFA vehicle). Statistical analysis was performed with Student's t test. Error bars represent SEM. N=2 with replication of 6.

Figure 6.2: ADAM10 levels are not affected by BFA treatment. (A) Representative Western blot analysis of cells treated with BFA only or BFA and L-685 in different combinations. Each "+" represents one hour of treatment with the indicated drug, each "-" represents one hour of treatment with vehicle only. No symbol means that no treatment was applied. Lane 1 represents 3 hours of BFA treatment and one hour of L-685 treatment while lane 3 represents contemporary treatment of BFA and L-685 for 4 hours. Actin was used as a loading control. (B) Quantification of ADAM10 levels in cells treated as reported above. The levels of ADAM10 for each treatment were normalized to the corresponding vehicle control. There was no significant difference in ADAM10 levels between treatments and corresponding controls. N=2 with replication of two. Statistical analysis was performed with Student's t-test.

Figure 6.3: BFA treatment causes an increase in APP immature levels and a decrease in APP processing. Representative Western blot analysis of cells treated with BFA only or BFA and L-685 in different combinations. Each "+" represents one hour of treatment with the indicated drug, each "-" represents one hour of treatment with vehicle only. No symbol means that no treatment was applied. Lane 1 represents 3 hours of BFA treatment and one hour of L-685 treatment while lane 3 represents contemporary treatment of BFA and L-685 for 4 hours. Actin was used as a loading control.

Figure 6.4: BFA treatment causes an increase in APP levels but a decrease in C83 levels. (A) Quantification of APP total levels in BFA treated as in figure 6.3 or control cells. (B) Quantification of C83 levels in cells treated as reported in Figure 6.3. There was no statistical difference between C83 levels in cells treated with BFA for 3 hours and then with L-685 and vehicle only control. Each treatment was normalized to the corresponding vehicle control. N=2 with replication of two. Statistical analysis was performed with Student's t-test.

Figure 6.1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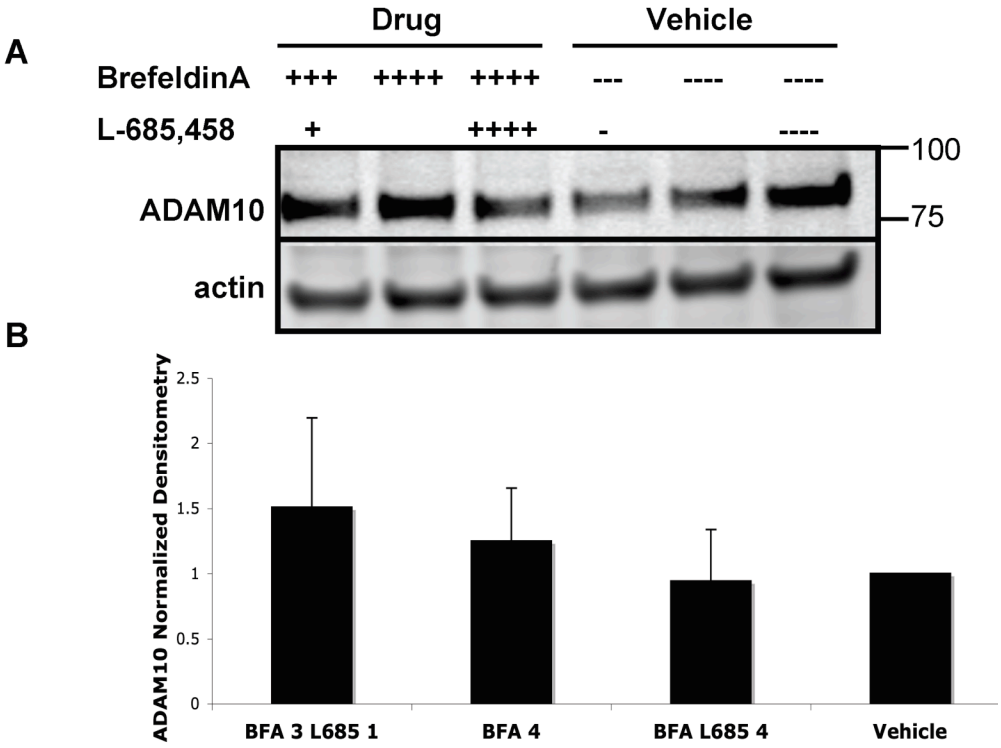
p<0.0001
p<0.0001

Figure 6.2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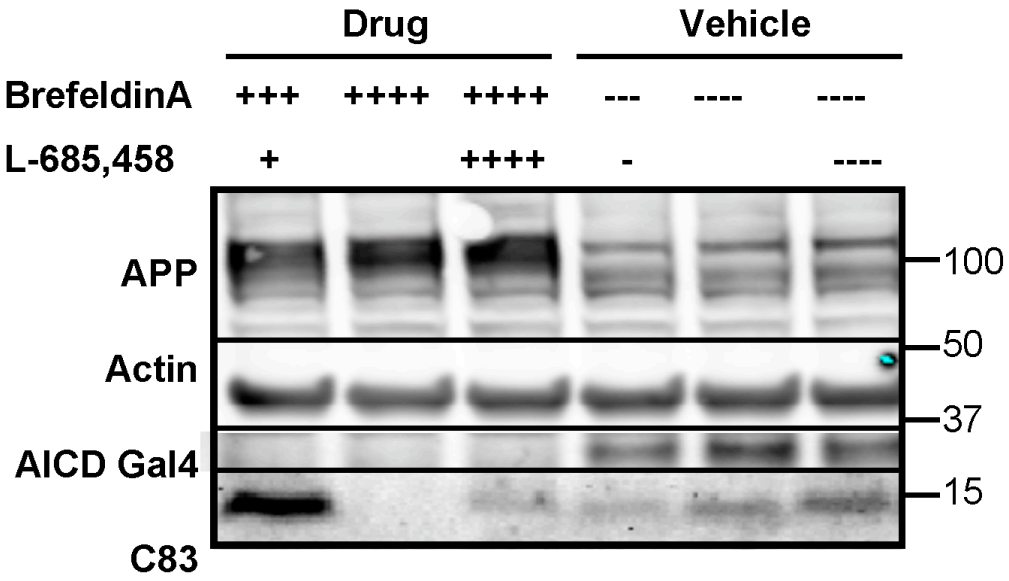
Drug
Vehicle
BrefeldinA
+++
++++
++++

L-685,458
+
++++
-

100
75
ADAM10
actin
B
ADAM10 Normalized Densitometry
2.5
2
1.5
1
0.5
0
BFA 3 L685 1
BFA 4
BFA L685 4
Vehicle

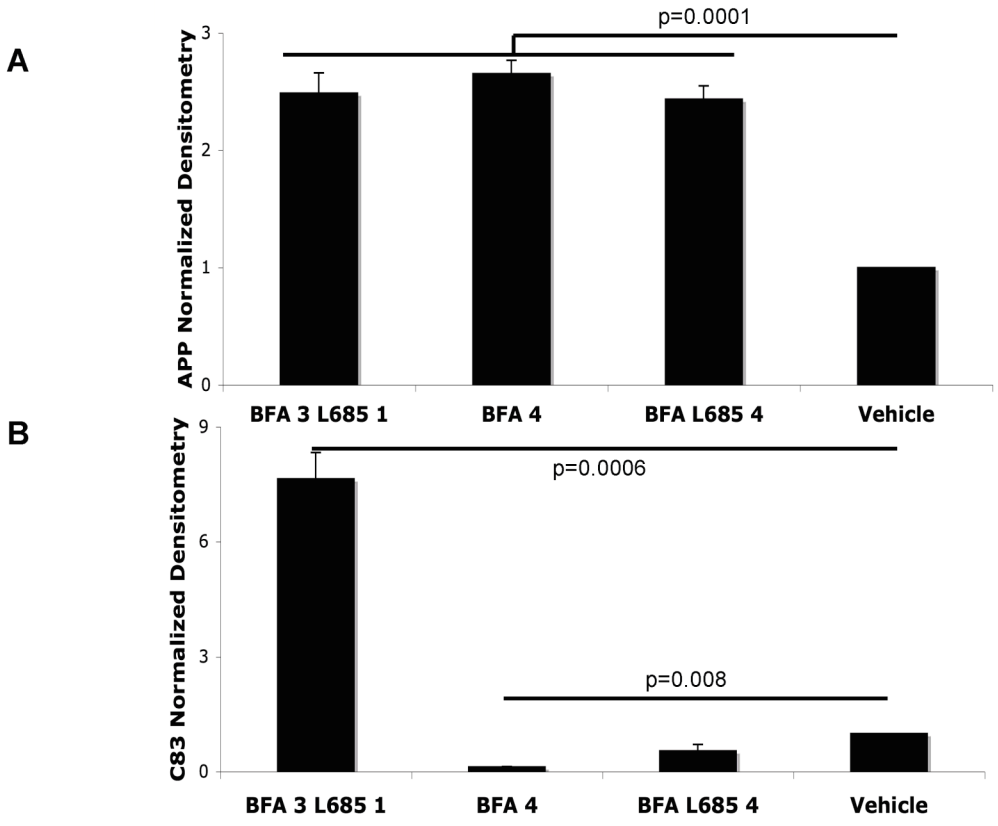
Figure 6.3


Drug
Vehicle
BrefeldinA
+++
++++
++++

L-685,458
+
++++
-

APP
Actin
AICD Gal4
C83
100
50
37
15

Figure 6.4


A
APP Normalized Densitometry
p=0.0001
0
1
2
3
BFA 3 L685 1
BFA 4
BFA L685 4
Vehicle
B
C83 Normalized Densitometry
p=0.0006
p=0.008
0
3
6
9
BFA 3 L685 1
BFA 4
BFA L685 4
Vehicle

7. APPENDIX II: TrkB isoforms and APP localization in HEK293 cells

We have noticed effects of TrkB isoforms on APP metabolism and we hypothesize that some of these effects are due to a different trafficking of APP that leads to altered processing. For example BACE1 levels were increased by transfection of TrkB FL in HEK293 APP^{Swe} cells but the C83/C99 ratio was decreased. This would suggest that APP in TrkB FL transfected cells is not available to BACE1 for cleavage probably due to a different intracellular localization.

We performed preliminary fluorescence microscopy experiments to test the hypothesis that APP localization is different when TrkB isoforms are transfected into the cells.

We employed TrkB constructs GFP tagged and an APP-RFP tagged construct and co-transfected them in equimolar ratios in HEK293 cells cultured on glass coverslips. 48 hours post-transfection, cells were fixed in paraformaldheyde and mounted on glass slides for confocal microscopy observation. Magnification and laser intensity of the appropriate excitation wavelengths were kept constant through all image acquisitions. Single cells in the acquired images were scored for:

1. TrkB pattern of distribution (diffused fluorescence; clustered puncta; Golgi accumulation).

2. APP distribution.

3. Co-localization of APP and GFP, estimated based on extent of yellow signal in the images.

All these assessments are highly qualitative and preliminary as subcellular organelles markers were not employed and 3D imaging also was not employed to verify accurate co-localization. Images were quantified blindly for all the aforementioned characteristics and scored at three levels: 1, 2 and 3 as measures of appearance of each characteristic. The arbitrary scores were then averaged for the number of cells counted and finally associated to the transfected constructs for data analysis and discussion.

In Figure 7.1 we show three representative confocal images of TrkB/APP transfected HEK293 cells. We noticed that the truncated constructs tended to highlight more the rim of the cells suggesting an association with lamellipodia and the cell membrane (Figure 7.1). This characteristic was annotated but not quantified with the scoring system and is in agreement with previous published findings (Haapasalo et al., 2002). TrkB FL Y515F receptors seemed to be more aggregated in puncta compared to the wild type TrkB FL while the TrkB SHC isoform displayed a more even distribution patter. We could speculate that this phenomenon is partially due to SHC association to the intracellular domain and possible interactions with other membrane proteins.

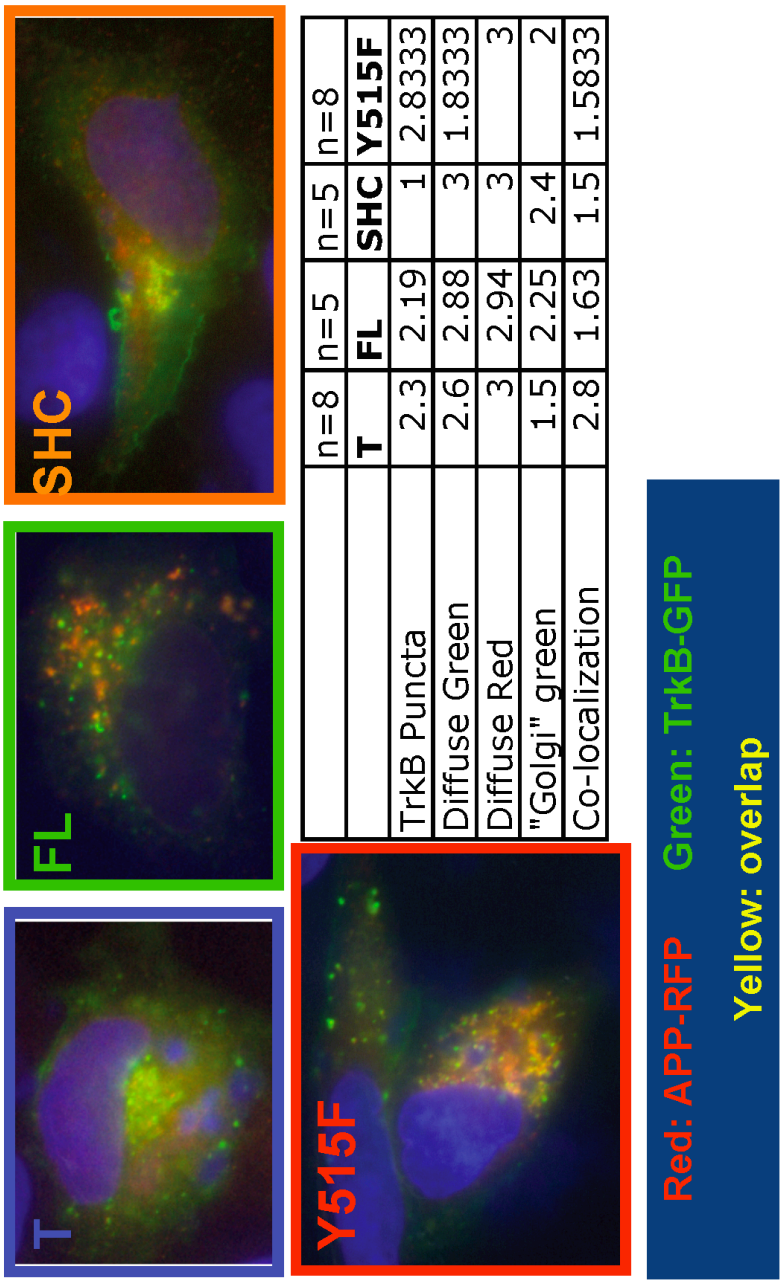
TrkB T was less associated with “Golgi-like” accumulation suggesting that the potential of this receptor to disrupt protein glycosylation is lower than the tyrosine kinase counterparts. Surprisingly, even the TrkB SHC isoform was found to be accumulated in the Golgi more than initially expected from a superficial observation. We have to remember that transfection of this isoform alone did not cause any glycosylation effects on APP. APP signal seemed to be evenly distributed in cells

independently of TrkB transfected construct. Finally TrkB FL and TrkB SHC isoforms and the TrkB Y515F mutant seemed to co-localize to a lesser extent with APP than the TrkB T isoform.

In conclusion our preliminary data suggests that TrkB have different cellular localizations and different effects on Golgi structure. Also the co-localization of the TrkB isoforms with APP seems to differ among isoforms. Further experiments are needed to identify the specific subcellular organelles affected and to explore the biological significance of TrkB and APP residence in the same cellular compartments.

Figure 7.1: Fluorescence microscopy images of HEK293 cells transfected with TrkB GFP tagged constructs and APP-RFP. The transfecetd TrkB construct is indicated in the panels in the top left corner. The table reports arbitrary assigned scores for the indicated characteristics. N is the number of cells that were scored and which scores were averaged to get the values reported in the table.

Figure 7.1



	n=8	n=5	n=5	n=8
	T	**FL**	**SHC**	**Y515F**
TrkB Puncta	2.3	2.19	1	2.8333
Diffuse Green	2.6	2.88	3	1.8333
Diffuse Red	3	2.94	3	3
"Golgi" green	1.5	2.25	2.4	2
Co-localization	2.8	1.63	1.5	1.5833

8. APPENDIX III

This appendix is a published paper that provides a streamlined method to subclone shRNA constructs in lentiviral vectors. This method is very useful in creating RNAi tools that are suitable for use in neuronal primary cultures and *in vivo*.

TECHNOLOGY REPORT

A streamlined sub-cloning procedure to transfer shRNA from a pSM2 vector to a pGIPZ lentiviral vector

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ABSTRACT

RNA interference (RNAi) is a widely used molecular biology technique to investigate the importance of specific genes in molecular pathways. Since mammalian cells are equipped with endogenous RNAi processing machinery, it has become common practice to transfect constructs that encode for short hairpin RNAs that are then cleaved to form the active RNAi sequences that bind to target mRNAs. Given the profit potential of this research approach, companies have developed retroviral libraries of shRNA constructs targeting the majority of the human genes. Recent technologic advances have allowed the rapid improvement of the vectors carrying the shRNA constructs while the silencing sequences remain the same. Therefore, sub-cloning of shRNA sequences from more obsolete vectors to newer vectors is a straightforward way to take advantage of newer delivery technologies. We describe here a streamlined procedure to transfer shRNA sequences from the pSM2 retroviral vector to a newer pGIPZ vector that is more stable, contains a GFP cassette and allows the preparation of high titer viral particles for transduction of cells and *in vivo* use. We demonstrate that our protocol provides a cost-effective and fast method to successfully sub-clone shRNA from a pSM2 retroviral vector to a pGIPZ lentiviral vector making it a useful tool for the investigators that have purchased pSM2 vectors in the past and wish now to upgrade their constructs by inserting them in more versatile vectors.

KEYWORDS: Sub-cloning, shRNA, RNA interference, lentivirus, retrovirus, pSM2, pGIPZ, pTRIPZ

INTRODUCTION

RNA interference (RNAi) is an endogenous system that regulates gene expression. Since its discovery (Fire et al, 1998) it has been exploited to silence specific genes and has become an important experimental method utilized in cellular and *in vivo* studies. RNAi based therapies are in development (Castanotto and Rossi, 2009). Given this promise and utility, many current investigations aim at better understanding the molecular mechanisms of RNAi and to find effective delivery methods for RNAi reagents.

There are several approaches to introduce silencing RNAs into cells. One of them is to directly introduce short interfering RNAs, 21-23 nucleotide duplexes targeting specific mRNAs, into the cells or tissue under investigation (Elbashir et al, 2001). The disadvantage of this approach is that silencing is dependent on the amount of siRNA administered. A sustained silencing requires a constant and expensive siRNA supply. This shortcoming is eliminated when vectors containing sequences encoding short hairpin RNAs (shRNAs) are utilized (Paddison et al, 2002). shRNAs are processed in the cells to produce

siRNA. Cells transfected with these vectors can sustain RNAi-mediated gene silencing for 48 hours or longer under antibiotic selection.

The first large library of shRNA constructs targeting human and mouse genes was created in a retroviral vector, pShagMagic2 (pSM2) (Paddison et al, 2004). This vector is subject to frequent recombination, does not contain a GFP marker and has inefficient viral packaging that limits the use in hard to transfect cells and *in vivo*.

Aware of these problems, companies have transferred the shRNAs into more stable GFP-tagged lentiviral vectors that produce high-titer viruses (Moffat et al, 2006). Lentiviruses are suitable for transduction of hard to transfect cell lines, primary cells and *in vivo* applications. The latest generation of lentiviral constructs includes inducible shRNA production (TRIPZ Lentiviral Inducible shRNAmir Library®, www.openbiosystems.com).

All the researchers who purchased the now outdated retroviral libraries cannot take advantage of these improvements. It is very expensive for laboratories to buy a complete lentiviral library, and the single lentiviral constructs range from $209 to $428. One inexpensive way to "upgrade" the shRNA without purchasing new ones is to sub-clone them to an appropriate lentiviral vector. Open Biosystems® provides a protocol for sub-cloning shRNA constructs from pSM2 into the lentiviral vector pGIPZ but it requires expensive kits and multiple steps. Therefore, if an investigator wants to sub-clone a high number of shRNA constructs, it may be cheaper and more convenient to just purchase the constructs.

We developed a protocol that greatly simplifies the transfer of shRNA sequences from the retroviral pSM2 vector into the pGIPZ lentiviral vector. The improvements in the protocol are reported in Table 1. This sub-cloning protocol can be applied to sub-clone shRNA from pSM2 to newer lentiviral vectors and possibly to sub-cloning schemes that involve plasmids and fragments of the same sizes reported here.

MATERIALS AND METHODS

Vector preparation and restriction digestion

pSM2 and pGIPZ plasmids were purified with Qiagen Miniprep kits and DNA concentration was measured spectrophotometrically. All the restriction enzymes, the T4 DNA ligase and the molecular weight markers were purchased from New England Biolabs. The Open Biosystems protocols were followed for bacterial culture growth and for recombination checks.

pGIPZ vector (5-10µg) was digested with *Mlu* I/*Xho* I (1-2U/µl) and the 13,087kb band was gel purified (QIAquick® gel extraction kit, Qiagen). The elution step was performed with 50µl HPLC water. The purified vector was used immediately after heat-inactivation, or stored frozen at -20°C until further use. The pSM2 vector (4µg) containing the shRNA of choice was also digested with *Mlu* I/*Xho* I (1-2U/µl) to generate a ~350bp fragment containing the shRNA, in a 20µl reaction volume (final insert concentration of 200ng/µl). After heat-inactivation (65°C for 20min) the cut pSM2 was used directly in ligation without purification.

Table 1. Comparison between the Open Biosystems® and the proposed streamlined sub-cloning protocols for insert preparation. Cost- and time-savings are detailed for steps in insert preparation that are significantly different and are estimated for the smallest sized kit/reagent. In summary, an investigator with pSM2 vectors available can sub-clone a single construct for $631 using the Open Biosystems® protocol or $256 using our streamlined protocol. For both protocols there is an additional one-time expense of purchasing the empty pGIPZ vector ($321). In summary, our simplified procedure provides approximately 60% cost savings compared to the protocol provided by Open Biosystems® and is faster. Alternatively, an investigator can purchase the pGIPZ shRNA clones for $209 each, as well as the required pGIPZ control shRNAs ($428 each).

Original Open Biosystems® Protocol			Streamlined Protocol		
Step Materials / Reagents	Time	Cost	Step Materials / Reagents	Time	Cost
Primers for pSM2 shRNA PCR: pSM2 forward - 5' aagccctttgtacaccctaagcct 3' pSM2 reverse - 5' actggtgaaactcacccagggatt 3'	1-3d	$14	Not required		
4 PCR amplifications per shRNA to be sub-cloned KOD Hot start Polymerase Kit®, Novagen®	2-3hr	$50	Not required		
Restriction digest *Mlu* I/*Xho* I	3hr	$121	Restriction digest *Mlu* I/*Xho* I	3 hr	$121
Agarose gel Electrophoresis	45min		Not Required		
Gel purification/quantitate fragment concentration Wizard SV Gel /PCR Clean-up System column, Promega®	30min	$83	Not required		
Ligation (250ng destination vector)	3hr	$63	Ligation (100 ng destination vector)	3hr	$63
Transformation Prime+ competent *E. coli*, Gentaur®		$300	Transformation DH5-alpha *E. coli*, InVitrogen®		$72
TOTAL COSTS		$631			$256

Ligation and transformation

Experimental and control ligation mixes were set up in 10µl final volume using 1:1 molar ratios of the destination vector:insert (100ng vector and 2.6ng insert). 100ng of the uncut pGIPZ vector was used as a positive control diluted in ligation buffer, 100ng of the *Xho* I/*Mlu* I pGIPZ cut vector without ligase was used to control for cutting efficiency of both enzymes and 100ng of the *Xho* I/*Mlu* I pGIPZ cut vector with 1µl of ligase were used to control for re-ligation of partially cut pGIPZ vector. In addition we had a ligation reaction without DNA added to control for DNA contamination in our reagents.

Ligation was performed at room temperature for 3hr to overnight and was followed by heat-inactivation at 65°C for 20min. 1µl of ligation mix was transformed in DH5α cells (InVitrogen). 300µl of the bacterial suspension were plated onto LB-ampicillin plates. Plates were incubated over night at 37°C. The next morning, colonies on the plates were counted to verify successful ligation. Typical observed colony counts were as follows: More than 100 colonies on the pGIPZ positive control and 20-100 colonies on the experimental ligation or ligations. The pGIPZ vector cut without ligase had generally 0-10 colonies while the pGIPZ cut vector added with ligase had 0-20 colonies.

Clone selection and verification

The ratio between the number of colonies on the experimental plates and the control plate (cut pGIPZ vector with ligase) was calculated and multiplied by three, the resulting number of colonies was selected for screening to validate our method. Each colony picked was dissolved in 10µl sterile HPLC water. 5µl of this suspension were used to inoculate a 5ml liquid culture of LB-ampicillin, 2µl were dotted on a master LB-Amp plate, and 2µl used as template for PCR to confirm shRNA insertion. The PCR amplification products are 600bp if the shRNA sequence is present and 500bp if it is not. The following PCR parameters were used: 5min at 94°C; then 30 cycles of, 94°C for 15sec, 56°C for 30sec, 72°C for 25sec. The pGIPZ-specific primers used were:

X76 Forward: 5'ACGTCGAGGTGCCCGAAGGA

M100 Reverse: 5'AAGCAGCGTATCCACATAGCGT

This PCR reaction only amplifies shRNA in pGIPZ vectors.

In addition to the PCR check, the pGIPZ vectors containing the sub-cloned shRNA were also digested to check for recombination of the vectors and successful insertion of the shRNA sequence. *Sac* II digestion was used to test for recombination of the pGIPZ vectors containing the sub-cloned shRNAs (expected bands: 7927bp, 2502bp and 1259bp). Successful ligation of the shRNA sequence was verified by excising it from the recipient pGIPZ vector using *Mlu* I/*Xho* I double digestion (expected bands: 350bp and ~10Kb).

Knock-down and western blotting procedure

SH-SY5Y cells over-expressing APP-Gal4 were transiently transfected or transduced as previously described (Zhang et al, 2007). Transfection was performed with pSM2 plasmids targeting APP or a non-silencing control (Open Biosystems), and with pGIPZ plasmids containing the same shRNA sequences as the pSM2 vectors that were sub-cloned following our protocol. Transfection was performed with Arrrest-In® (Open Biosystems®) transfection reagent. Whole cell lysates were prepared in ice-cold radio immuno-precipitation buffer **(**150mM NaCl**,** 1%, v/v, NP40**,** 0.5%, v/v, DOC**,** 1%, w/v, SDS**,** 50mM Tris, pH 8.0) supplemented with Halt cocktail of protease and phosphatase inhibitors (ThermoFisher). The western blotting procedure has been previously described (Zhang et al, 2007). We used anti-APP antibody clone A8717 (Sigma) and anti-actin antibody (Sigma) at 1:2000 and 1:15,000 dilution, respectively. The same cell line was transduced with viruses obtained following the packaging protocol provided by Open Biosystems® but using the same sub-cloned pGIPZ vectors previously employed for the transfection.

RESULTS AND DISCUSSION

Our protocol was successfully used to sub-clone shRNA constructs into pGIPZ vectors. As an example, one experimental transformation plate had approximately fifty colonies, the control cut pGIPZ vector with ligase had 6 colonies so the ratio of clones containing the insert to the empty ones was 50/6=8.3. Twenty-five colonies were screened with PCR using pGIPZ specific primers that amplify the shRNA sequence. In Figure 1A we show 16 of the 25 PCR reactions with the appropriate controls run on an agarose gel stained with ethidium bromide. Two clones did not show amplification (not shown), one clone resulted empty (Figure 1A; lane 4) and three clones showed sub-optimal amplification (Figure 1A; lanes 13, 14, 15). All the remaining clones showed the correct band size with efficient amplification. In summary, out of the 25 colonies screened, only 3 did not show correct amplification confirming a ratio of 25/3=8.3 clones containing insert to the false positive ones. Following PCR we checked the recipient vectors for both recombination and the presence of the insert. In Figure 1B we show the results of *Sac* II restriction digestion of 16 clones that showed the correct amplicon following PCR. These 16 clones did not show signs of recombination. After checking for recombination of the recipient plasmid, we also checked for correct ligation of the shRNA sequence. In Figure 1C we used restriction digestion to excise the inserted shRNA from the clones and we confirmed the correct ligation in the new vector. All the clones that showed the correct PCR amplicon also showed correct restriction patterns making restriction digest unnecessary. Insertion of the shRNA sequences was also verified by sequencing.

In order to ensure that our sub-cloned constructs maintained silencing activity, comparable to the original pSM2 plasmids, we transfected, using the same procedure, both vectors in the same cell line. Moreover, we assessed if viral transduction of the cells also showed effective silencing. Figure 2 shows the detection of Amyloid Precursor Protein (APP) by western blotting in SH-SY5Y APP-Gal4 cells following transfection of shRNA constructs in pSM2 or pGIPZ vectors or transduction with pGIPZ containing viral

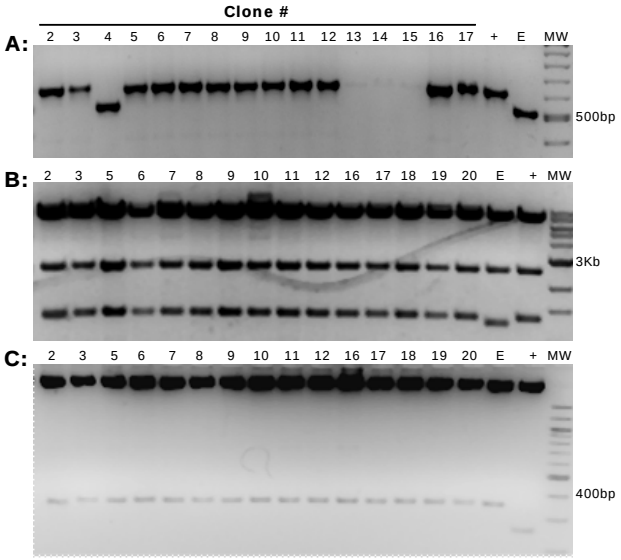

Figure 1. Gel electrophoresis analysis of PCR and restriction endonuclease-digested products of the putative clones. **A.** Successful subcloning was assessed by colony PCR, using pGIPZ specific PCR primers. 16 putative clones were picked from a transformation plate. The last two samples are a positive control (+) (a pGIPZ vector containing a shRNA sequence that had been previously verified by nucleotide sequencing) and the pGIPZ empty vector (E). Clone 4 does not contain the insert while clones 13, 14 and but showed inefficient PCR amplification. These PCR results confirm that the shRNA is inserted in the pGIPZ destination vector. **B.** *Sac* II restriction endonuclease digest of positive clones identified in panel (A) shows no signs of pGIPZ recombination. We observe the expected bands: 7927bp, 2502bp and 1259bp. **C.** *Mlu* I/*Xho* I restriction digest shows the inserted 350bp band corresponding to the shRNA excised from the destination pGIPZ vector.

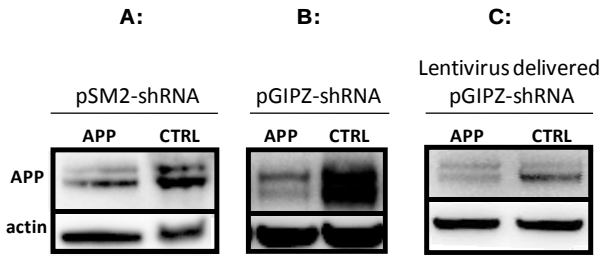

Figure 2. Western blot analysis of cells transfected or transduced with shRNA constructs in different vectors. Knock-down of the Amyloid Precursor Protein (APP) is assessed in the human neuroblastoma cell line SH-SY5Y. APP is over-expressed in this cell line and is detected as two bands that are fully-glycosylated (top migrating band) and partially-glycosylated (lower migrating band) states of APP, respectively. The anti-human APP antibody A8717 was used. Actin levels were used as a loading control. **A** and **B**. Transfection of either pSM2 vectors (A) containing the shRNA targeting APP or pGIPZ vectors (B), containing the same shRNA construct, efficiently decreases APP total levels compared to non silencing control shRNA. **C.** APP levels are also efficiently decreased through transduction of the cells with viral particles obtained with the subcloned shRNA pGIPZ vectors. APP: Amyloid Precursor Protein; CTRL: non-silencing shRNA.

particles. The silencing efficiency of the shRNA constructs in their original pSM2 vectors (Figure 2A) is comparable with the one of the shRNA subcloned in the pGIPZ vector (Figure 2B) showing that the construct was successfully inserted and does not loose targeting ability. Finally, we show that the pGIPZ construct can also efficiently decrease APP levels when delivered via viral particles (Figure 2C).

CONCLUSIONS

The RNAi Resource Center at Drexel University purchased from Open Biosystems the pSM2 retroviral library targeting ~65,000 human genes. Our sub-cloning method has been applied successfully to ~100 shRNA sequences of interest that were transferred from the pSM2 vector into the pGIPZ empty vector. The protocol proved successful in the hands of different operators and 80% of the shRNA constructs were sub-cloned successfully on the first attempt. Unsuccessful sub-cloning was largely due to the presence of recombination in the pSM2 donor vectors.

pGIPZ vectors containing shRNAs obtained from our subcloning procedure produced high titer viruses (10^6 infectious units/ml) that were used to transduce hard-to-transfect cell lines and primary neurons. We believe that our protocol will be helpful to all the investigators who have invested in the now outdated pSM2 libraries allowing them to efficiently upgrading their shRNA sequences into the new lentiviral vectors. Given the simplicity of the steps involved, it would probably be possible to implement high throughput methods to automate the rapid transfer of many shRNA simultaneously. This procedure offers approximately 60% cost savings and substantial time savings compared to the suggested protocol.

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COMPETING INTERESTS

None declared

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Zigman WB, Lott IT (2007) Alzheimer's disease in Down syndrome: neurobiology and risk. Ment Retard Dev Disabil Res Rev 13:237-246.

VITA

Sara Ansaloni

Born in Pavullo Nel Frignano August 13 1978, Italy

EDUCATION

PhD
Department of Biology, Drexel University, Philadelphia, PA, USA
March 2005-anticipated completion December 2010
GPA: 4.0
Thesis: "Characterization of *NTRK2* as a modulator of APP metabolism"

BS/MS Biotechnology (Diploma di Laurea)
University of Modena and Reggio Emilia, Modena, Italy
October 1997-2002
Summa cum laude
Thesis: "Poly-phenol-oxidases in cell cultures of *Vitis vinifera* for biotransformation"

HONORS/AWARDS

- Graduate Student Outstanding Service Award, Drexel University, June 2010
- Travel grant, Graduate Studies Office, Drexel University, May 2010
- Joseph S. Mozino Scholarship, Drexel University, 2006-2010
- Travel Grant, Graduate Studies Office, Drexel University, July 2008
- Highly Commended, Graduate Student Teaching Awards Competition, Drexel University 2007-2008
- Travel grant, Graduate Studies Office, Drexel University, August 2007
- Travel fellowship from the Committee of the Life Science Symposium LSS07 at EPFL August 2007
- Yearly full tuition waiver and scholarship, University of Modena and Reggio Emilia, 1997-2002
- Yearly Outstanding Student Scholarship, University of Modena and Reggio Emilia, 1997-2002

APPOINTMENTS

Research Assistant
Dr. Saunders laboratory, Department of Biology, Stratton Hall, Drexel University, 2005-present.

- Characterization of the *NTRK2* gene product, TrkB, as a potential modulator of APP metabolism. Knock-down, over-expression and retinoic acid induced up regulation were used to investigate a hypothesized TrkB dependent α-secretase activity regulation.
- Investigation of mechanisms of axonal transport in live primary neuronal cultures using video-microscopy and fluorescence microscopy techniques.
- Developed a streamlined protocol for sub-cloning of shRNA constructs into pGIPZ lentiviral vector.
- Mentoring and supervision of undergraduate students working in the laboratory.
- Collaboration with other laboratories on different projects:
  - Lentiviral mediated knockdown of histidine decarboxylase in human mast cells.
  - Study of the severing protein levels in brain tissues from AD models.
  - shRNA-mediated knockdown of the cannabinoid receptor 1 *in vivo*.
  - shRNA-mediated knockdown of the TLR4 receptor on THP1 cells.
  - Developing a sensitive, piezoelectric detection method of Aβ in serum and CSF.

Teaching Assistant
Biology Department, Drexel University, Philadelphia, March 2005-2008.

- Taught laboratory techniques, lectured, held recitations, tutored, prepared exams for students and graded.
- Laboratory Instructor: Developmental Biology, Advanced Immunology for graduate students, Medical Microbiology for nursing students, Cells and Genetics for BS/MD students, Food Safety for nutrition and food science students, Physiology and Nutrition for biology major students.

Visiting Scholar
Ultrasound Laboratory, Department of Radiology, Stemmler Hall, University of Pennsylvania, 2004-2005.

- Used a mouse melanoma model to evaluate the potential anti vascular activity of low energy ultrasound. Project conducted with Dr. Andrew Wood under the supervision of Dr. Chandra Sehgal.
- Developed a phantom model to evaluate the potential use of ultrasound imaging for determining blood flow and perfusion. Project conducted independently under the supervision of Dr. Chandra Sehgal.

Visiting Scholar
Protein Chemistry Laboratory, Department of Pathology and Laboratory Medicine, Stellar Chance Laboratories, University of Pennsylvania, 2003-2004.

- Development of an animal model to study metabolism and pharmacokinetics of compstatin, a peptide inhibitor of the complement system. Independent research project conducted under the supervision of Dr. John Lambris.

COURSEWORK

- Biometry (MatLab), Data Analysis (SAS), Plant Genetics and Genomics, Research Methods, Advanced Genetics, Molecular Mechanisms of Neurodegeneration, Ethics in Research.
- Biomedical Engineering courses: Bionanotechnology (focus: drug delivery), Tissue Engineering I, II and III.

PUBLICATIONS

Sara Ansaloni, Nadav Lelkes, Jonathan Snyder, Charles Epstein, Aditi Dubey and Aleister Saunders "A streamlined subcloning procedure to transfer shRNA from a retroviral vector to a lentiviral vector". *"Journal of RNAi and Gene Silencing."* 2010, 6 (2): 411-415.

Sara Ansaloni, Brian Leung, Neeraj P. Sebastian, Rohini Samudralwar, Mariana Gadaleta and Aleister Saunders "TrkB isoforms differentially affect AICD production through their intracellular functional domains". *Accepted, "International Journal of Alzheimer's Disease."*

Sara Ansaloni, Brian Leung, Mariana Gadaleta and Aleister J. Saunders. "TrkB isoforms differentially regulate APP metabolism". *Submitted to "Journal of Biological Chemistry."*

Sara Ansaloni, Martin Zhang, Preeti Khandelwal, Aleister J. Saunders "Identification of APP Metabolism Regulators". *In preparation for submission to "Human Molecular Genetics."*

Sara Ansaloni, Martin Zhang, Shivangi Inamdar, Jeff Thomas, Gregg Johannes, He Zhao, Bahrad A. Sokhansanj, Rob Moir, Daniel R. Marenda, Aleister J. Saunders. "Characterization of Ubiquilin 1 mediated metabolism and its interaction with the proteosome system". *In preparation*.

Ranjita Chakraborty, Vidya Vepuri, Siddhita Mhatre, Sarah J. Michelson, Radha Delvadia, Arkit Desai, Sean Miller, Marianna Vinokur, David Melicharek, **Sara Ansaloni**, Robert Moir, Aleister J. Saunders, and Daniel R. Marenda. "Characterization of a Drosophila Alzheimer's Disease Model: Pharmacological Rescue of Cognitive Defects". *Submitted to PLoS ONE.*

Neha Patel, David Hoang, Nathan Miller, **Sara Ansaloni**, Quihong Huang, Jack T. Rogers, Jeremy C. Lee and Aleister J. Saunders. "MicroRNAs can regulate human APP levels." *Molecular Neurodegeneration* 2008; 3(10).

Ralph M. Bunte, **Sara Ansaloni**, Chandra M. Sehgal, William M-F. Lee, and Andrew K.W.Wood. "Histopathological Observations Of The Antivascular Effects Of Physiotherapy Ultrasound On A Neoplasm." *Ultrasound in medicine and Biology* 2006; 32 (3): 453– 461.

Andrew Wood, **Sara Ansaloni**, Lisa Zeimer, William M-F. Lee, Michael Feldman and Chandra Sehgal. "The Antivascular Action Of Physiotherapy Ultrasound On Murine Tumors." *Ultrasound in Medicine and Biology* 2005; 31(10): 1403-10.

Andrew K.W.Wood, Ralph M. Bunte, **Sara Ansaloni**, William M-F. Lee, and Chandra M. Sehgal. "The Antivascular Actions of Mild Intensity Ultrasound on a Murine Neoplasm." Published on the ISTU proceedings, 5th International Symposium on Therapeutic Ultrasound (Boston, USA, October 2005).

Sara Ansaloni, Peter Arger, Theodore Cary and Chandra Sehgal. "Evaluation of contrast-enhanced power Doppler imaging for measuring blood flow." Presented by Sara Ansaloni at the International Conference of Ultrasonic Imaging and Signal Processing and published on the SPIE proceedings (San Diego, USA, February 2005).

POSTERS

Sara Ansaloni, Brian Leung, Anna Vorobyeva, Aleister Saunders. "TrkB isoforms differentially affect APP metabolism." Cellular and Molecular Neurobiology Gordon Research Conference (Hong Kong, China, June 2010).

Sara Ansaloni, Peter Baas, Robert Nichols and Aleister Saunders. "Microtubule Severing proteins expression in Alzheimer's disease animal models." International Conference on Alzheimer's Disease (Chicago, USA, July 2008).

E S Schulman, M.D., S C Pugliese, M.D., **S Ansaloni**, MS. , P Mannam, M.D, H Nishi, Ph.D., M Bouchard, Ph.D. and A J Saunders, Ph.D. "RNA Interference-Induced Gene Silencing Of Histidine Decarbarboxylase Produces Human Mast Cells Deficient In Histamine". American Thoracic Society (San Diego, CA, USA, May 2009).

Sara Ansaloni, Peter Baas, Robert Nichols and Aleister Saunders. "Severing proteins expression in an animal model of Alzheimer's disease." Second Annual EPFL Life Sciences Symposium, Neuroscience: Molecules, Systems and Diseases (Lausanne, Switzerland, August 2007).

Sara Ansaloni, Nadav Lelkes, Nick DiPatrizio, Kenneth Symanski, Aleister Saunders. "Cloning of shRNA constructs in a lentiviral vector: advantages and applications." Drexel University RISC (Research, Innovation, Scholarship and Creativity) Day (Philadelphia, USA, April 2007).

Sara Ansaloni, Jason De Vito, Dan Pagano, Peter W. Baas, Jeremy C. Lee, Aleister Saunders. "Investigating the interaction between amyloid precursor protein and Eg5 for a potential role in Alzheimer's disease."
Drexel University Seventh Annual Research Day (Philadelphia, USA, April 2006).

Sara Ansaloni, Lisa S. Ziemer, Andrew K.W.Wood, Michael D. Feldman, William M-F. Lee, Chandra M. Sehgal. "Measurements Of Tumor Antivascular Response Using Ultrasound Contrast Agent."
Penn/GSK Drug Discovery Initiative (Philadelphia, USA, December 2004).

ORGANIZATIONAL INVOLVEMENT/PERSONAL DEVELOPMENT

2008/2009

- **President** of the Graduate Student Association, Drexel University.

  - *Obtained Health Insurance coverage for all PhD students.*
  - *Increased Graduate Student Association budget by ~30%.*
- **Chair** of the Mentor Award Committee, Drexel University.
- **Member, Student Health Advisory Committee,** Drexel University.
- **Member,** Committee for Teaching Assistant Awards, Drexel University.
- **Advanced training for leadership excellence** based on Dr. John Maxwell's book "The 21 Irrefutable Laws Of Leadership".

2007/2008

- **President,** Biology Graduate Student Association, Drexel University.
- **Member**, Student Health Advisory Committee, Drexel University.
- **Member,** Committee for Teaching Assistant Awards and preparation course.
- **Personal Leadership Certificate,** Creating Excellent Organizations Program for development of personal and professional skills, Drexel University.
- **Professional Development Workshops**: "Delegating for Results" by UNISYS, "Political Savvy", Frank Perras, Career Concepts consulting company.

2006/2007

- **Secretary,** Biology Graduate Student Association, Drexel University.

2005/2006

- **Social Chair,** Biology Graduate Student Association, Drexel University.