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Delineating the mechanism(s) of BDNF/TrkB mediated proliferation in Neuroblastoma

Timothy C. Graham

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Delineating the mechanism(s) of BDNF/TrkB mediated proliferation in Neuroblastoma

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Delineating the mechanism(s) of BDNF/TrkB mediated proliferation in Neuroblastoma

A

THESIS

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MASTERS of SCIENCE

by

Timothy Christopher Graham

Houston, Texas

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DEDICATION

To my friends and family who have supported my interests and goals every step of the way.

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Foremost I'd like to thank my mentor and advisor, Dr. Patrick Zweidler-McKay. If not for his unwavering dedication to always help and guide me in my project, I would have never gotten anywhere. I would also like to thank Dr. Peter Zage as well as the members in my lab for all of their support and advice as well as keeping me sane throughout the years. Lastly, I would like to thank everyone in the Pediatrics department as well as all the friends I made along the way who helped me through the tough times.

Delineating the mechanism(s) of BDNF/TrkB mediated proliferation in Neuroblastoma

Timothy Christopher Graham, B.S.

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Neuroblastoma is the most common extra-cranial solid tumor in children, arising from neural crest precursor cells. The neurotrophin receptors (TrkA/B/C) have been implicated as important prognostic markers, linking the biology of the tumor to patient outcome. High expression of TrkA and TrkC receptors have been linked to favorable biological features and high patient survival, while TrkB is expressed in unfavorable, aggressive tumors. Several studies suggest that high levels and activation of TrkB by its ligand brain-derived neurotrophic factor (BDNF) stimulates tumor cell survival, proliferation, and chemoresistance. However, little is known about the molecular mechanisms that regulate proliferation. The TrkB signaling pathway in neuroblastoma cells has been difficult to evaluate due to the loss of TrkB expression when the cells are used *in vitro*. Here we determined the role of proximal signaling pathways downstream of TrkB on neuroblastoma proliferation. By analyzing a panel of neuroblastoma cell lines, we found that the SMS-KCN cells express detectable levels of protein and mRNA levels of TrkB as analyzed by western, RT-PCR, and surface expression by flow cytometry. By the addition of exogenous human recombinant BDNF, we showed that activation of TrkB is important in the proliferation of the cells and can be repressed by inhibiting TrkB kinase function. By BDNF stimulation and use of specific kinase inhibitors, the common pathways involving PLC γ , PI3K/AKT, and MAPK were initially investigated in addition to PI3K/MTOR and FYN

pathways. We demonstrate for the first time that Fyn plays a critical role in TrkB mediated proliferation in neuroblastoma. Constitutively active and over-expressed Fyn reduced neuroblastoma proliferation, as measured by PCNA expression. Knockdown of Fyn by shRNA was shown to cooperate with activated TrkB for an enhanced proliferative response. Although TrkB activation has been implicated in the proliferation of neuroblastoma cells, little is known about its effects on cell cycle regulation. Protein levels of pRB, CDK2, CDK4, CDC25A, cyclin D1, and cyclin E were analyzed following BDNF stimulation. We found that BDNF mediated activation of TrkB induces multiple common proximal signaling pathways including the anti-proliferative Fyn pathway and drives cell cycle machinery to enhance the proliferation of neuroblastoma cells.

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Chapter 1
Introduction

Neuroblastoma

Neuroblastoma is the most prevalent extra-cranial solid tumor in childhood, accounting for 15% of all pediatric oncology deaths (1, 2). It arises from the pluripotent sympathicoadrenal precursor cells in the neural crest, which are the primordial cells of the sympathetic nervous system. Tumorigenesis starts by the failure of stem cells to differentiate properly followed by uncontrolled proliferation (3). This is evidenced by previous research showing that within many types of solid tumors exists a population of cells responsible for the initial growth of the tumor called tumor initiating cells. These cells in neuroblastoma exhibit stem cell properties to sustain tumor growth by the ability for self renewal, however also possess the ability to differentiate into normal neurons or regenerate phenotype copies that are characteristic of the primary neuroblastoma tumor (4, 5). The disease predominantly arises in children younger than 4 years old with a median age of 22 months. Neuroblastoma has an incidence rate of about 1 out of 7,000 live births favoring males to females 1.2:1. An autosomal dominant pattern of inheritance also has been found, however it is thought to only affect approximately 1-2% of the patient population. The more common occurrence is the sporadic form, which is associated with a wide variety of factors such as gain of alleles and oncogene activation (6). The majority of neuroblastoma tumors are found in the abdominal area with over 50% of cases originating in the adrenal medulla. Additional sites frequently include brain/neck, chest, and pelvis. The clinical presentation of the disease is very broad, depending on primary tumor site, the presence of metastatic disease, and the occurrence of para-neoplastic syndromes (7, 8).

Since the clinical manifestation of the disease is highly variable between individual patients, the International Neuroblastoma Risk Group (INRG) has developed a staging

system based on tumor burden and spread. The idea of risk stratification is to ensure proper decisions to manage the disease by determining which tumors require an aggressive multimodal therapy versus those that can be managed more conservatively (9). Patients with stage 1, 2, or 4S or the disease presenting in the first year of life have a good prognosis. This is typically associated with a locoregional tumor in an otherwise healthy child. In contrast, a patient presenting with stage 3 or 4 or is past the age of one has a poor prognosis. Children presenting with stage 4 metastatic disease and are also over the age of 1 have an overall survival rate of 10-20%. The child can have an identical presentation to the locoregional disease, but has a much higher tumor burden along with metastatic lesions (8).

Neuroblastoma is an enigmatic disease due to its diverse clinical and biological behavior. In some patients less than 12 months of age, even metastatic disease has been shown to spontaneously regress on its own or following limited treatment, noted as stage 4S. Interestingly, neuroblastoma is the only disease that has ever been shown to demonstrate spontaneous regression from an undifferentiated phenotype to a completely benign one. A potential mechanism to explain the phenomenon is through the regulation of NGF (nerve growth factor) and the activation of its neurotrophin receptor, TrkA, suggesting an important role growth factors may play in neuroblastoma biology (10). However, for half of the patients with stage 4S, particularly those over 12 months of age, the progression of the disease will continue despite intensive therapy (6, 11). Due to the heterogeneous nature of the disease, a better understanding of its genetic, biological, and morphological behavior as well as identifying inherent tumor markers would improve patient survival rate. Further knowledge of prognostic markers would help stratify patients into regimens that can be better tailored to their needs on a case by case basis.

Many prognostic tumor markers that have been identified are associated with the regulation of proliferation, differentiation, and apoptosis of the cells. *MYCN*, an oncogenic transcription factor, is among several important molecular markers that are used to predict the outcome of the disease. Amplification of *MYCN* has been shown to be associated with advanced stages of the disease with rapid tumor progression, seen in about 25% of patients with neuroblastoma. Even patients with low stages of the disease with localized tumors have a worse prognosis when *MYCN* is amplified (12, 13). Other markers that are important for dictating the severity of the disease include deletion or loss of heterozygosity of chromosome 1p, gain of chromosome 17q, and notably the expression of the Trk receptors.

Trk receptors

Trk receptors have been identified as important prognostic factors that influence the clinical behavior of neuroblastoma (14). Favorable, lower stage neuroblastomas express high levels of TrkA and C along with their corresponding ligands NGF and NT-3 respectively, which highly correlate with favorable outcomes and overall patient survival by promoting tumor cell differentiation (15, 16). However, expression of full length TrkB and elevated levels of its ligand, BDNF, are mainly found in unfavorable, aggressive neuroblastomas with amplification of the *MYCN* (17). Additionally, full length TrkB over expression has been shown to protect neuroblastoma cells from chemotherapy-induced apoptosis in cell culture systems (18, 19). It has also been shown to promote cell dissemination and invasive potential, which contributes to the metastatic phenotype commonly seen (20).

The field of Trk receptors originally started by the discovery of neurotrophins in the 1940s by Cohen and Levi-Montalcini. However, it was many decades later when their respective receptors were identified (21). The first member of the Trk family of receptor tyrosine kinases was initially discovered from a colon carcinoma-derived oncogene. When the oncogene was isolated and cloned, it was found to consist of the first seven of eight exons of tropomyosin fused to the transmembrane and cytoplasmic domains of a novel tyrosine kinase (22). Consequently, this single pass, cell surface transmembrane molecule was named tropomyosin-related kinase (Trk) and is now commonly referred to as TrkA or neurotrophin tyrosine receptor kinase 1 (NTRK1). Based off of their high sequence homology to TrkA, TrkB/NTRK2 and TrkC/NTRK3 genes were identified as well (23, 24). However, the functional relevance of Trk receptors first came to light whenever they were identified as a gene capable of transforming immortalized fibroblasts. Once Trk receptors were linked with their neurotrophin counterparts and implicated as being important for the development of the nervous system, research on them intensified among neurobiologists (25).

Neurotrophins

Neurotrophins were originally identified as promoters of neuronal survival, but have since been appreciated to regulate many aspects of neuronal development such as survival and differentiation. The first neurotrophin, nerve growth factor (NGF), was found while searching for the reason how motor and sensory neurons survive after ablation and transplantation studies of target tissues (26). This profound discovery had validated the theory of neurotrophic factor action, which suggested that targets of neuronal innervation secrete limiting amounts of survival factors, balancing the size of a target tissue with the

number of innervating neurons. NGF has since been shown to be internalized by receptor-dependent processes and transported along axons in membranous vesicles to the cell soma by cytoskeletal and energy dependent processes (27). Local signaling is shown to regulate growth cone motility as well as in the soma control cell survival and gene expression. The neurotrophin is then recycled by a process controlled by lysosomes called ligand turnover. Even though an abundance of NGF-regulated phenotypes were characterized, a specific correlating receptor was never found. However in 1991, nerve growth factor was found to bind to and activate the tyrosine kinase activity of TrkA (28, 29). Subsequently TrkB has been shown to be activated by BDNF and NT-4/5 and TrkC by NT-3. However some promiscuity exists in that NT-3 can also bind to both TrkA and TrkB in some cell types, but less efficiently (30, 31) (Fig.1).

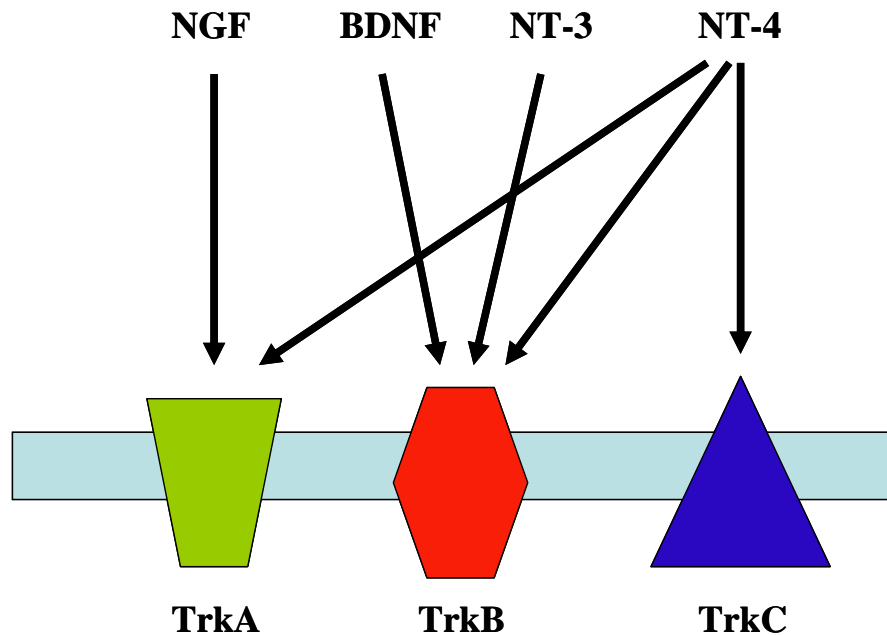


Fig 1. Representation of Trk receptors with their corresponding ligands.

Differential splicing of exons encoding portions of the Trk intracellular domains can lead to truncated isoforms of the receptor. Truncated isoforms of TrkB and C have been

shown to have comparatively short cytoplasmic motifs without a tyrosine kinase motif. Expression of these isoforms inhibits productive dimerization of kinase-containing Trk receptors, thereby preventing responses to neurotrophins (32).

The major ligand-binding domain for the three Trk receptors has been localized to the Ig-C2-like domain, proximal to the membrane, which also works in the prevention of spontaneous dimerization. In addition by point mutation analyses against the Trk receptors, the domain closest to the transmembrane region was found to play a more prominent role in activation (33-35). The N termini of neurotrophins have been shown to be important in controlling binding specificity. Once attached to the Ig-C2 domain of the Trk receptor, the structure of this region is reorganized, permitting neurotrophins such as NT-3 the ability to activate more than one type of Trk receptor (36).

TrkB cell signaling in neuroblastoma

Much like other receptor tyrosine kinases, Trk receptors homo-dimerize in response to ligand binding. The dimeric receptors then phosphorylate one another in *trans*, leading to the auto-phosphorylation of three tyrosine residues on the receptor (Y₆₇₀, Y₆₇₄, and Y₆₇₅) to potentiate an activation loop of the kinase domain. In addition, the active receptor phosphorylates two main tyrosine residues at Y₄₈₄ and Y₇₈₅, which serve as docking sites for SHC and PTB domain adapter proteins as well as PLC γ respectively (37, 38). Subsequently, the recruitment of these adapter proteins leads to the activation of multiple growth factor-regulated signaling pathways, which include phosphoinositide-3 kinase (PI3K)/ AKT pathway (39), release of calcium ions by phospholipase C- γ (PLC- γ) (40), and RAS/ mitogen activated protein kinase (MAPK) (41). In neuroblastoma cells, these pathways

have important roles that regulate cell proliferation, differentiation, and survival.

Specifically it has been shown that the activation of PI3K/AKT and PLC- γ pathways have been shown to mainly enhance cell survival, whereas activating the RAS/MAPK pathways promotes cell differentiation in neuroblastoma (42).

Recent data by the Thiele lab suggests that TrkB over expression plays a role in the survival and increased resistance to chemotherapy. The PI3K/AKT axis was identified as the mediator for BDNF protection in neuroblastoma from chemotherapy induced apoptosis (19). This survival mechanism has been shown to be initiated by the phosphorylation of BCL-2-associated death promoter (BAD), a pro-apoptotic member of the BCL-2 gene family. When BAD is dephosphorylated, it forms a heterodimer with BCL-2 and BCL-XL, inactivating them and allowing the proteins Bax or Bak to trigger apoptosis. However, when BAD is phosphorylated, this causes the formation of the BAD-(14-3-3) protein homodimer, leaving BCL-2 free to inhibit Bax-triggered apoptosis. Additionally activated AKT has also been shown to signal to and phosphorylate the Forkhead transcription factor (FKRL1), thus preventing its ability to translocate to the nucleus, and thereby decreasing the transcription of the pro-apoptotic members Bim and FASL (43). Since AKT is the head of many critical signaling pathways for cell survival and chemo-resistance, targeting and inhibiting its activity along with courses of chemotherapy could help treat the disease more effectively. However, the Serine/Threonine kinase domain of AKT is very similar to the domains of other proteins, so it has been difficult to develop AKT-specific inhibitors. Similar strategies are being developed to attack other therapeutically relevant targets along the PI3K/AKT axis, however very few have shown any promise. This has led researchers to start looking in other directions for AKT independent mechanisms that may also regulate cell survival and

growth of neuroblastoma cells. One of those pathways is regulated by a member of the Src family kinase, Fyn, which has been shown to be important in the development of neuronal cells (44).

Src family kinases

Src family kinases (SFKs) are nonreceptor tyrosine kinases that phosphorylate tyrosine residues of various cytosolic, nuclear, and membrane proteins. Srcs were discovered in the early 1900s, originally described as the oncogene responsible for the tumor progression of sarcomas in chickens, eventually known as the Rous Sarcoma Virus. Later they were discovered to be associated with malignant transformations, opening the field of oncogenesis (45). Since their discovery, an entire family of members has been discovered that regulate a wide variety of phenotypes ranging from development of neurons (46) to the oncogenesis of prostate cancer (47). Three distinct members of the Src family of kinases have been shown to be broadly expressed in the adult CNS: Src, Fyn, and Yes (48). Much like Src, the over expression of Fyn has been shown to drive the morphologic transformation in normal cells, as seen in NIH 3T3 fibroblast cells displaying a cancer like phenotype along with increase anchorage independent growth (49). However, both Src and Fyn have also been implicated in neuronal differentiation. They were shown to be initially expressed in neuroectodermal cells during neural fold formation in chickens, which then enrich the growth cone portion of neural processes upon neuronal differentiation. Their expressions regulate cell adhesion molecules such as neural cell adhesion molecule (N-CAM), important for axonal outgrowth and guidance (50, 51).

Fyn plays a critical role in neuroblastoma differentiation

In addition to the developmental role Fyn plays in neurons, the Eilers group published an article in *Cancer Cell* in 2002 showing that over expression of Fyn in neuroblastoma led to a differentiated phenotype and correlated with low risk tumors (44). Their results showed that the expression of Fyn protein is correlated with tumor stage in a panel of patient samples; high expression of Fyn with lower tumor stage and low expression of Fyn with higher tumor stages. In addition to looking at Fyn expression levels, they performed a kinase activity assay, which suggested that high Fyn kinase activity was consistently observed in stage 1 tumors biopsies and that stage 4 tumors mainly expressed low kinase activity. They showed that high expression of Fyn is a good prognostic marker for long term survival of neuroblastoma patients and that this trend is independent of MYCN expression (44).

Overall, their results suggest that Fyn could play an important role in neuroblastoma differentiation, however the pathways responsible for the activation and signaling of Fyn are still unknown. Several publications have shown the interaction between Fyn and TrkB. The Chao research lab found a novel role for Fyn in mediating Trk activation by adenosine in hippocampal neurons. They show that G-protein coupled receptor (GPCR) activates Fyn by adenosine, which then co-localizes and activates Trk receptors by phosphorylation (52). In addition, the interaction between Fyn and TrkB was shown to occur through their SH2 domains in cultured neurons (53). Furthermore, Fyn has also been shown to determine the localization of TrkB receptors in lipid rafts in neurons (54). These findings help put together a picture of a potential pathway that characterizes the activity of Fyn through TrkB, however this interaction has still never been described in neuroblastoma or even in regards to proliferation or differentiation.

TrkB plays a critical role in anoikis

To complement the characterization of TrkB mediated proliferation of adherent neuroblastoma cultures, we also evaluated its role in a suspension environment. A major contributor to the severity of the disease is the ability for cells to disseminate from the primary tumor and metastasize to another part of the body. For normal cells, encountering foreign environments would trigger a process called anoikis, which induces apoptosis of the cells due to a lack of signaling from the extracellular matrix and unfamiliar cell adhesion signals. However many types of tumors such as neuroblastoma are anoikis resistant. The Peeper lab recently used a functional genomic screen to identify TrkB as an onco-protein responsible for the promotion of metastasis. *In vitro*, they discovered that over expression of TrkB caused nonmalignant epithelial cells to become anoikis resistant. Similarly in nude mice, the TrkB expressing cells also produced highly invasive and metastatic tumors with short latencies (55). Despite all of their work, the mechanism behind TrkB regulated anoikis resistance still remains unknown. Furthermore, they show that TrkB plays an important role in anoikis resistance, but only by an over expressed system in epithelial cells. A model system that naturally expresses an abundant expression level of TrkB such as neuroblastoma would enhance the physiological relevance of their finding.

Significance

Although the treatment effectiveness of neuroblastoma has progressed through the years, high risk patients are still treated with intensive multimodal therapies that produce similar, suboptimal cure rates similarly seen in the past. The discovery of Trk receptors has been an important advancement in the ability to study specific signaling pathways based on the phenotypes of the neuroblastoma. Although the analysis of the Trk structure as well as identifying down-stream signaling targets has come a long way, the field is still incomplete. Further research is needed in order to fully understand the Trk concept from a mechanistic standpoint.

Hypothesis

Considering the following:

- TrkB receptors have been identified as an important prognostic marker in neuroblastoma that dictate poor prognosis of the disease
- TrkB has been previously shown to regulate proliferation, cell survival, and chemoresistance
- The mechanism behind TrkB mediated proliferation is still unknown
- Fyn expression is important for the differentiation of neuroblastoma cells

The research presented in this dissertation was guided by the hypothesis that **BDNF-mediated activation of TrkB utilizes common proximal signaling pathways to drive cell cycle machinery to enhance the proliferation of neuroblastoma cells.**

Chapter 2

TrkB characterization in neuroblastoma

RATIONALE

In previous Trk research, a common problem is the sensitivity of reagents to detect the TrkB receptor. The TrkB signaling pathway in neuroblastoma cells has been especially difficult to evaluate as a suitable cell line to measure TrkB signaling has not been available due to the loss of TrkB expression when the cells are used *in vitro*. Several labs have turned to using an exogenous over-expressed TrkB model to research specific phenotypes, however this method may produce misleading results. The super physiological expression of TrkB receptors could induce a phenotype that isn't normally seen with a normal expression level. In addition, if a pathway of interest was shown to be highly activated in the over expressed system, it would be difficult to determine whether the results were real or were actually due to the over abundance of TrkB receptors regulating something that wouldn't normally be activated. Furthermore, the relevance of the finding would be called into question if eventually moved into an *in vivo* setting because that level of TrkB expression would never be expressed normally. To avoid the confusion associated with an over expressed system, we decided to direct our attention to find a suitable cell line that would express a detectable level of TrkB.

From a panel of 8 neuroblastoma cell lines, SMS-KCN, SMS-KCNR, SK-N-SH, CHP-134, SHEP, LA-155N, NGP, and SY5Y, we conducted a Taqman Quantitative Reverse Transcriptase - Polymerase Chain Reaction (qRT-PCR) to evaluate the expression of TrkB mRNA relative to a GAPDH control (Fig.2a). Although 7 of the 8 cell lines expressed detectable levels of TrkB mRNA transcript, the SMS-KCN cell line had the most abundant TrkB expression, 16 fold over the next highest which was SMS-KCNR. Interestingly, both of these cell lines were derived from the same patient: SMS-KCN derived

from the primary tumor at diagnosis and SMS-KCNR from a bone marrow biopsy at relapse. This finding suggests that endogenous over-expression of TrkB plays a major role in the primary tumor, however still may have an important role in metastasis.

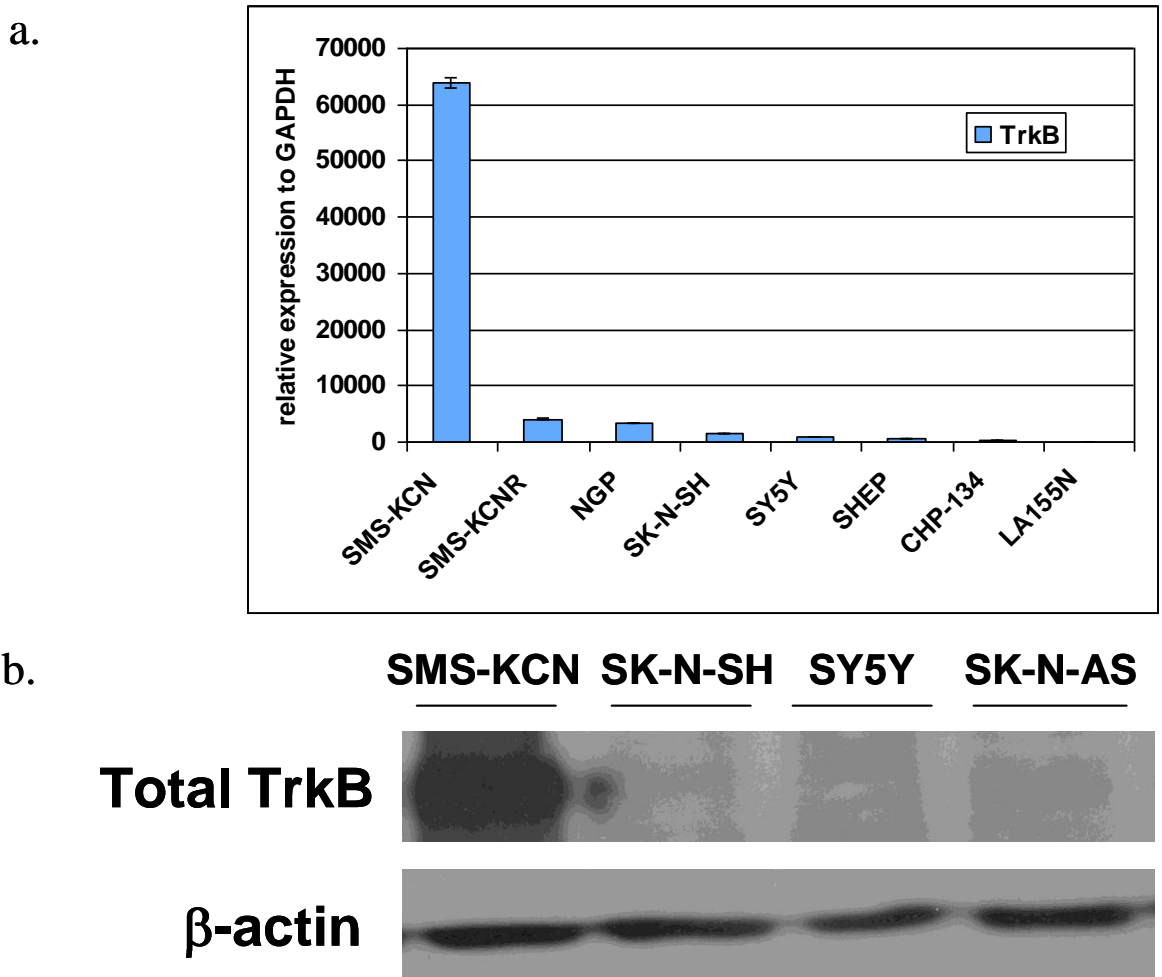


Fig 2. TrkB expression in a panel of neuroblastoma cell lines. a) Taqman qRT-PCR and b) Western blot show that SMS-KCN cells have a higher expression level total full length TrkB compared other neuroblastoma cells lines

However, high levels of mRNA expression may not correlate with high protein levels, so next we wanted to determine if the mRNA expression of TrkB translated to a detectable

amount of protein by western blotting. Whole cell lysate was isolated from a panel of cell lines and probed with total TrkB antibody, which detects all TrkB isoforms, and Beta Actin as a loading control. Consistent with our RT-PCR results, we found that the protein expression of SMS-KCN had an easily detectable TrkB protein expression (Fig.2b).

SMS-KCN is an MYCN-amplified neuroblastoma cell line that expresses readily detectable endogenous levels of both TrkB and BDNF, making this cell line useful for analyzing the TrkB mechanism from a physiological setting. SMS-KCN cells exhibit an autocrine BDNF loop that maintains a high level of basal TrkB activation, shown to be important in stimulating cell survival and neurite extension and its inhibition decreases cell proliferation (56). Furthermore, Brodeur in 1994 showed that this cell line also expresses TrkA, but not the low affinity neurotrophin receptor, p75NTR, or its ligand NGF. However, when the cells were induced with exogenous NGF, TrkA signaling was shown to be defective due to a lack of kinase function (17). In our experiments, the expression of TrkC and p75NTR mRNA by RT-PCR was found to be significantly lower than TrkB (~5,300 fold and ~2,000 fold less respectively) suggesting a lack of dependence on those receptors compared to TrkB (Fig.3). These reports taken together suggest that when exogenous BDNF is added to the system, the only main Trk signaling pathway activated would be TrkB.

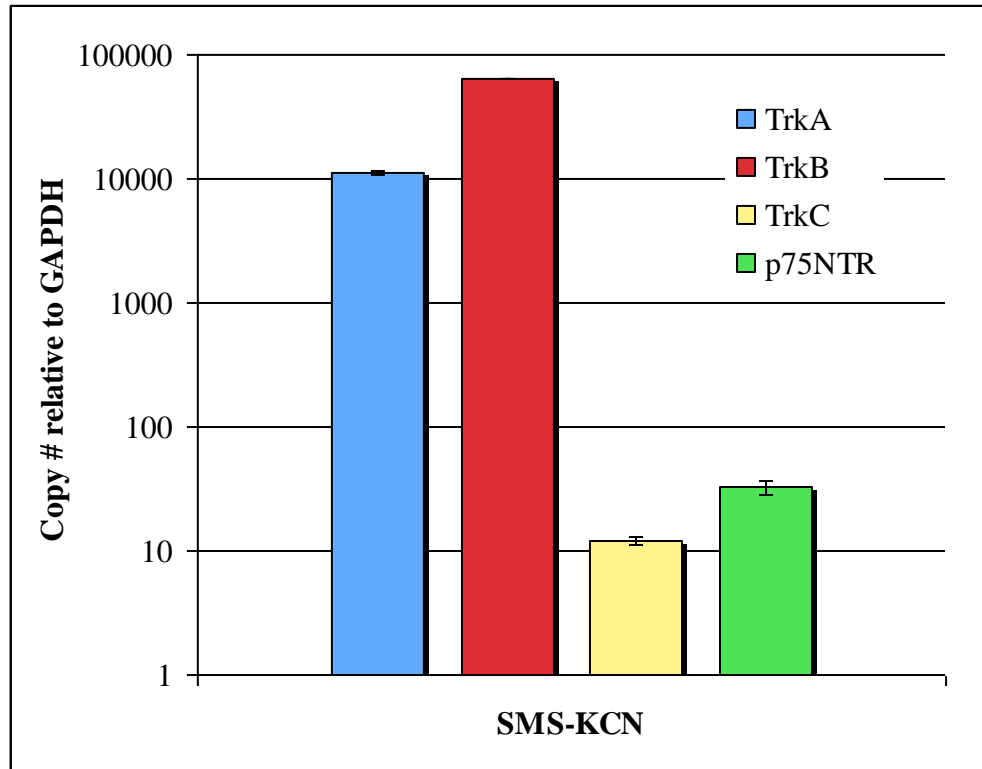


Fig. 3. mRNA levels of TrkA, B, C and p75NTR in SMS-KCN cells by Taqman qRT-PCR.

Although SMS-KCN cells express an abundant level of TrkB mRNA and protein shown by RT-PCR and western blot respectively, the results don't indicate the location in the cells. If the expression of cell surface receptors were low, stimulating the cells by exogenous BDNF wouldn't provide an adequate signal over basal levels in order to elucidate downstream signaling targets. However, if there is a high expression of cell surface receptors, then this would further suggest that the SMS-KCN cell line would be a good model system to characterize the TrkB mechanism.

By flow cytometry, we examined the cell surface expression level of TrkB receptors using a primary antibody for total extracellular TrkB with a phycoerythrin (PE) conjugated secondary antibody to detect as a fluorescent tag. SY5Y was used as a negative control cell

line because TrkB was not detectable by western blotting and didn't have significant levels of TrkB mRNA. To quantify, the mean fluorescence intensity was taken from each measurable group and normalized relative to an unstained control. SMS-KCN cells showed a ~12 fold higher cell surface TrkB expression when compared to the SY5Y control cell line. (Fig.4 a,b). This result is consistent with our RT-PCR and western data, showing a higher TrkB mRNA and protein expression in SMS-KCN cells compared to SY5Y cells. This finding suggests that SMS-KCN cells do have a high TrkB surface expression that would be suitable to use as a model to stimulate using exogenous BDNF. However, SMS-KCN cells have been previously shown to have an autocrine loop that activates TrkB receptors at basal level (17). Although the BDNF autocrine loop might result in a high baseline of activation for TrkB, we believed we could still stimulate a measurable difference in proliferation by the addition of exogenous BDNF. Next we wanted to analyze whether these cells could respond to exogenous BDNF to produce a phenotypic response over the basal activation level of TrkB.

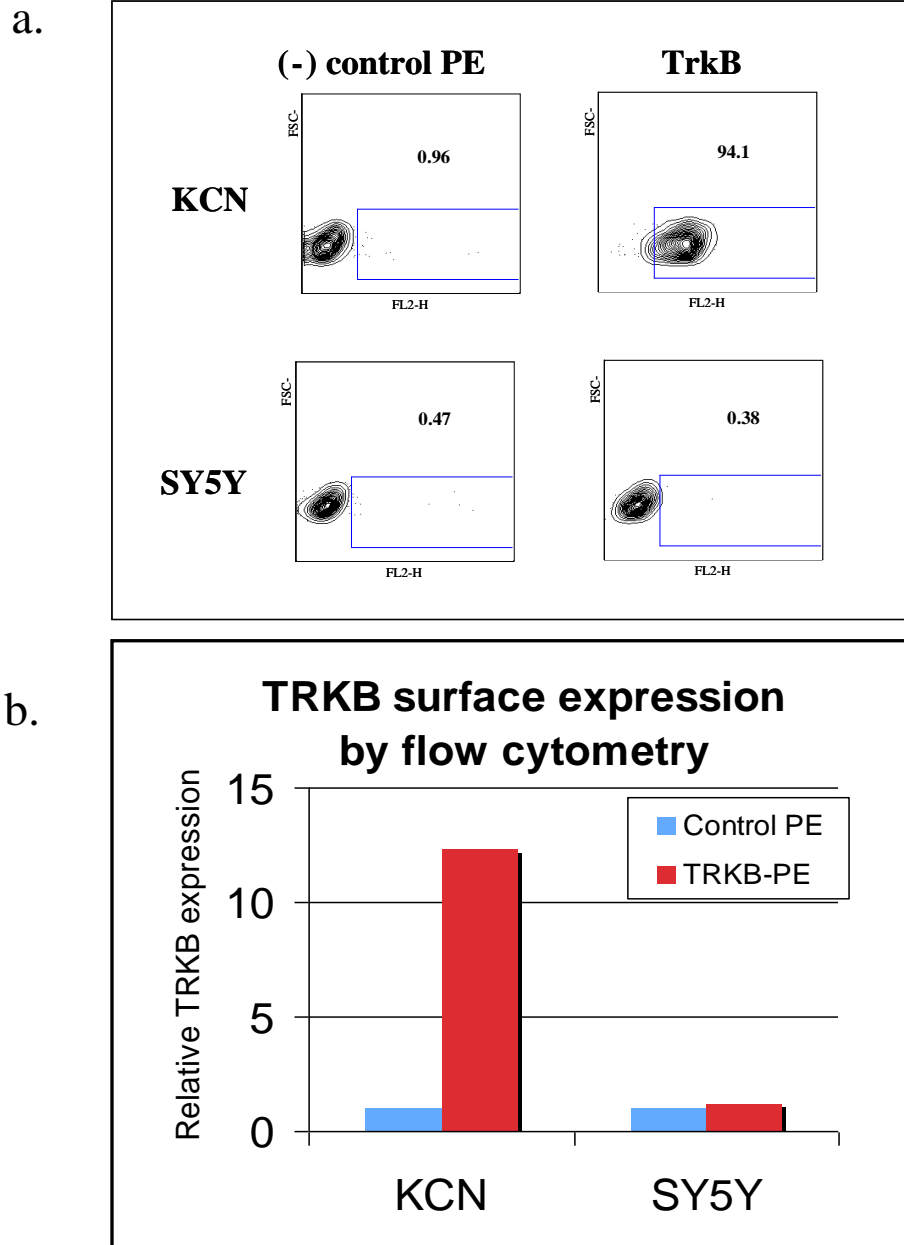


Fig 4. Surface expression of TrkB by flow cytometry a and b) SMS-KCN cells express 12 fold more cell surface TrkB receptors than SY5Y cells

In a panel of neuroblastoma cell lines (CHP-134, IMR-32, KCN, SHEP, SK-RG, SY5Y, and SMS-KCN), we wanted to see whether we could produce a BDNF dose response using an Alamar blue proliferation assay. The cells were plated in a 96-well plate and

treated with a range of BDNF concentrations (0 – 100ng/mL) in triplicate for 72 hours. The Alamar blue dye is a cell viability indicator that uses the reducing power of viable cells to convert the active ingredient, resazurin, to the fluorescent molecule, resorufin. Only live cells will continuously produce resorufin, thus providing a quantitative measure of viability and surrogate for proliferation. Compared to other *in vitro* proliferative assays that use MTT indicators, the presence of Alamar blue doesn't interfere with the reactions of the electron transport chain, allowing the cells to remain healthy and intact (57). The panel of 7 neuroblastoma cell lines produced a dose-dependent effect with as low as 10ng/mL of BDNF, for a mean proliferative increase of 43% (Fig.5). This result suggests that all of the neuroblastoma cell lines exhibit a functional expression level of TrkB to elicit a response. However, the SMS-KCN cell line, with the most abundant expression of TrkB, was the most responsive, providing an 80% proliferation increase at only 6.3ng/mL of BDNF. None of the other cell lines produced such a dramatic effect, even at 100ng/mL of BDNF. The most reasonable explanation for the BDNF sensitivity is because of the difference in TrkB expression levels. The difference between adding 100ng/mL of BDNF compared to a lower concentration is shown to be minimal due to TrkB receptors being the limiting factor. In addition, another possible explanation is that the BDNF sensitivity of SMS-KCN cells is controlled by the rate of TrkB recycling. Since SMS-KCN cells are consistently activated by its autocrine BDNF loop, the rate at which receptors are recycled could be much faster than a cell line that is not used to constitutive TrkB activation. This in turn would provide continuous BDNF mediated proliferation in SMS-KCN cells over the 72 hour period. Regardless, these results corroborate with the TrkB protein and mRNA data found previously, but it also suggests that those TrkB receptors are functional.

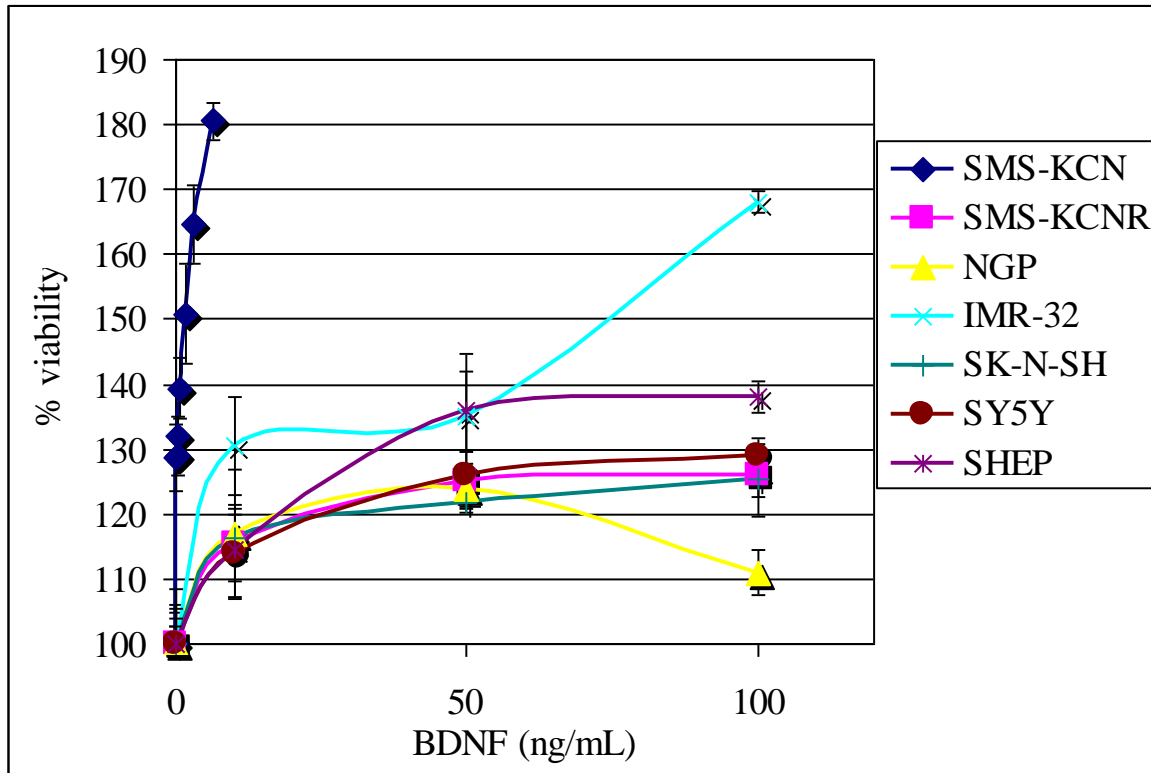


Fig 5. BDNF induction stimulates the proliferation of a panel of neuroblastoma cell lines shown by an Alamar blue proliferation assay

Analyzing the data more closely, we saw that SMS-KCN cells were extremely responsive to BDNF; as low as 0.1ng/mL of BDNF producing a 29% increase in proliferation (Fig.6). This is important to note because the use super physiological doses of 50-100ng/mL of BDNF to elicit a phenotypic response could lead to irrelevant, non-physiological results. Finding that these SMS-KCN cells can react at 0.1ng/mL of BDNF suggests that this is a specific TrkB event.

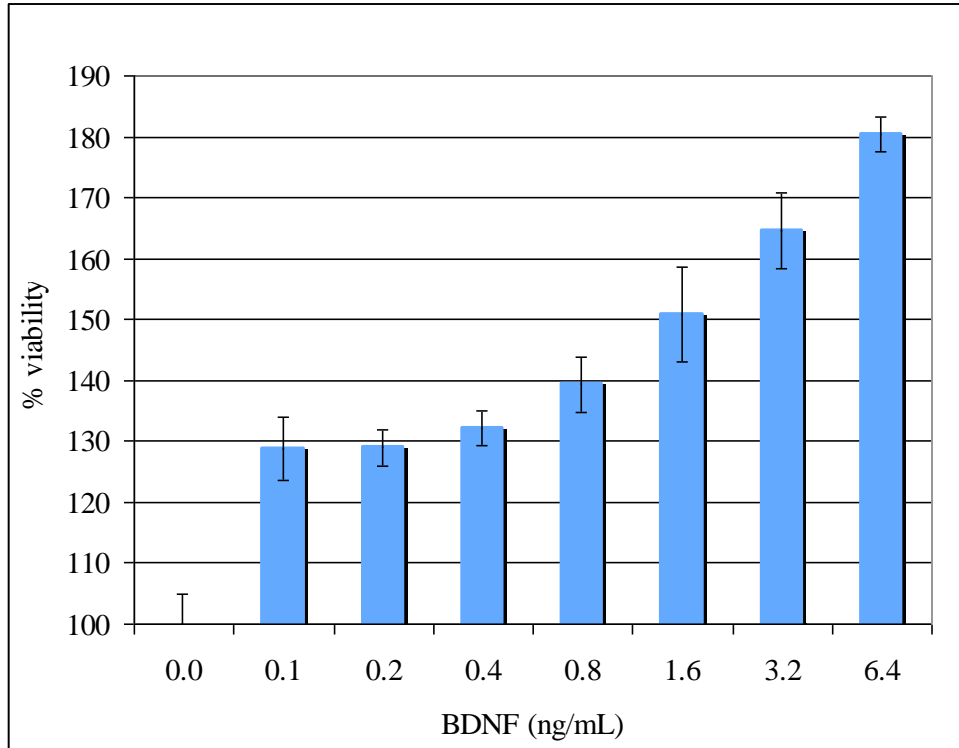


Fig 6. As little as 0.1ng/mL of BDNF can stimulate proliferation of SMS-KCN cells shown by an Alamar Blue proliferation assay

After showing that TrkB plays a critical role in the proliferation of SMS-KCN cells, we next wanted to analyze how important these receptors were to the cells by inhibiting their kinase activity. The Trk inhibitor, AZ23, has been noted to be selective for Trk kinases, compared to other Trk kinase inhibitors such as K252a and CEP-701 that have been shown to act against multiple targets (58). AZ23 is a potent, small molecule inhibitor of the Trk kinase that works by ATP competition. In a panel of cell lines, SMS-KCN, SMS-KCNR, SK-N-SH, SK-N-AS, CHP-134, and LA-155N, cells were treated with a range of AZ23 concentrations and incubated for 72 hours in an Alamar blue proliferation assay. We found that proliferation was prevented in an AZ23 dose-dependent manner in all

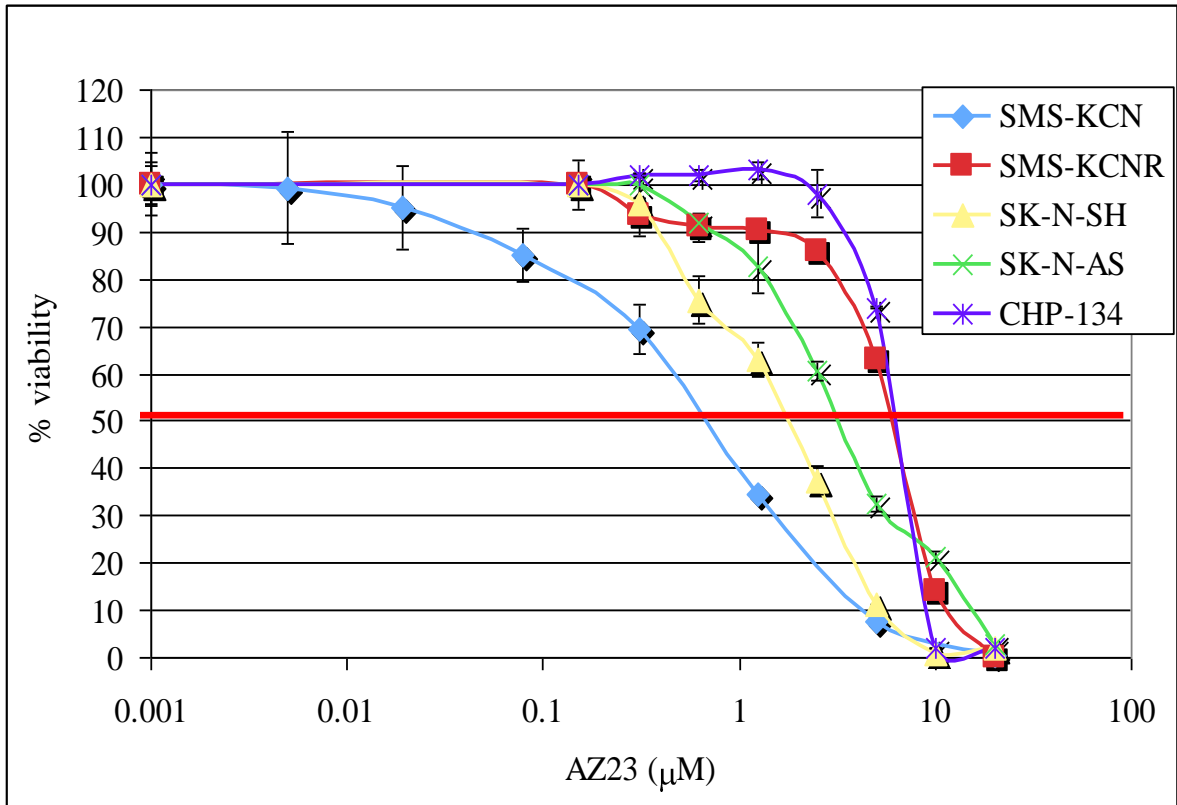


Fig 7. TrkB inhibition leads to decreased proliferation in a panel of neuroblastoma cell lines, shown by a 72 hour time course proliferation assay treated with/without the Trk kinase inhibitor, AZ23.

cell lines with IC50s as high as 7μM. At micro-molar concentrations of the inhibitor, other Trk targets are most likely being affected as well, suggesting that TrkB alone may not be critical in the proliferation of some of these cell lines. However, the SMS-KCN cell line was shown to be sensitive to the inhibitor with an IC50 at 0.8μM (Fig.7). This result suggest the importance of TrkB to survival and proliferation in a number of neuroblastoma cell lines, but particularly to the SMS-KCN cell line.

To show that AZ23 prevents the activation of TrkB, we measured the phosphorylation status at Tyr₈₁₇ using varying concentrations of BDNF by western blotting

(Fig. 8a). The intracellular phosphorylation sites for TrkB include Tyr₅₁₆, Tyr₇₀₂, Tyr₇₀₆, Tyr₇₀₇, Tyr₈₁₇, however it was previously suggested that Tyr₈₁₇ and Tyr₅₁₆ were the main residues important for transmitting signals that induce pathway stimulation in SMS-KCN cells (59). First we show that as little as 25nM of AZ23 was enough to inhibit BDNF-induced phosphorylation of TrkB in SMS-KCN cells, suggesting the high potency the inhibitor has for TrkB. Second, as little as 5ng/mL of BDNF was sufficient to activate TrkB beyond basal level. This result confirms our previous proliferation assay describing the sensitivity of SMS-KCN cells to BDNF. Third, the blot also shows that a super physiological dose at 100ng/mL of BDNF doesn't produce any additional level of activation compared to the 25 and 5ng/mL doses.

Our results also show that AZ23 could prevent BDNF mediated proliferation, as analyzed by an Alamar blue proliferation assay (Fig. 8b). A range of AZ23 concentrations (0.1 – 10 μ M) were shown to block the proliferative response of a range of BDNF (0.1 – 10ng/mL) in a dose dependent manner. Although I was able to show that 25nM of AZ23 was sufficient to inhibit BDNF stimulated TrkB activation by western blotting, the results of the Alamar blue proliferation assay dictated the need for 1 μ M of AZ23 to block induced proliferation. This finding suggests that even though TrkB may play a critical role in proliferation, other pathways need to be stimulated in conjunction to produce the phenotype. It is possible that the expression of the low affinity BDNF receptor, p75NTR, may play a role in promoting BDNF activation of TrkB as suggested in other studies (60, 61), which would warrant the need for a higher concentration of AZ23 for competitive inhibition. As a way to further characterize the BDNF mediated proliferation as described, we analyzed how the cell cycle was affected.

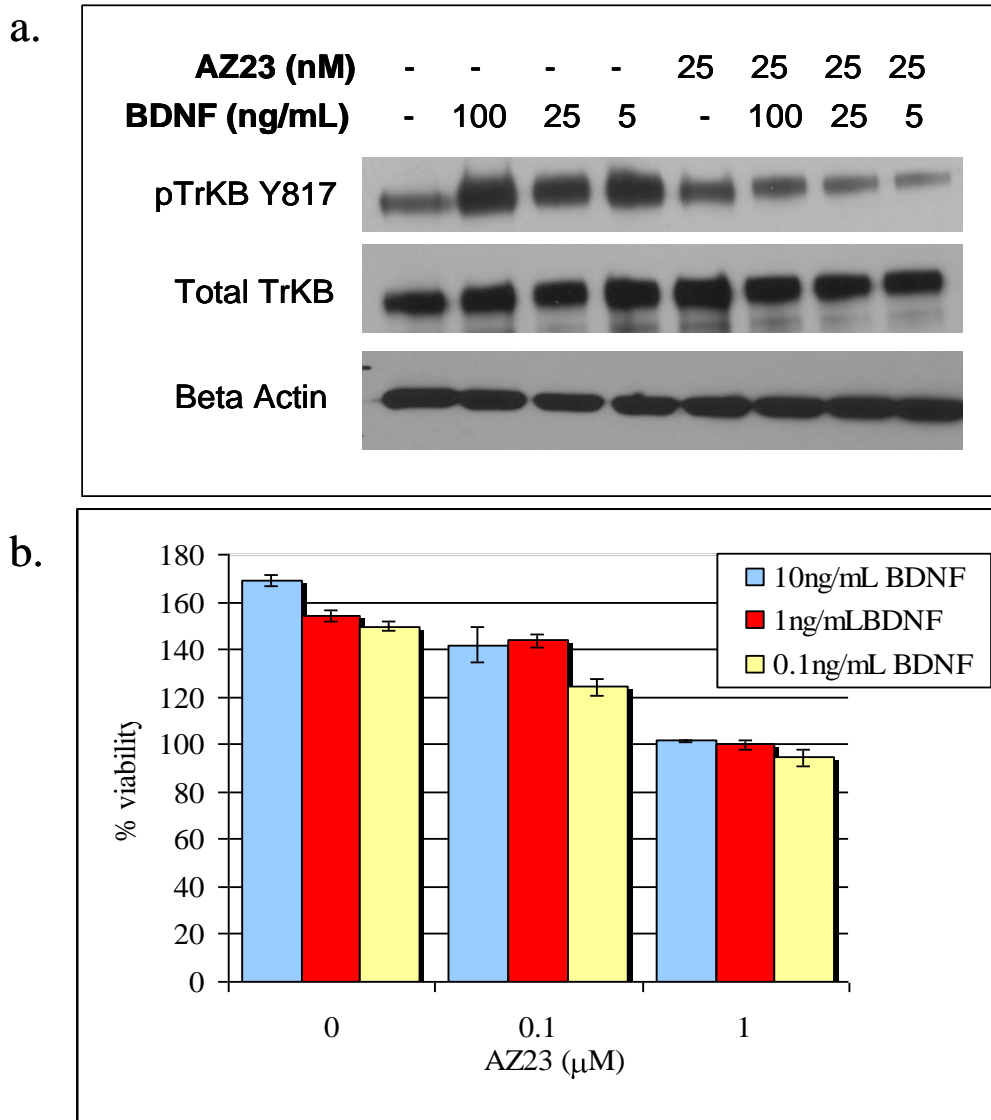


Fig 8. BDNF activation/AZ23 inhibition of TrkB a) AZ23 blocks BDNF mediated TrkB activation in a dose dependent manner shown by Western blot b) and BDNF mediated proliferation shown by an Alamar blue proliferation assay

To further test whether the inhibition of TrkB has an anti-proliferative effect in neuroblastoma, we analyzed the cell cycle of the IMR-32 cell line. SMS-KCN cells proved to be difficult to work with by flow cytometry due to its nature to aggregate easily. Since IMR-32 cells responded to BDNF efficiently in my previous Alamar blue proliferation assay, we used them to model cell cycle expression during TrkB inhibition. However, since this cell line had a much lower expression of TrkB as seen by protein and mRNA levels, a higher range of doses of AZ23 was needed in order to see an effect compared to SMS-KCN cells. Triton permeabilization was used to incorporate the intercalating agent, Propidium Iodide (TPI) into the cells to stain DNA. From there, the status of DNA can then be analyzed to see what phase of the cell cycle the cells are in based on the total amount of DNA, which is represented as FL3-area in the graph (Fig. 9). The lowest FL3-area from ~0-75 units of fluorescence correlates with DNA in the sub G0 phase, ~75-125 units is the G0/G1 phase, ~125-150 units is S phase, and ~150-200 units is the G2/M phase. The results show an AZ23 dose dependent decrease of fluorescent intensity levels at the S and G2/M phases (cycling phases for cell division) suggesting a G2/M arrest of cell cycle (Table 1). Since the G0/G1 phase did not exhibit a dose dependent decrease, this suggests an increase in cell death, shown in the sub G0 phase (Fig. 10). Studies by the Theile lab (62) and confirmed by our findings indicate the important role that TrkB plays in survival and proliferation in neuroblastoma cells. In addition, Thiele shows that TrkB signaling stimulates neuroblastoma disaggregation and invasion, which taken together with cell survival are characteristics of metastatic cells. We decided to investigate the role TrkB may play in metastasis.

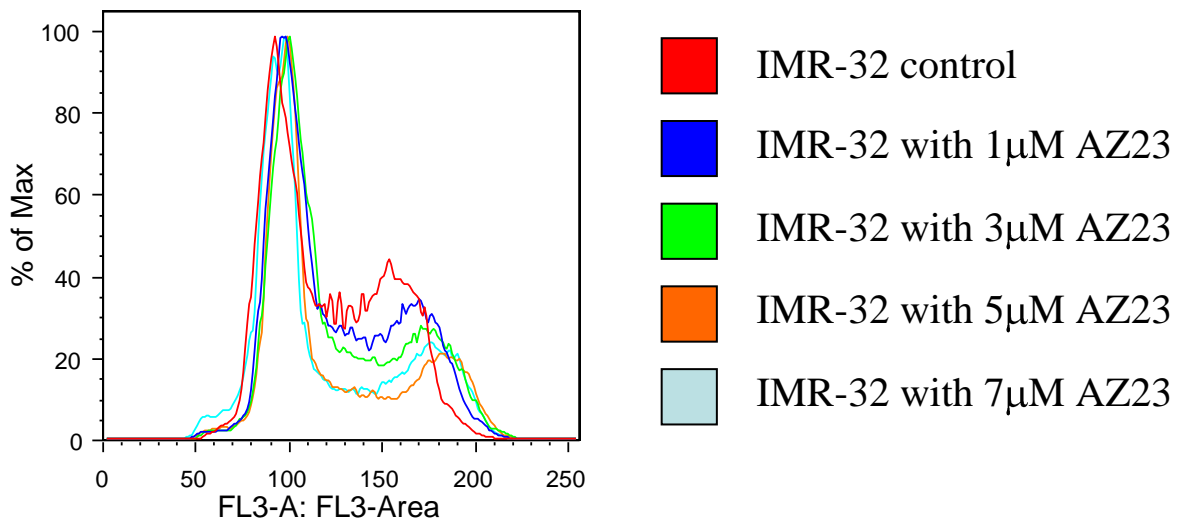


Fig 9. TrkB inhibition arrests the progression of the cell cycle in IMR-32 cells.

	% G0/G1	% Cycling
control	34.92	47.6
1mcM AZ23	34.0	44.5
3mcM AZ23	36.3	41.1
5mcM AZ23	33.9	37.9
7mcM AZ23	33.6	36.3

Table 1. The reduction of cycling cells is shown to be dose dependent on AZ23

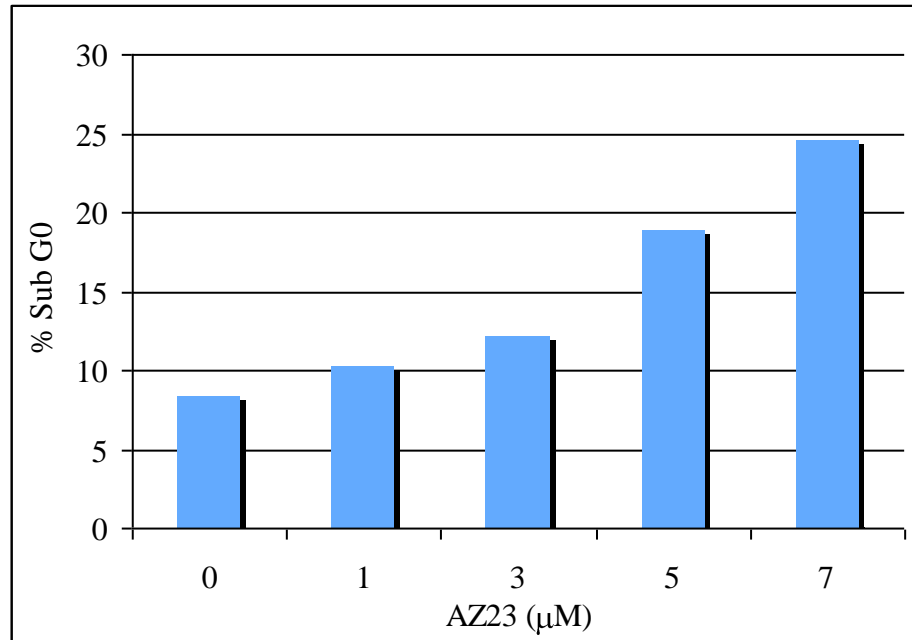


Fig 10. TrkB inhibition increases the sub-G0 cell population in a dose dependent manner

TrkB promotes anoikis resistance

Recent studies suggest that TrkB could play a role in the suppression of anoikis, which is the self-induced death of the cell (apoptosis) that is caused by the loss of cell-matrix interactions. In 2004, the Peeper lab conducted a genome wide screen and identified TrkB as a potent and specific suppressor of caspase-associated anoikis of non-malignant epithelial cells (55). Since TrkB is an important signaling target in neuroblastoma, this suggests that the aggressive nature and high metastatic potential of the disease could be attributed to the increased TrkB activation seen in more severe stages of the disease. Other human malignancies with also a natural over-expression of TrkB, such as prostate (63) and ovarian cancer (64) have tried to relate this phenomenon to their model. However, little research has been done to apply this finding to neuroblastoma.

Our previous results indicate that SMS-KCN cells have an abundant level of TrkB protein and mRNA shown by western and qRT-PCR, so we wanted to test whether their activation would induce anoikis resistance. SMS-KCN cells were grown in either an attached environment to simulate primary tumor growth or detached environment in plates coated with POLY-HEMA to prevent adhesion. As demonstrated previously, BDNF increases proliferation of neuroblastoma cells when in an attached environment. Since SMS-KCN cells have an autocrine BDNF loop that is constantly activating TrkB, these cells were expected to have a basal level of resistance to anoikis. The addition of exogenous BDNF should further promote this phenotype. Using an Alamar blue proliferation assay, we first reproduced our results showing 20ng/mL of BDNF promoting the proliferation of attached SMS-KCN cells as an internal control. Suspension SMS-KCN cells in POLY-HEMA coated plates showed a 20% decrease in proliferation due to an anoikis effect.

However, when the cells in suspension were stimulated with 20ng/mL of BDNF, the population of cells lost due to anoikis were rescued, shown by a 20% increase in viability that is similar to the attached control (Fig. 11). The results suggest a full recovery that parallels the baseline of attached SMS-KCN cells, thus correlating with the previous studies suggesting that TrkB could play a role in neuroblastoma anoikis resistance.

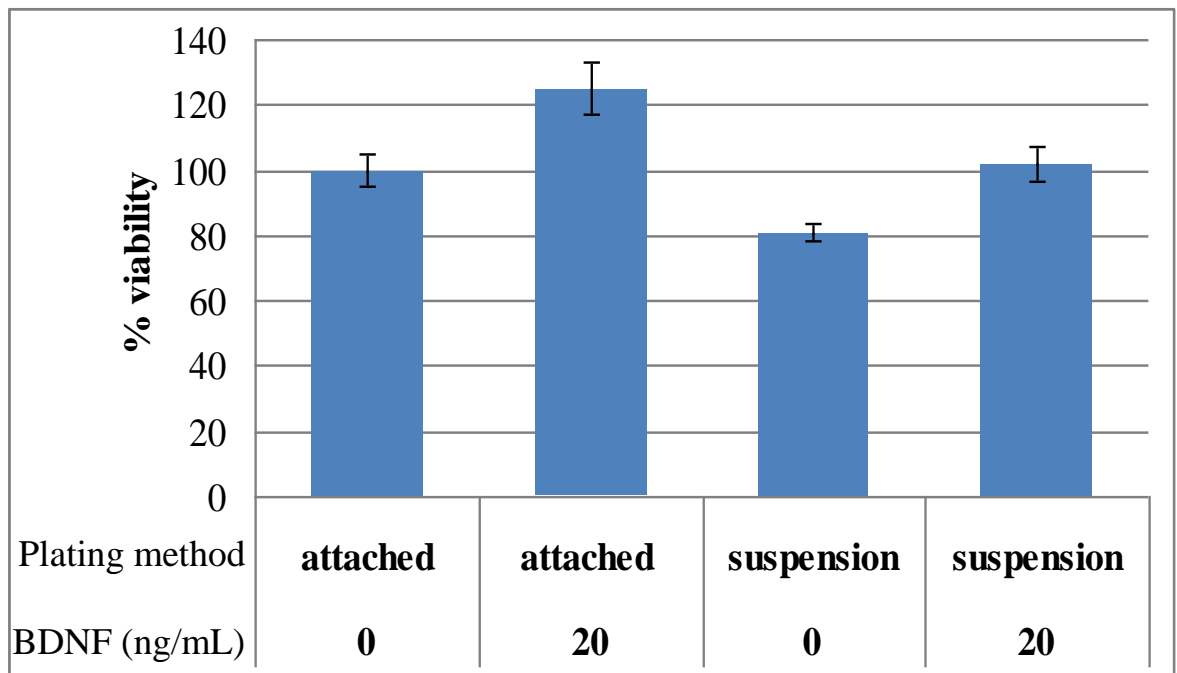


Fig 11. TrkB activation promotes anoikis resistance shown by an Alamar blue proliferation assay

In order to further assess that anoikis resistance can regulated by TrkB expression in neuroblastoma, a time course assay was studied to analyze how long after BDNF induction did it take for SMS-KCN cells to provide the maximal anoikis resistance effect. The SY5Y cell line, which expresses a minimal level of TrkB mRNA and protein previously seen in Fig. 3, was used as a negative control for the experiment. Cultured SMS-KCN and SY5Y

cells were grown in a POLY-HEMA coated 96-well plate and treated on day 0 with 20ng/mL of BDNF. BDNF stimulated samples were controlled for by a non-treated condition at each time point. The results showed a maximal effect in SMS-KCN cells at 24 hours of BDNF stimulation, inducing a 30% rescue rate that had a sustainable signal for up to 104 hours (Fig. 12). However in SY5Y cells, BDNF stimulation didn't produce a similar rescue effect, however were able to show a proliferative response in an adherent environment. A possible reason to explain our results for SY5Y cells could be that proliferation in suspension may require a higher level of TrkB stimulation in order to compensate for the apoptosis due to anoikis. The high expression level of TrkB in SMS-KCN cells could counteract the effects of anoikis by its robust proliferative response. These results together suggest that TrkB activation is important in anoikis resistance, and that the effect can sustain long after the initial BDNF stimulation.

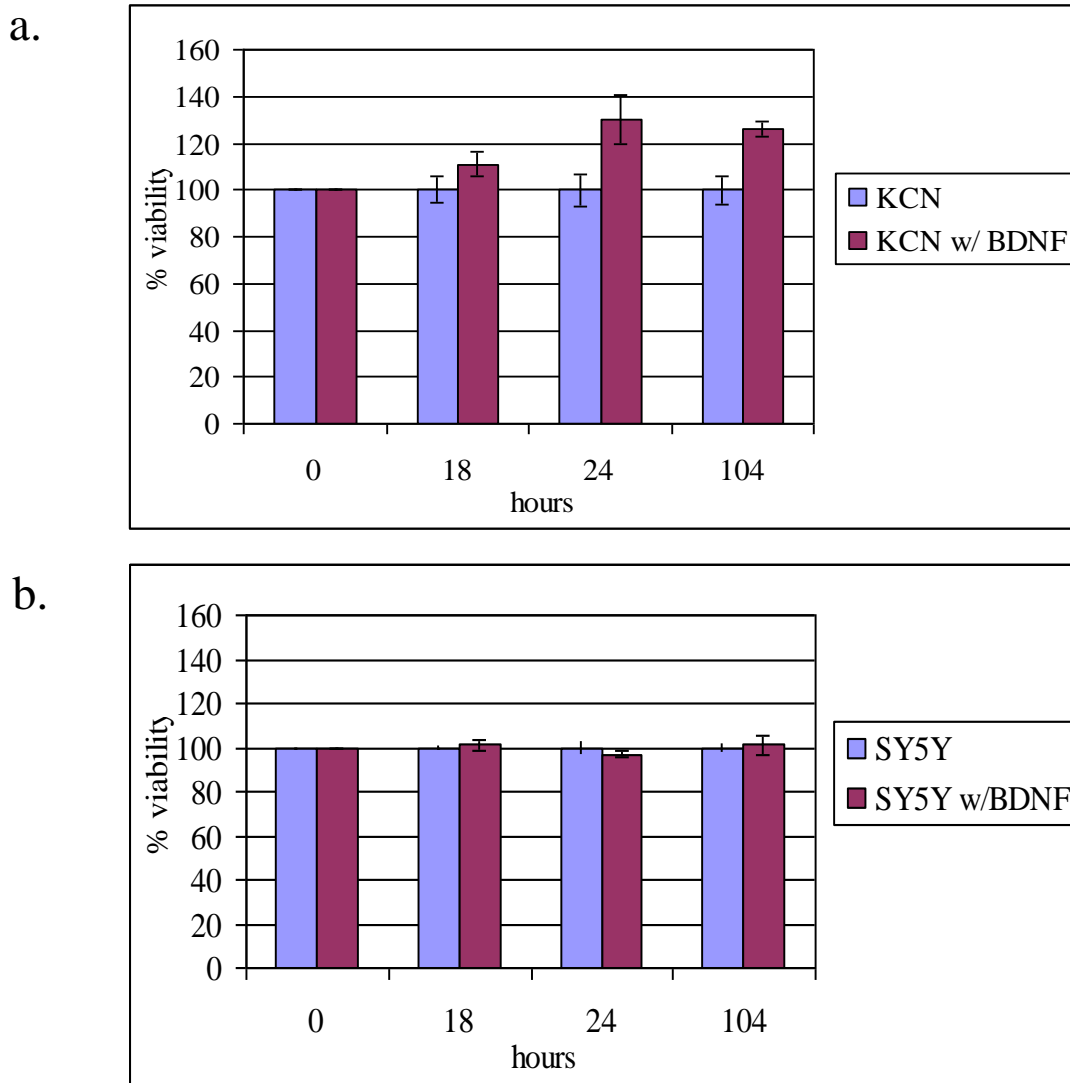


Fig 12. Maximal anoikis resistance was seen at 24 hours upon 20ng/mL of BDNF stimulation in a) SMS-KCN cells compared to the b) negative control cell line SY5Y.

Summary

Several studies have shown that TrkB plays an important role in anoikis resistance, however this is the first report to demonstrate this phenotype in neuroblastoma cells. Due to the high expression of TrkB receptors in aggressive stages of the disease, TrkB mediated anoikis resistance could explain the robust metastatic behavior commonly associated with

those patients. More work however is needed in order to further elucidate the mechanism involved in order to better identify additional targets that regulate the resistance.

TrkB mediated cell signaling

We then began to evaluate downstream signaling pathways of TrkB in SMS-KCN cells to find a potential mechanism of BDNF/TrkB mediated proliferation in neuroblastoma cells. Trk receptors resemble other receptor tyrosine kinases by dimerizing in response to ligand stimulation. The active tyrosine kinase phosphorylates specific intracellular tyrosine residues which serve as attachment sites for Shc and PLC. Once bound to the active receptor they become phosphorylated by the Trk tyrosine kinase. PLC catalyzes the breakdown of lipids to (DAG) diacylglycerol and (IP3) inositol 1,4,5 tri-phosphate. IP3 in turn promotes the release of calcium from intracellular stores while DAG stimulates PKC activation to modulate cell adhesion and migration. Activation of the SH2 domain of SHC activates either the Ras/MAPK or PI3K/AKT pathway, which have been shown to lead to phenotypes such as neuronal development and cell survival respectively (21).

We started by looking at the PI3K/AKT pathway, stimulating SMS-KCN cells with increasing doses of exogenous BDNF up to 20ng/mL for 10 minutes (Fig. 13). Based on our previous Alamar blue data in Fig 5, this range of doses were shown to stimulate TrkB effectively. Initially, we wanted to confirm that activated phospho-TrkB (pTrkB) was BDNF dose dependent, then whether pPI3K (Tyr₁₉₉), pAKT (Ser₄₇₃), and pBAD (Ser₁₁₂) followed the trend. Our results confirm previous data shown by Thiele's lab that the PI3K/AKT/BAD axis regulates survival in our neuroblastoma model system (19).

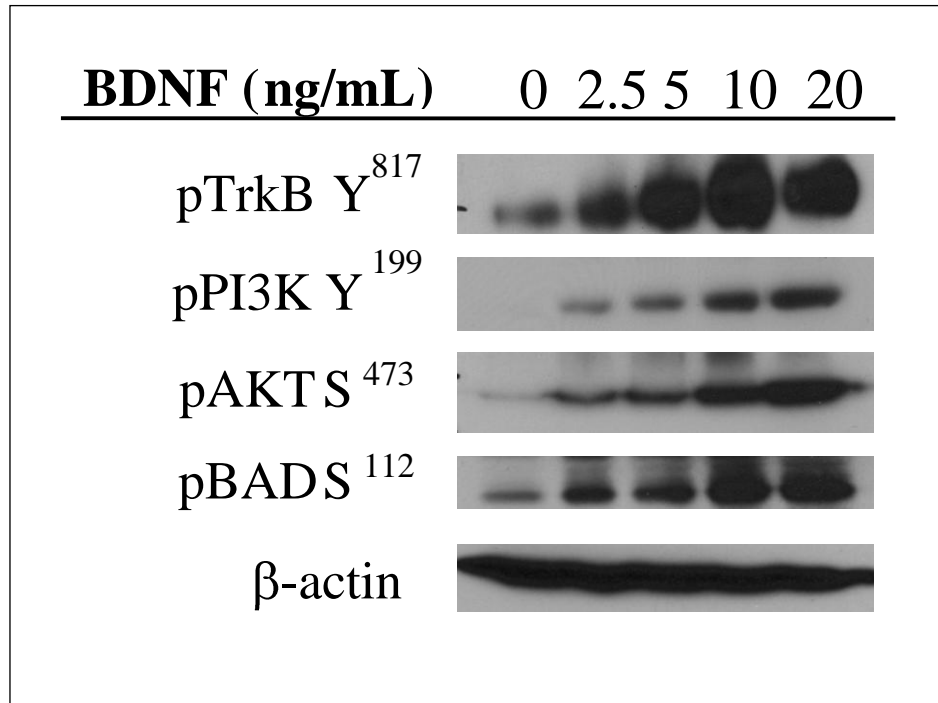


Fig 13. BDNF stimulation activates the PI3K/AKT pathway in a dose dependent manner in SMS-KCN cells

AKT has been identified as the critical starting point of many important pathways involved in proliferation, apoptosis, differentiation, and metabolism. In many cancers such as acute myeloid leukemia, one critical target of AKT signaling is the mammalian target of rapamycin (mTOR), which has been implicated as important for the proliferation, growth, and survival of the cells (65). This novel axis however has not been fully researched in neuroblastoma.

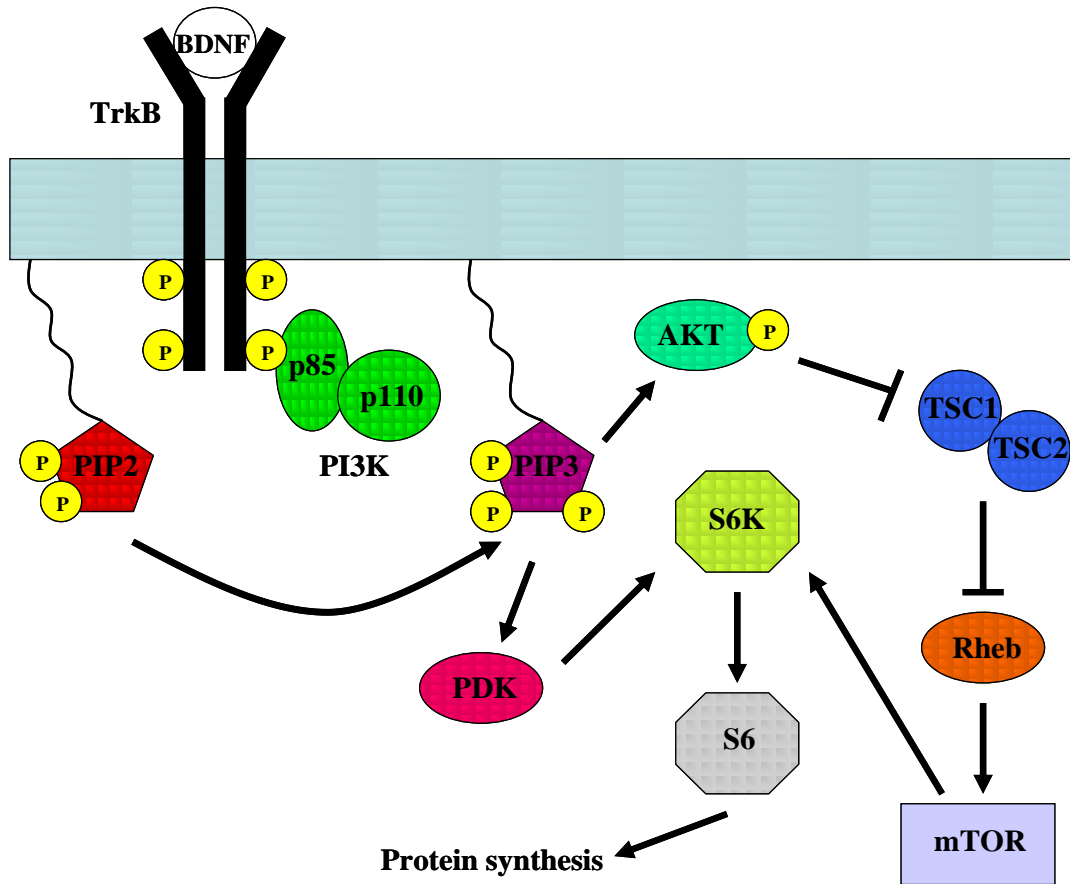


Fig. 14. Model of tyrosine kinase activated PI3K/AKT/mTOR axis

mTOR is a serine/threonine protein kinase that belongs to the phosphatidylinositol 3-kinase-related kinase protein family, implicated as the nutrient sensor responsible for growth and proliferation. Upon TrkB activation, AKT becomes phosphorylated. AKT acts to prevent TSC1 and 2 inhibition of Rheb, which in turn activates mTORC1. However, another pathway exists where PI3K activated PDK1 can also act directly on p70S6K to regulate the mTOR phenotype without the help of AKT. (Fig 14) To elucidate the potential mTOR mechanism, we assayed p-mTOR at Ser₂₄₄₈ and its known downstream targets p70S6k at Thr₃₈₉ and Thr₄₂₁/Ser₄₂₄ and RPS6 at Thr₂₃₅ (Fig. 15).

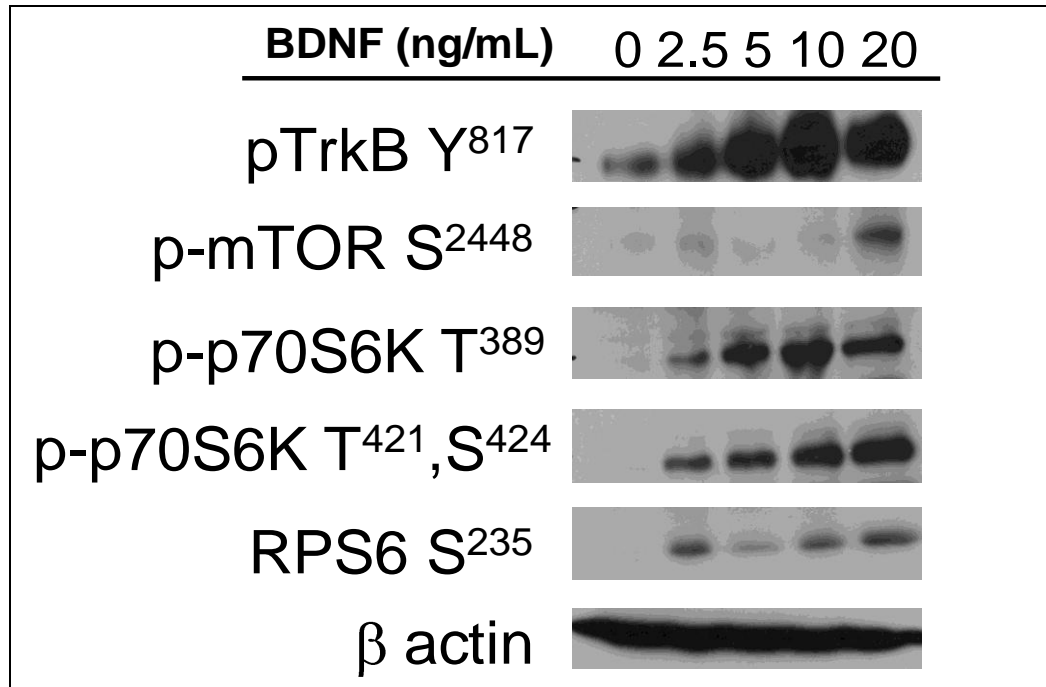


Fig 15. BDNF induces a dose response mediated activation of the mTOR pathway

The known downstream targets of mTOR, p70S6K and S6, followed a BDNF dose dependent trend, shown to become active by 2.5ng/mL of BDNF. mTOR wasn't shown to be phosphorylated until 20ng/mL of BDNF stimulation, suggesting that PDK1 may play a direct role in regulating the mTOR proliferative phenotype. To test whether the TrkB mechanism signaled to mTOR by means of PI3K/PDK1 or PI3K/AKT pathways, we used the PI3K inhibitor LY294002 and the AKT inhibitor Triciribine. LY294002 acts as a competitive inhibitor for ATP binding site of the enzyme, shown previously to inhibit the proliferation of choroidal melanoma cells (66). Triciribine is a tricyclic nucleoside that has been shown to selectively inhibit the phosphorylation and activation of AKT, and not its upstream activators PI3K or PDK1 nor any other parallel pathways such as ERK1/2, STAT3, PKC, or JNK. Triciribine has been previously shown to effectively inhibit the growth of AKT-overexpressing melanoma cell lines in vitro and in vivo (67). Both of these

inhibitors would be used to test whether BDNF mediated proliferation could still take place when either the function of AKT or PI3K would be inhibited. The experiment would show that if inhibiting the function of AKT doesn't prevent BDNF mediated proliferation, but inhibiting PI3K does, it would further suggest that the PI3K/PDK1 pathway might signal directly to p70S6K instead of going through AKT. A range of relevant concentrations (0 – 10 μ M) of each inhibitor was used in an Alamar blue proliferation assay with SMS-KCN cells +/- 10ng/mL of BDNF. The BDNF concentration was chosen based from my previous western blot data to provide the most profound stimulation of pTrkB, thus giving the inhibitors the best chance to decrease the proliferation induced by activated TrkB. SMS-KCN cells were plated over night and a concentration range of each inhibitor was used (0 – 10 μ M) +/- 10ng/mL of BDNF. After 72 hours of incubation, our results showed that 10ng/mL of BDNF was still able to promote proliferation with both inhibitors, seen by the sustained increase of proliferation over the non BDNF treated control (Fig. 16a,b). To verify whether 10 μ M of the AKT inhibitor Triciribine was actually preventing the phosphorylation and activation of AKT, we analyzed its efficacy by western blot (Fig. 16c). The decrease in pAKT signal from BDNF alone to the Triciribine/BDNF combination showed that the inhibitor worked efficiently. In addition, the western blot also showed no cross reactivity with the close by signaling pathway MAPK, shown as having no significant change in protein expression between BDNF alone and Triciribine/BDNF combination. These results suggest that neither AKT nor PI3K are the main factors important for BDNF mediated proliferation. Furthermore, this also suggests that the mTOR pathway through either PDK1 or AKT direct stimulation does not play an important role in the proliferation of SMS-KCN cells either.

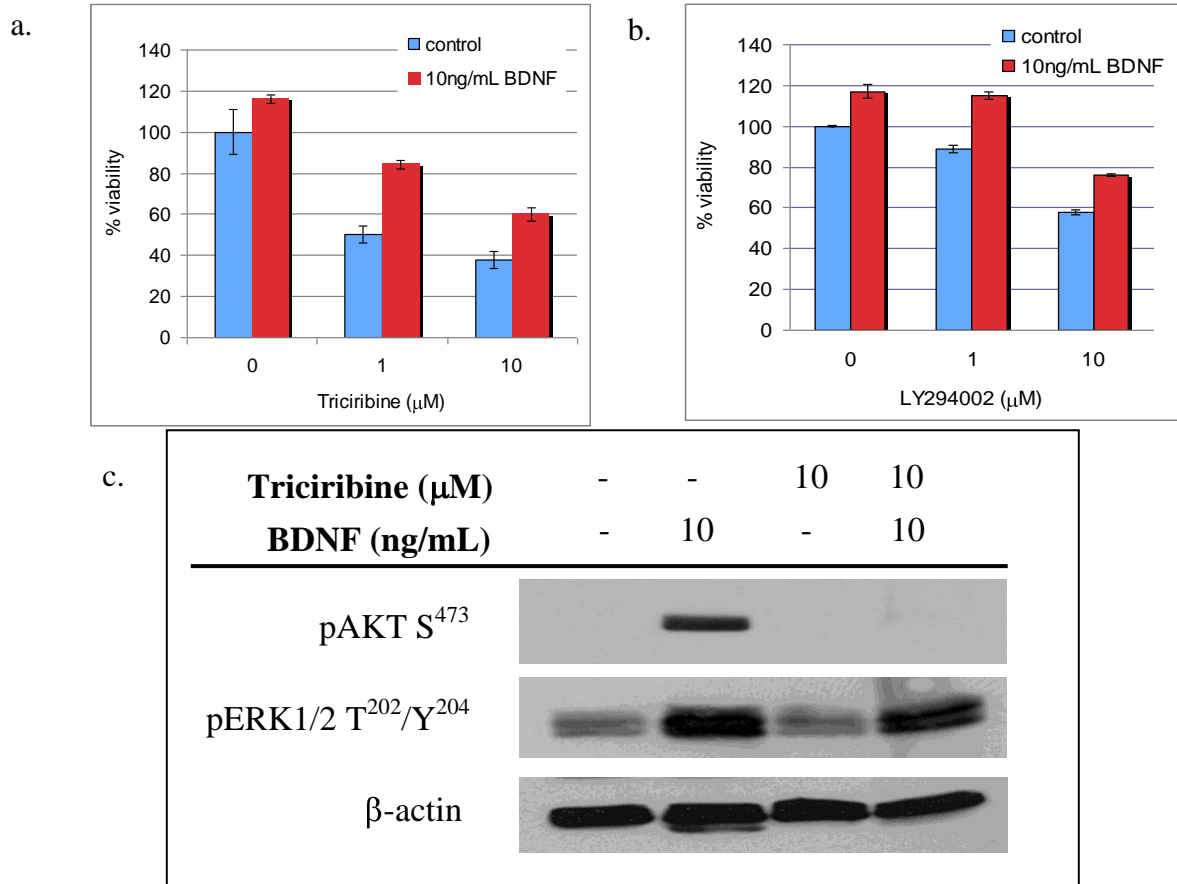


Fig 16. TrkB mediated proliferation is not regulated through a) AKT or b) PI3K signaling. c)

The efficiency of the AKT inhibitor, Triciribine, was measure by western blot

Another pathway that has been extensively explored downstream of TrkB is the MAPK pathway, which has also been shown to be important to the development of neurons. We first wanted to test whether targets of the MAPK pathway could be stimulated using exogenous BDNF.

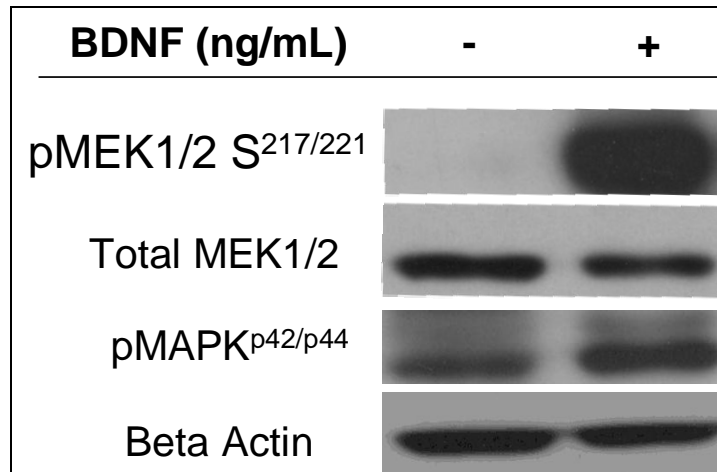


Fig 17. Exogenous BDNF stimulates MAPK pathway in SMS-KCN neuroblastoma cells

By stimulation with 100ng/mL of BDNF for 10 minutes, we showed that we could stimulate the pathway as seen by the increase in activation of pMEK (Ser_{217/221}) and pMAPK

(Thr₂₀₂/Tyr₂₀₄) compared to basal levels using Beta Actin as a loading control (Fig. 17).

From here, we then wanted to see if this could be the major regulator in BDNF mediated

proliferation. However, using the common MEK inhibitor, MEK inhibitor I (0 - 10 μ M) +/-

10ng/mL of BDNF over a 3 day incubation period, BDNF-mediated proliferation was not

prevented, shown by an Alamar blue proliferation assay (Fig 18).

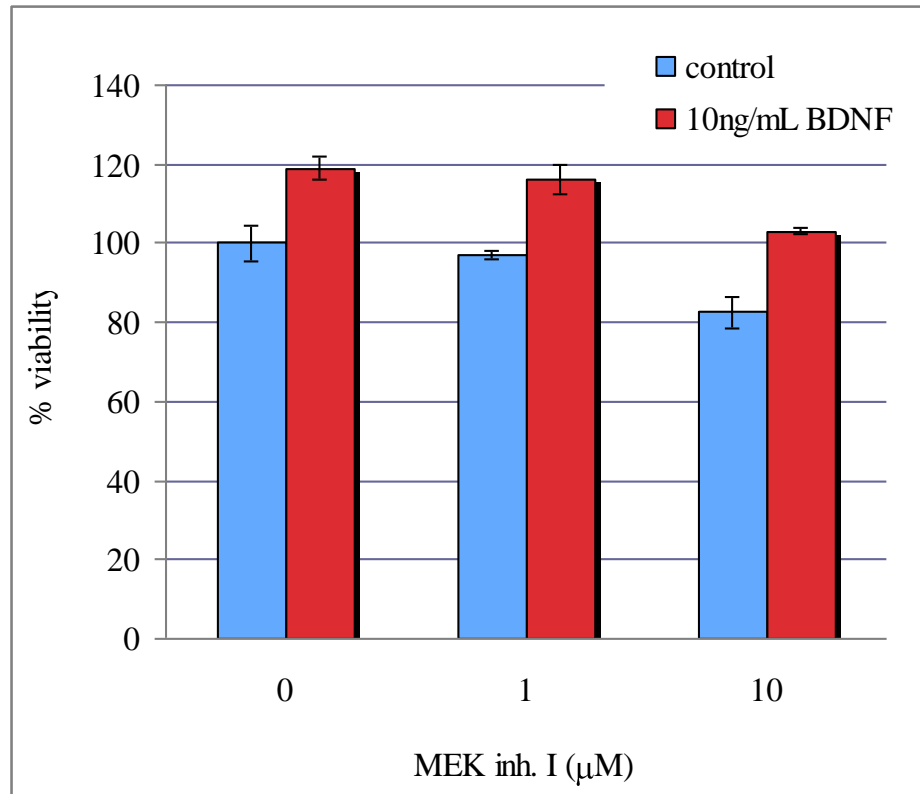


Fig 18. MAPK pathway inhibition through MEK doesn't prevent BDNF mediated proliferation

Although PLC γ has been implicated as important for mainly cell adhesion and migration of neurons, we wanted to test whether it could play a role in BDNF mediated proliferation in neuroblastoma (68). Using the PLC γ inhibitor, U73112, we analyzed BDNF mediated proliferation using +/- 10ng/mL of BDNF in an Alamar blue proliferation assay. We found similar results to the AKT, PI3K, and MEK1 inhibitors, namely that BDNF-mediated proliferation was not inhibited. This suggests that on its own, PLC γ is not important in BDNF mediated proliferation (Fig 19).

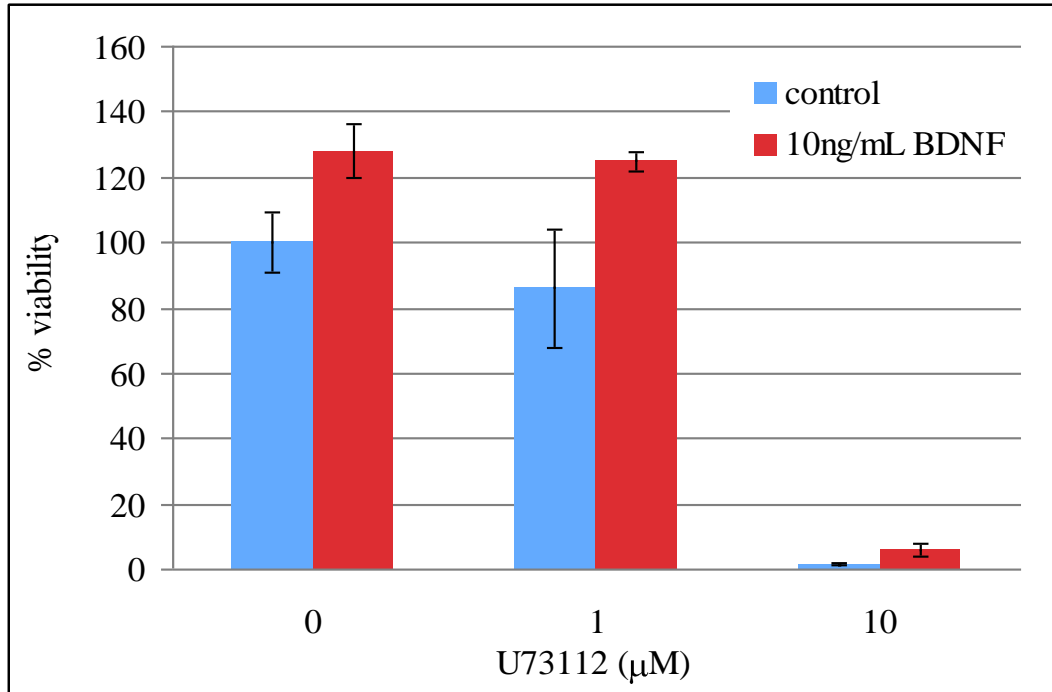


Fig 19. PLC γ inhibition doesn't prevent BDNF mediated proliferation

Summary

Our results suggest that after analyzing the common pathways for TrkB mediated signaling (AKT, MAPK, and PLC γ), none of the pathways were individually responsible for BDNF mediated proliferation. This was seen by the inability of each kinase inhibitor for the three common proximal signaling pathways to prevent BDNF induced increase of proliferation (10ng/mL of BDNF), as analyzed by an Alamar blue proliferation assay. Therefore, we examined other potential single pathways of TrkB that haven't been fully elucidated in neuroblastoma, starting with the Src family kinases.

Chapter 3

Fyn regulates TrkB mediated proliferation

RATIONALE

The Src family of kinases is a family of non-receptor tyrosine kinases that have been implicated in various phenotypes that promote carcinogenesis. Interestingly, the Src family member, Fyn, has been shown to be an important pro-differentiation prognostic marker in neuroblastoma: up-regulated in lower stages and down-regulated in high stages of the disease. However, the mechanism to explain this Fyn controlled phenotype is unknown. Previous research has linked Fyn activation with TrkB in neuronal cells, such as in the process of enhanced learning and memory during chronic multiple stress (69). However, the role that Fyn might play in TrkB signaling to induce neuroblastoma proliferation still remains unknown.

To determine the importance of Fyn to neuroblastoma cells, we acquired lentiviral GFP constructs for constitutively active Fyn, over-expressed wild type Fyn, and dominant negative Fyn, generously provided by Dr. Joya Chandra (MD Anderson Cancer Center). She had previously used these constructs to analyze how oxidative stress promotes the up-regulation of Fyn in chronic myelogenous leukemia cells (70). By analyzing a variety of methods to transfect the Fyn constructs, we ultimately found that from a panel of neuroblastoma cell lines, transient transfection of NGP cells by the lipid based reagent produced the highest transfection efficiency. The transfection efficiency was measured by GFP expression by flow cytometry. SMS-KCN cells would have been the optimal cell line to continue working with, however the cells were not permissive in accepting the constructs. However, the transfection efficiency would not be suitable enough to analyze by methods such as RT-PCR or western blotting. We needed a strategy to look at Fyn expression in the few cells that were transfected, amidst a large population of non-transfected cells. To

address this issue, we established an assay called phospho-flow using flow cytometry, which would analyze markers on a single cell basis. We analyzed the subgroup of cells have been previously transfected with GFP tagged Fyn constructs by probing with the primary antibodies for phospho-Src family (Tyr₄₁₆) and phospho-Fyn (Tyr₅₃₀). Due to similar structural motifs of their activation loops, the Src family antibody detects the endogenous phosphorylation at Tyr₄₁₆ of all the family members to show an upregulation of enzyme activity. The phospho-Fyn specific antibody recognizes endogenous levels of Fyn when phosphorylated at the Tyr₅₃₀ site. The expression of activated Fyn as well as a general activation of the Src family members was measured in relation to GFP produced by the transfected constructs. After the transfection, we waited for 48 hours to achieve maximal expression, then fixed the cells and permeabilized them using a Cytotfix/Cytoperm kit. The primary antibodies for phospho-SFK and phospho-Fyn were used, followed by a PE-conjugated secondary antibody. A secondary antibody only negative control was used for each sample to give a non-specific binding fluorescence level. We compared the activation of Fyn and general Src family members by creating a ratio between the mean fluorescence intensity of PE signal in the GFP⁺ subpopulation compared to the GFP⁻ population (Fig 20).

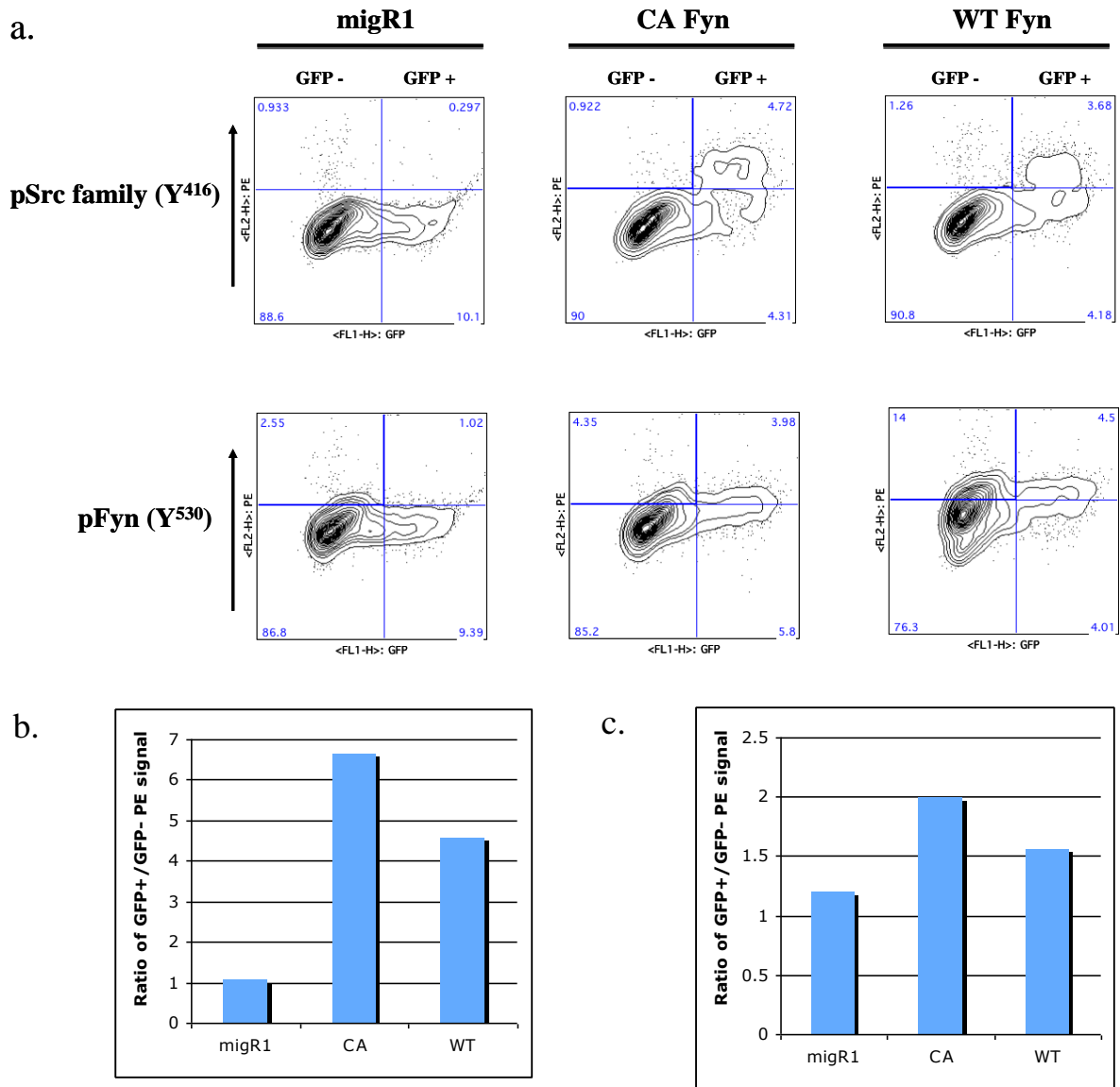


Fig 20. a) Both constitutively active (CA) and over expressed wild type (WT) Fyn constructs promoted an increase in PE signal for pSrc and pFyn expression, analyzed by flow cytometry. The mean fluorescence intensity for b) pSrc and c) pFyn was analyzed relative to a migR1 control.

Compared to a migR1 GFP expression control, we found that the constitutively active construct produced ~7 fold higher activation of Src family members and ~2 fold higher activation of Fyn. Surprisingly, the over-expressed wild type construct produced a similar result, ~5 fold higher in phospho-Src family and ~1.5 fold higher in phospho-Fyn expression than compared to migR1 control. This trend shows that when the cell is over-expressed with the wild type Fyn construct, the expression of activated Fyn is nearly as high as that by the constitutively active construct, suggesting that Fyn becomes readily activated whenever available.

To test whether Fyn is important to neuroblastoma proliferation, we probed the transfected cells for PCNA, a well established marker of proliferation. Proliferating cell nuclear antigen (PCNA) is a member of the DNA sliding clamp family of proteins that assist in DNA replication. Again using intracellular flow cytometry, we stained cells transfected with the Fyn constructs using an antibody to PCNA and a secondary antibody conjugated to PE. We found that both over expressed wild type and constitutively active Fyn inhibited proliferation as seen by the decrease of PCNA expression; the constitutively active construct producing a 70% decrease and the wild type a 20% decrease (Fig. 21). This suggests that high expression of activated Fyn plays an inhibitory role in neuroblastoma proliferation.

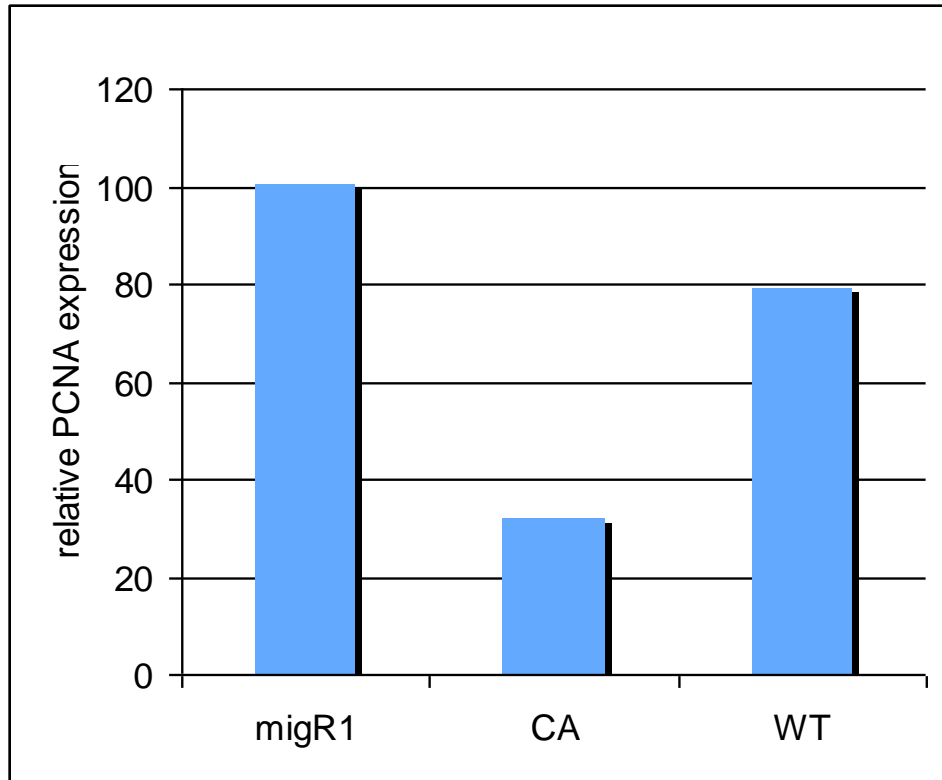


Fig 21. PCNA expression decreases relative to GFP+ expression from constitutively active (CA) and over expressed wild type (WT) Fyn constructs compared to a migR1 control.

To determine the role of wildtype and constitutively active Fyn on neuroblastoma proliferation and survival, we began by transfecting NGP cells with the Fyn constructs. Cells were then collected on days 1 and 4 and analyzed by flow cytometry for GFP expression. Results were normalized to the transfection efficiency of Day 1 (Fig. 22). We show that migR1 expression increased from day 1 to day 4, showing the time for maximal GFP expression following transfection. However, a rapid decrease of GFP expression was seen in cells with wild type and constitutively active Fyn on day 4, suggesting that the Fyn transfected cells did not proliferate as quickly compared to the migR1 control. This finding

correlates with previous studies suggesting that high expression and activity of Fyn causes a growth inhibitory phenotype in neuroblastoma.

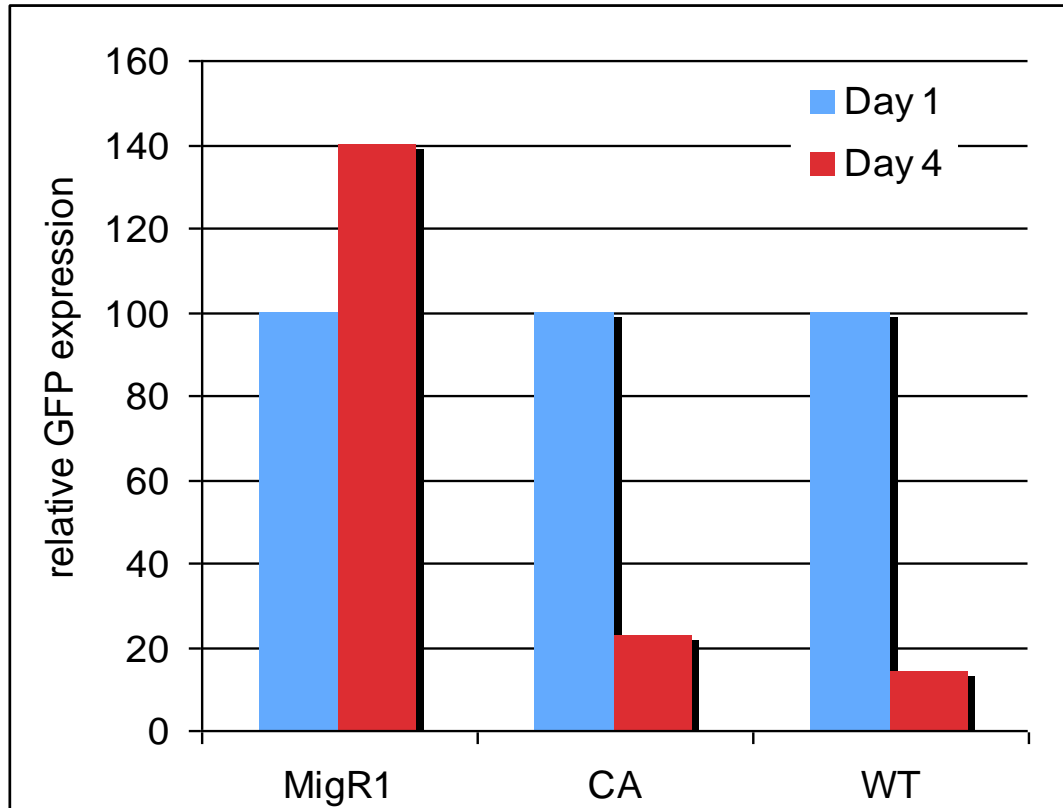


Fig 22. GFP expression of both Fyn constructs decreases faster over time compared to a migR1 control

We have shown that Fyn is important to neuroblastoma cell proliferation, however whether this phenotype has a connection with TrkB is still unknown. Previous research has shown a potential role for the interaction between Fyn and TrkB as a regulator of hippocampal NMDA receptor activation as a way to enhance spatial learning (71). However, the interaction between the two proteins has never been studied in regards to neuroblastoma proliferation. As an initial test, we used the general Src family kinase

inhibitor, PP2, to test whether the inhibition of Src family kinases would promote the proliferation of SMS-KCN cells. Cells were grown in a 96-well plate and treated with +/- 20ng/mL of BDNF and/or PP2 for 72 hours in an Alamar blue proliferation assay (Fig. 23).

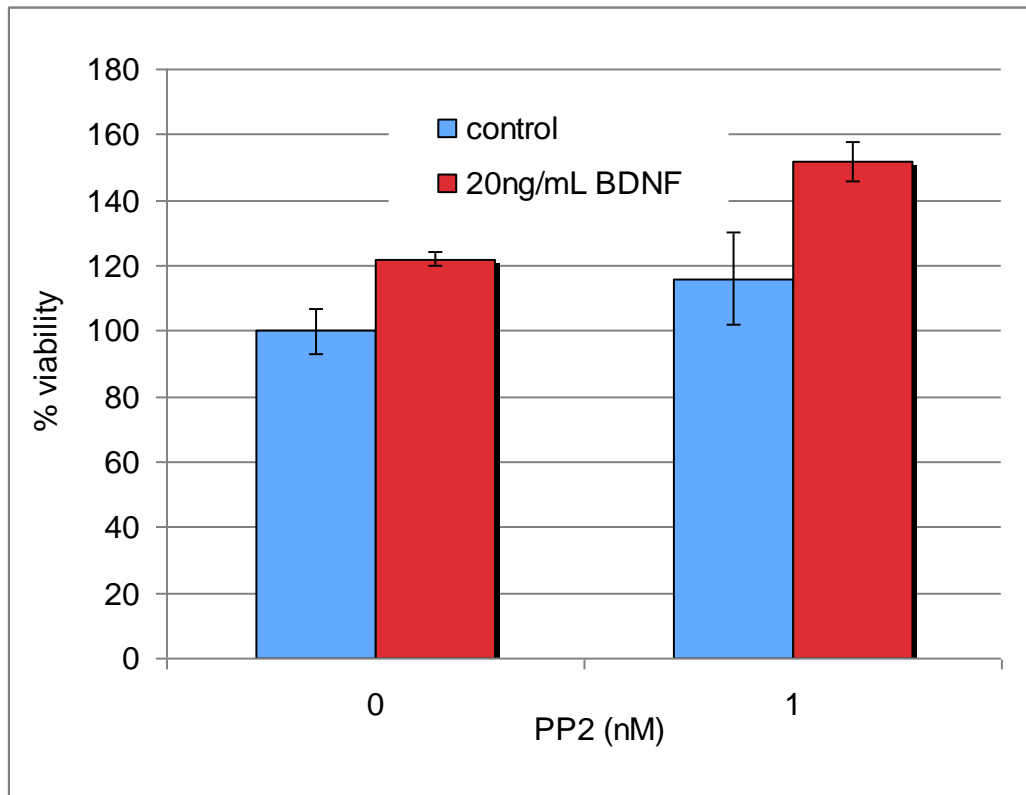


Fig 23. Inhibition of Src family kinases cooperates with TrkB activation to promote neuroblastoma proliferation shown by an Alamar blue proliferation assay

As a control test, we show that BDNF (20ng/mL) induced a 20% increased in proliferation of SMS-KCN cells, seen previously in our past results. With the use of 1nM of PP2, our results show that the Src family inhibitor increased the proliferation of SMS-KCN cells to a similar level of BDNF stimulation (~16% increase). To test whether Src family members played a role in BDNF-induced proliferation, a combination of PP2 to BDNF

treatment of the cells led to a 30% increase in proliferation compared to BDNF induced cells with no PP2. This result suggests that the combination of TrkB activation with Src family inhibition promoted increased proliferation. These trends were confirmed by cell counts; 1nM of PP2 +/- 10ng/mL of BDNF in SMS-KCN cells (Fig. 24). These data suggest that inhibiting Src family kinases cooperates with TrkB activation as a mechanism to promote neuroblastoma proliferation.

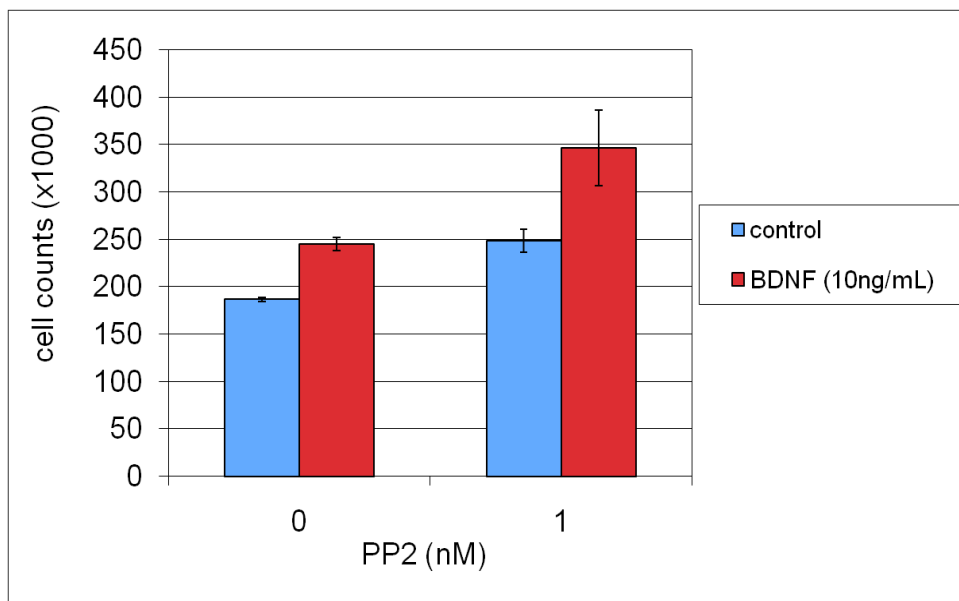


Fig 24. Inhibition of Src family kinases (1nM) cooperates with TrkB activation to promote neuroblastoma proliferation shown by cell counts

To determine if Fyn is the member of the Src family kinases responsible for cooperating with TrkB to induce proliferation, a shRNA to Fyn was used to directly inhibit activity by silencing its gene expression. NGP cells were transiently transfected with the Fyn GFP tagged shRNA, an empty GFP tagged vector control (negative), and a GFP tagged scrambled control using Lipofectamine 2000. Phospho-flow was conducted in order to

validate that the shRNA could knock down Fyn activation by probing with a p-Fyn (Tyr₅₃₀) antibody and a PE conjugated secondary antibody. The mean fluorescent intensity of Fyn-PE expression was taken for GFP⁺ and GFP⁻ groups for each vector. The background fluorescence was determined by the secondary only control, and pFyn results were calculated relative to its expression. We show that for the scrambled and vector (negative) controls, the mean fluorescent intensity for PE produced nearly the same value between GFP⁺ and GFP⁻ cells. However, the Fyn shRNA decreased the expression of p-Fyn by 15% in GFP⁺ cells compared to GFP⁻ cells, suggesting this shRNA works properly (Table 2).

	<u>mean PE of GFP⁺</u>	<u>mean PE of GFP⁻</u>	
negative control	28.2	23.3	
negative secondary only	44.1	44.6	
negative pFyn	176	176	
scrambled control	27.6	23.3	
scrambled secondary only	34.2	32.3	
scrambled pFyn	225	224	
shRNA control	28.2	23.9	
shRNA secondary only	38.6	39.7	
shRNA pFyn	179	206	→ 15% decrease in pFyn expression

Table 2. Validation of Fyn shRNA by phospho-flow shown by the 15% decrease of the mean fluorescence intensity of pFyn in the shRNA GFP⁺ subset of cells compared to the GFP⁻ cells. This system was controlled for by an empty vector (negative) and scrambled shRNA vector.

Next we wanted to analyze whether Fyn plays an important role in TrkB mediated proliferation. NGP cells were transfected with the Fyn shRNA or a scrambled control at a similarly high efficiency (Fig. 25a), as shown by GFP signal relative to the size of the cells, dictated as side scatter (SSC). These cells were then directly subjected to an Alamar blue proliferation assay with a range of BDNF concentrations (0 – 10ng/mL) for 72 hours (Fig 25b). At each BDNF concentration, the Fyn shRNA reading was calculated relative to a corresponding scrambled control. The results show that by silencing Fyn gene expression by the shRNA, BDNF mediated TrkB activation further induced the proliferation of the cells in a dose-dependent manner. This result confirms previous reports that TrkB and Fyn cooperate together, and also suggests a potential mechanism behind TrkB proliferation in neuroblastoma. We believe that upon activation of TrkB by BDNF, Fyn becomes recruited and forms a complex through its SH2 domain to the activated receptor. The BDNF mediated complex then goes on to promote the proliferation of the cells.

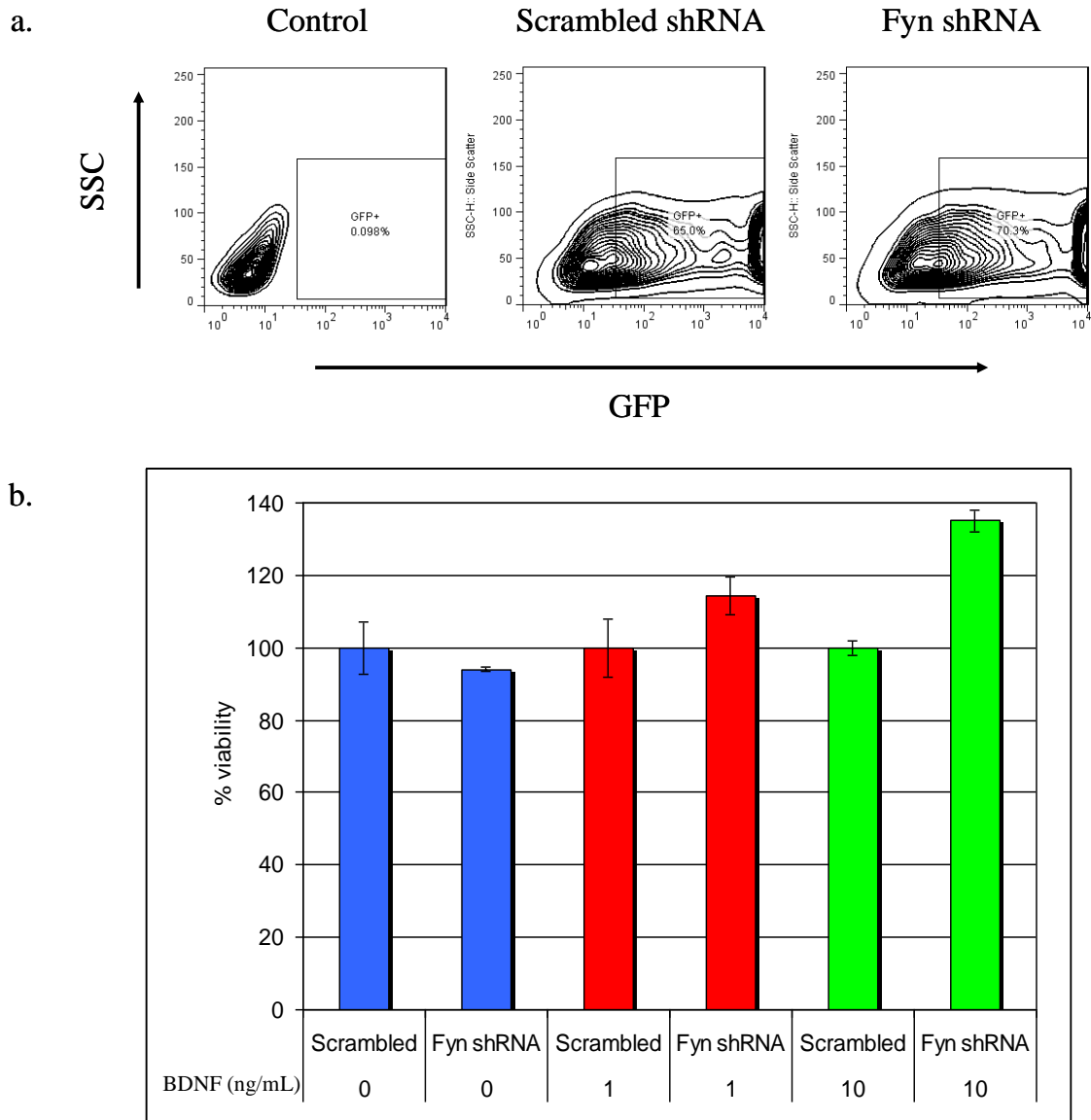


Fig 25. a) Transfection efficiency between the Fyn shRNA and scrambled shRNA are similarly high in NGP cells b) Fyn cooperates with TrkB to induce proliferation, shown by a BDNF dose response effect

Summary

Previous research has identified several common signaling pathways that may explain the roles that TrkB receptors play in various systems; from chemo-resistance and survival to neuronal development. In addition, here we show that the promotion of Fyn expression and activity promotes the differentiation of neuroblastoma cells. Constitutively active and wildtype Fyn expression inhibited proliferation of the cells seen by the reduced expression of PCNA. Conversely, the inhibition of the Src family members by PP2 treatment along with BDNF stimulation enhanced proliferation of neuroblastoma cells. This effect was found to be a Fyn specific event, shown by a shRNA knockdown of Fyn expression cooperating with BDNF stimulation to promote proliferation.

Chapter 4

BDNF mediated cell cycle regulation

Rationale

Although TrkB activation has been implicated in the proliferation of neuroblastoma cells, little is known about its effects on cell cycle regulation. We wanted to identify important downstream cell cycle regulators to further characterize the mechanism behind the phenotype. To decipher the progression of which specific targets are activated upon TrkB activation, we conducted a 27 hour time course study of BDNF stimulation in SMS-KCN cells.

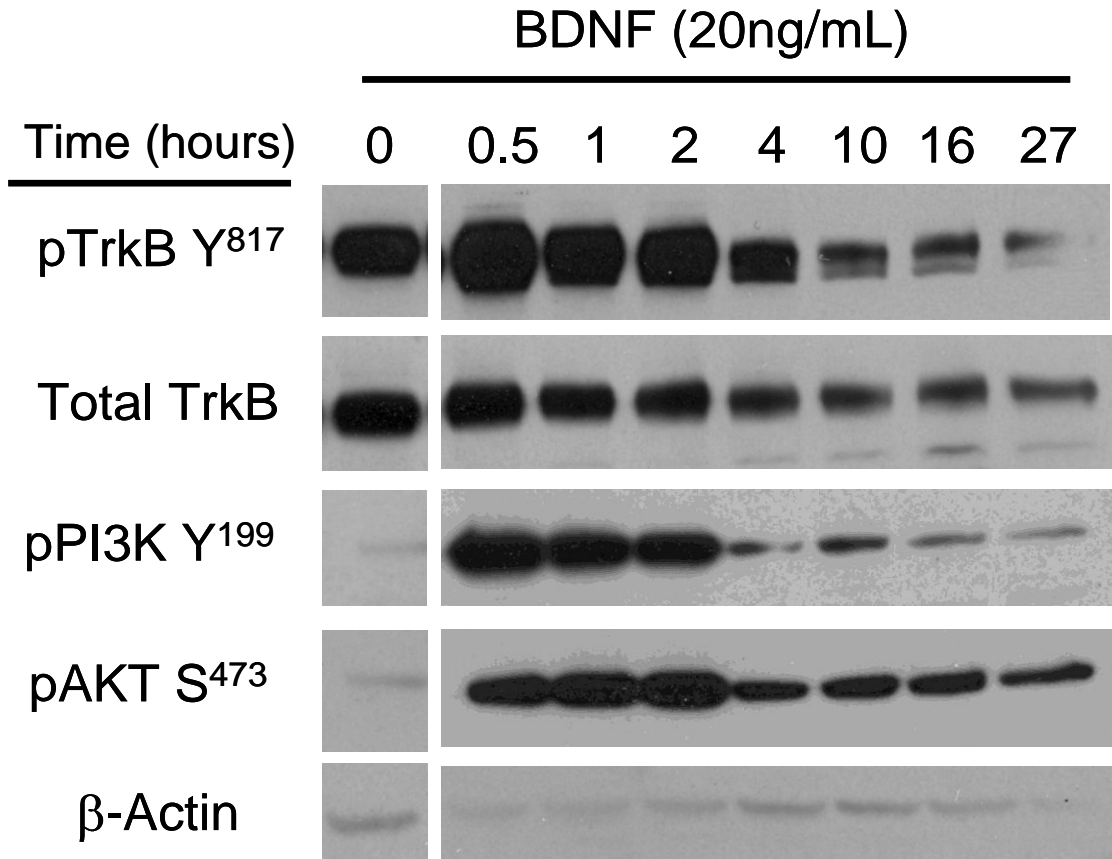


Fig. 26. BDNF stimulation maximizes the activation of TrkB and its downstream targets

PI3K and AKT until 4 hours.

Exogenous BDNF stimulation can be seen by the initial phosphorylation of TrkB. Protein expression of pTrkB was shown to be increased over baseline until 4 hours where its expression decreases to under baseline (Fig. 26). We believe that when there is no exogenous BDNF in the system, there exists a controlled feedback loop between TrkB activation through its autocrine BDNF loop and receptor turnover rate. This accounts for the continuous high expression of pTrkB seen in non-stimulated SMS-KCN cell. However, when exogenous BDNF is added, the increase in TrkB activation disrupts this loop by activating more TrkB receptors at once. This in turn causes a greater number of receptors to be recycled, limiting the activation over a longer period of time. Potentially this could be studied by fluorescently tagging TrkB receptors and stably transfecting them into a cell line that has a minimal level of TrkB, such as the SY5Y cell line. The fluorescent tag can then be tracked after the initial BDNF stimulation, comparing cell surface expression to intracellular expression. In addition to the TrkB activation length, we also expected to see a similar trend with PI3K (Y₁₉₉) and AKT (Ser₄₇₃), since they are just downstream of TrkB. The results showed that their activation was also maximized until 4 hours, but their activation persisted significantly above the 0 time point baseline. This suggests that even after the initial BDNF stimulation of TrkB, the signal persists through various pathways that can lead to beneficial phenotypes such as cell survival and chemoresistance.

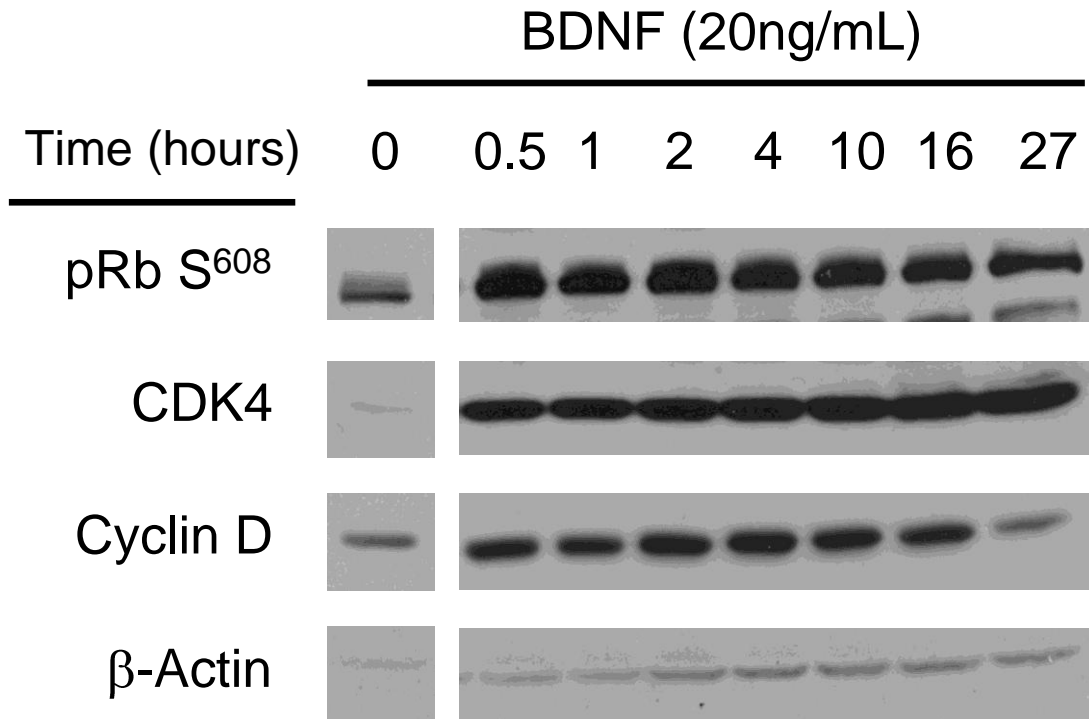


Fig 27. BDNF stimulation initially activates Rb and its upstream target CDK4/cyclin D complex during the cell cycle process

After confirming BDNF induction promotes the activation of TrkB targets as an internal control, we wanted to establish the effect on the retinoblastoma tumor suppressor protein, (Rb). Rb has been elucidated as an important regulator of neuronal differentiation by the decrease the phosphorylation level of Rb as well as cyclin-dependent kinase (CDK) activity (72). Conversely, the proliferation of cells would promote the opposite trend: increasing the phosphorylation of Rb and formation of CDK complexes. Since BDNF has shown to promote proliferation of neuroblastoma cells, we wanted to analyze the status of these cell cycle regulators. Upon phosphorylation, Rb becomes inactive and subsequently detaches from E2F to promote a G1/S-phase gene expression that dictates growth and division of the cell. A number of proliferative signals such as PCNA are then activated by

E2F-mediated transcription. Our results show that Rb (Ser₆₀₈) responds to BDNF induction quickly by 0.5 hours and persists throughout the time course experiment, confirming that Rb is important to BDNF-mediated proliferation in neuroblastoma cells (Fig. 27). A potential pathway that regulates Rb is the cyclin dependent kinase 4 (CDK4)/ cyclin D pathway, which affects the cell's entry into S-phase of the cell cycle. Our results show that the early increase in expression of CDK4 and cyclin D correlate with Rb activation, suggesting a potential mechanism for early entry into the cell cycle.

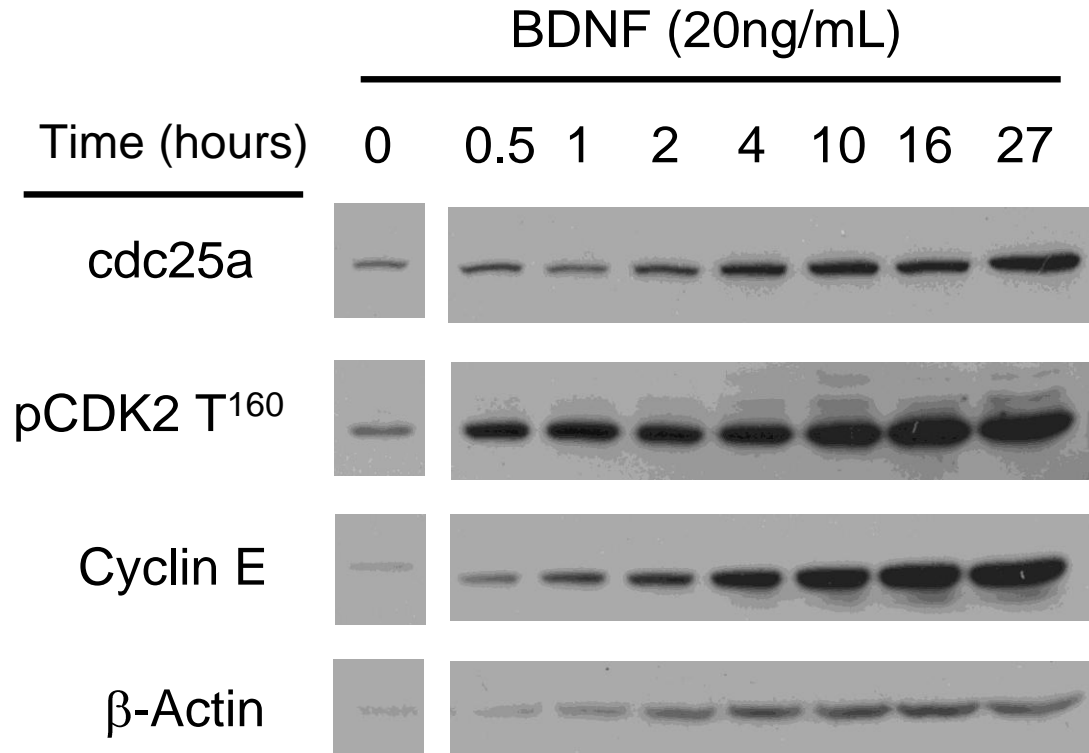


Fig 28. BDNF stimulation upregulates cdc25a and cyclin E and activates CDK2 after 4 hours.

Another direct regulator of Rb is the CDK2/cyclin E complex, shown to be important for the G1 to S transition of the cell cycle. During S-phase, the active CDK2/cyclin E

complex continues the phosphorylation of Rb once the CDK4/cylin D complex has dissociated, in order to further promote proliferation. *cdc25A* is known to promote the complex formation of CDK2/cyclin E by removing the inhibitory phosphates on CDK2 (Thr₁₄ and Tyr₁₅), which then leads to the phosphorylation at Thr₁₆₀ by a cyclin activated kinase (CAK). Our results show that the activation of *cdc25A* correlates with the expression level of cyclin E by an increase only after 4 hours (Fig. 28). Even though CDK2 is activated (Thr₁₆₀) early in the cell cycle when compared to basal levels, complex formation most likely doesn't take place till after 4 hours. This is suggested by the increase of pCDK2 expression at 10 hours and onward, which mirrors cyclin E expression at that point.

Summary

These results suggest that BDNF promotes the proliferation of neuroblastoma cells through the activation of both the CDK4/cyclin D and CDK2/cyclin E complexes. However, these complexes are shown to be important for the dissociation of Rb from E2F to promote proliferation through BDNF induction. CDK4/cyclin D complex prepares the cell to enter S phase by partially inactivating pRb in early G1 phase. Then the CDK2/cyclin E complex promotes the entry into S phase by completely inhibiting the binding of pRb to E2F, allowing for uninhibited E2F mediated proliferation (Fig. 29). Cell cycle deregulation plays a major role in the aggressiveness of a tumor, due to unrestrained proliferation. A variety of reasons may cause this such as a mutation in Rb that keeps it inactivated or an upregulation of Rb phosphorylation due to ligand stimulation. In the case for SMS-KCN cells, inhibiting the phosphorylation of Rb would serve to inhibit the growth and proliferation of the cells, since no known mutations of Rb exist for neuroblastoma. The CDK4/6 small molecule inhibitor, PD-0332991, has shown to induce a G1 arrest in primary

bone marrow myeloma cells. The inhibitor blocks CDK4, which prepares the cell to transition to S phase, as well as CDK6, which is important for the progression into S phase (73). We believe that if this inhibitor were used in neuroblastoma cells, proliferation of the cells could be inhibited by essentially preventing BDNF mediated phosphorylation of Rb.

1. CDK4/cyclinD complex prepares cell to go into S phase

2. cdc25A releases inhibitory phosphates from CDK2 to allow CDK2/cyclin E complex formation and promotion of cell to S phase

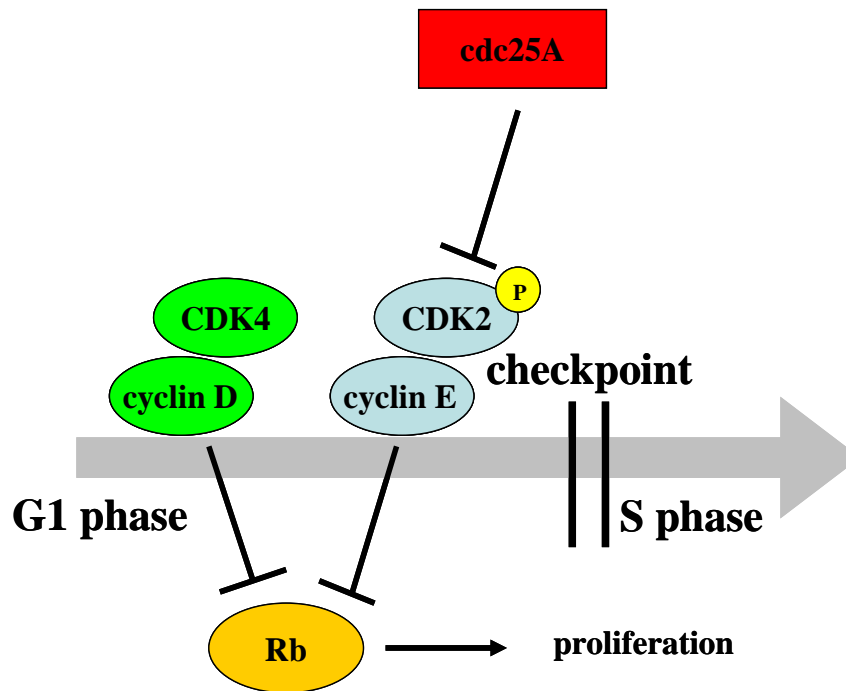


Fig 29. Summary of results for BDNF mediated proliferation through cell cycle regulation in neuroblastoma

Chapter 5
Discussion

Characterizing TrkB mediated proliferation in neuroblastoma

We characterize the role TrkB plays in neuroblastoma in a non-over expressed setting, which has been difficult to do in the past. We used the SMS-KCN cell line as a model for neuroblastoma. The high expression of TrkB and high sensitivity to BDNF allowed low BDNF concentrations to be used, thus minimizing non-specific binding. Previous findings confirmed by our results showed a minimal expression of the low affinity neurotrophic receptor, p75NTR (74). In addition, it was also previously shown that TrkA doesn't signal downstream in SMS-KCN cells, suggesting that BDNF stimulation would provide a clear, distinct signaling pathway (17). These characteristics combined make this cell line an excellent candidate to study TrkB signaling.

All of the neuroblastoma cells lines tested responded to BDNF stimulation with a mean proliferative increase of 43%, suggesting that TrkB plays a critical role in proliferation. However, SMS-KCN cells were shown to be more sensitive to BDNF stimulation as well as Trk inhibition by AZ23, suggesting a greater dependence on TrkB than other cells.

IMR32 cells were used in order to show that TrkB plays an important role in proliferation as analyzed by its effect on cell cycle regulation. A propidium iodide based flow assay demonstrated that inhibition of TrkB function promoted a G2/M arrest in the cells as well as an increase in the sub-G0 population. This result further suggests the importance of TrkB signaling at baseline by the presence of endogenous BDNF.

We then studied the role that TrkB played in suspension cells. A recent study identified TrkB expression as a direct suppressor of anoikis mediated apoptosis in non-malignant epithelial cells (25, 55). Using the high expression TrkB cell line, SMS-KCN, we tested to see whether this effect could be seen in neuroblastoma. Since an Alamar blue

proliferation assay measures the amount of live cells within a well, we used this technique to analyze the viability of SMS-KCN cells after 72 hours in a POLY-HEMA coated plate, where the cells could not attach to the bottom compared to cells that could. In addition, we treated unattached SMS-KCN cells with 20ng/mL of BDNF to see if we could promote anoikis resistance. Based on our results, we saw that BDNF increased the proliferation of the cells in an attached setting, as seen in our previous results. For the unattached SMS-KCN cells, we saw a full rescue effect due to BDNF induction compared to the attached control. In addition, we ran a time course study to analyze the maximal effect of BDNF mediated anoikis resistance in SMS-KCN cells compared to the low TrkB expression cell line, SY5Y. The results showed a maximal rescue effect of SMS-KCN cells at 24 hours by BDNF stimulation. These results together suggest that TrkB activation plays a critical role in promoting anoikis resistance, adding to the metastatic potential of the disease.

TrkB mediated cell signaling

Several signaling pathways have been identified that are important to TrkB function. The main pathway that has been shown to control chemo-resistance and cell survival in neuroblastoma is the PI3K/AKT axis (75). However, few targets have been identified downstream of AKT that may be responsible for the aggressive phenotype associated with high levels of TrkB in neuroblastoma. Previous research has linked the mTOR pathway as a potential downstream target of TrkB in leukemias (76), but this axis has not been thoroughly looked at in neuroblastoma. Our results correlate with previous findings that BDNF induces a dose-dependent activation along AKT axis. In addition, the downstream targets of mTOR, p70S6K and the ribosomal protein S6, were shown to be BDNF dose-dependent; stimulated

with as little as 2.5ng/mL of BDNF. This result suggests a potential link between AKT and mTOR pathways due to TrkB activation. Since mTOR is seen to be regulated by many different pathways, these results point to the fact that mTOR stimulation is most likely not a direct cause of TrkB activation, but is activated by downstream TrkB targets instead. Interestingly we found that the mTOR targets could be stimulated by PI3K/PDK1 directly, suggesting an AKT/mTOR bypass pathway. To clarify the mechanism, we used potent AKT and PI3K kinase inhibitors to test whether BDNF mediated proliferation could be repressed. However the results showed that neither of these inhibitors had any significant effect in preventing BDNF induced proliferation, suggesting that the PI3K/mTOR or AKT pathways were not critical for BDNF/TrkB induced proliferation. Additionally, the other two major pathways extensively studied in neuroblastoma, MAPK and PLC γ , did not have a significant effect on BDNF mediated proliferation when inhibited pharmacologically. A possible explanation for our results is that these common parallel pathways could be redundant in controlling similar signals that would lead to BDNF mediated proliferation. Thus these pathways would need to be inhibited in combination in order to see the decrease in cell proliferation.

Fyn regulates TrkB mediated proliferation

We explored alternate pathways that may serve as the important regulator of TrkB mediated proliferation. Although Src family kinases have not been extensively researched in neuroblastoma, they have been shown to play an important role in the carcinogenesis of many other different cancers. Interestingly, the Src family kinase member, Fyn, has been implicated as a pro-differentiation marker in neuroblastoma. The Eilers lab showed that Fyn

expression and activity levels could be used to dictate the severity of the disease (44), however a signaling mechanism was never associated with the finding. We wanted to test whether Fyn could be a critical factor in BDNF mediated proliferation. Previous reports suggest that Fyn and TrkB interact with to each other upon activation in hippocampal cells to promote spatial memory formation (50, 69), but this interaction has never been characterized in neuroblastoma cells.

We first reproduce previous results found by Eilers that high Fyn expression and activation elicited a differentiation phenotype in neuroblastoma. The activity for the Fyn vectors were acquired (constitutively active and over-expressed wildtype Fyn) were confirmed by flow cytometry, probing for pFyn and pSrc expression. Our results showed that the constitutively active and over-expressed wild type constructs increase both pFyn and pSrc expression, and also lowered PCNA expression (proliferative marker). This result correlates with the idea that high expression of Fyn promotes the differentiation of the cells, which in turn decreases their proliferation. In addition, the importance of high expression of activated or wild type Fyn was analyzed by measuring the loss of GFP over time. The idea was to see whether the Fyn constructs augmented the survival/ proliferation phenotype that would be “beneficial” to the cells. If they hindered the cells from being aggressive through proliferation and survival, then they would be selected against. Transfected cells would become differentiated, not divide as quickly, and eventually be eliminated out of the population, reducing the rate at which the GFP tag can be passed down through dividing cells. Our results show that cells with a high expression and activation of Fyn were selected against compared to cells transfected with a migR1 control, which proliferated like normal.

This further suggests that Fyn is an important regulator of differentiation in neuroblastoma cells.

However, inhibiting Fyn expression and activation should promote cells to elicit a greater proliferative response. Using a general Src family kinase inhibitor, PP2, to treat SMS-KCN cells in a proliferation assay, our results show an increase in proliferation compared to a non-treated baseline upon 1nM treatment. Since a number of Src family members are being inhibited simultaneously, this result could produce a mixed effect by promoting both proliferation and differentiation. Interestingly, the addition of BDNF induced a greater proliferative effect, even above BDNF induction alone, hinting at a possible control mechanism by TrkB. Furthermore, this trend was confirmed by cell counts in addition to the Alamar blue proliferation assay.

To show that this is truly a Fyn specific event, we obtained shRNA constructs for Fyn. By phospho-flow probing for pFyn, we show that the shRNA does repress its activity, identified by the decrease in the median fluorescent intensity of PE signal in GFP+ cells compared to GFP- cells. NGP cells were then transiently transfected with the shRNA DNA plasmids and treated with varying doses of BDNF (0, 1, 10ng/mL). When no BDNF was added, no significant change was seen between the scrambled control cells and the shRNA to Fyn cells. However, once 1ng/mL of BDNF was added to the system, the Fyn shRNA cells had a 15% increase over the scrambled control with the same BDNF induction. Furthermore, when 10ng/mL of BDNF was added, a greater proliferative effect was seen; a 35% increase over the scrambled control with the same BDNF induction. The results show that the repression of Fyn activity promotes proliferation in NGP cells in a BDNF dose-

dependent mechanism. These results suggest that Fyn specifically plays a critical role in TrkB mediated proliferation.

TrkB mediated cell cycle regulation

Our results confirm those seen previously suggesting that BDNF activated TrkB plays an important role in the proliferation of neuroblastoma cells. However, little research has been done to characterize the downstream cell cycle regulators that promote this phenotype. A time course western blot experiment using 20ng/mL of BDNF stimulation was conducted to elucidate the order of activated proteins targets within the pathway. We first reproduced our results showing TrkB, PI3K, and AKT activation using BDNF stimulation as a control. In addition, the blots provided insight as to how quickly the activation of these upstream targets took place, showing an increase of expression lasting up to 4 hours. Similarly, retinoblastoma protein tumor suppressor (Rb) became inactivated early on by phosphorylation, which is an important regulator in proliferation. Our results show that a known regulator of Rb, CDK4/cyclin D complex, has an expression that is also up-regulated early on as well, suggesting a potential mechanism that prepares cells to enter S phase shortly following BDNF induction. The CDK2/cyclin E complex also becomes increasingly expressed after 4 hours, which could play a role in promoting the cells that have already been prepared by the CDK4/cyclin D complex to go into S phase.

A common regulator of CDK2/cyclin E complex formation is *cdc25a*, which is known to degrade in response to DNA damage, resulting in cell cycle arrest. In addition to being a cell cycle regulator, it has also been implicated as an oncogene. The increased expression of *cdc25A* correlating with CDK2/cyclin E suggests continual promotion of S

phase entry, shown to occur long after TrkB has been activated. This trend proposes that if the micro-environment contains even a minimal concentration of BDNF, this could promote a proliferation signal that could sustain long after the initial signal, adding to the aggressive nature of the disease.

Conclusions

Trk receptors have been implicated in various systems, playing both positive and negative roles on oncogenesis depending on the tumor type. Although TrkB expression and activation in neuroblastoma have been shown to promote cancerous phenotypes and contribute to a poor prognosis of the disease, their signaling pathways have remained a mystery. Elucidating these mechanisms would better identify relevant targets that may be used in the treatment of the disease.

BDNF mediated signaling through TrkB has shown to be a critical factor in the regulation of proliferation in neuroblastoma cells, both in attached and suspension environments. Key cell cycle proliferative regulators such as Rb were shown to be directly regulated by BDNF. The interaction between TrkB and Fyn was found to be a critical link that helps explain the mechanism behind BDNF mediated proliferation.

We demonstrate that:

1. BDNF dose dependently induces proliferation in a panel of neuroblastoma cell lines

This was demonstrated inducing a panel of cell lines with a range of BDNF concentrations to produce a proliferative response.

2. TrkB expression level was correlated with sensitivity to BDNF

This was demonstrated by correlating the much higher TrkB protein/mRNA levels of SMS-KCN cells with the ability to respond to picogram concentrations of BDNF to induce a proliferative response against a panel of other cell lines.

3. Although TrkB induces the activity of PI3K/AKT, MAPK, and PLC γ , none of these pathways individually are critical for BDNF mediated proliferation

Pathways were shown to be activated through a BDNF dose response as seen by protein expression. Specific inhibitors of each pathway did not prevent the proliferative increase that BDNF induces in SMS-KCN cells.

4. Activation of TrkB may play an important role in promoting anoikis resistance

This was demonstrated by the full rescue of anoikis mediated apoptotic SMS-KCN cells with a maximal effect at 24 hours.

5. Expression of constitutively active and over expressed wild type Fyn decreases proliferation

This was demonstrated by a decrease of PCNA expression of cells when transfected with the constitutively active or over-expressed wild type Fyn constructs. GFP expression over time of transfected cells showed that these constructs were not beneficial to the health of the cells compared to a migR1 control.

6. The inhibition of Fyn cooperates with TrkB to increase proliferation of neuroblastoma cells

This was demonstrated by the cooperation between knocking out Fyn function by shRNA while stimulating with BDNF to produce a more robust proliferative effect in SMS-KCN cells.

7. BDNF induces the periodic activation and expression of CDK4/cyclin D and CDK2/cyclin E complexes, associated with increase in pRb.

This was demonstrated by the increase of protein expression of cell cycle regulators at specific times to promote the progression into S-phase by Rb activation.

Future Directions

To further elucidate the mechanism that regulated BDNF mediated proliferation in neuroblastoma, our next step would be to see how cell cycle regulators would be affected relative to the activity and expression level of Fyn. We predict that over expressed wild type and constitutively active Fyn would promote a G1 arrest, most likely affecting the phosphorylation of Rb by a decrease in CDK4/cyclinD and/or CDK2/cyclin E complex formation. Conversely, the inhibition of Fyn would promote the dissociation of Rb from E2F, thus leading to an increase in proliferation. Due to previous studies suggesting the interaction between TrkB and Fyn (52-54, 71), we believe the addition of exogenous BDNF could activate Fyn to promote a differentiation phenotype in neuroblastoma cells. The use of common differentiation markers such as synapsin and MAP2 would be compared to retinoic acid induced differentiation, which has been extensively studied in neuroblastoma (77). TrkB could play a dual role in balancing differentiation as well as cell survival and proliferation by the activation of multiple pathways. Logically, this makes sense because TrkB shares many proximal pathways with the pro-differentiation signaling of TrkA. The crossover between the two signaling pathways is possible, which may explain why the roles of TrkA and TrkB in some systems such as melanoma (78) and prostate cancer (79) are reversed; TrkB is pro-differentiation and TrkA is pro-proliferation.

Chapter 8
Materials and Methods

Neuroblastoma cell culture

Neuroblastoma cells were grown at 37°C in 5% carbon dioxide. RPMI-1640 (Invitrogen) supplemented with 10% heat-inactivated fetal bovine serum (FBS) (USB), 2mmol/L L-glutamine, 1mmol/L nonessential amino acids, 1 mmol/L penicillin-streptomycin, 1mM sodium pyruvate, and 10mM hepes (Sigma Chemical Company).

Therapeutic Agents

BDNF (Peprotech) stock solutions of 100mg/L were prepared in water and stored at 20°C. AZ23 was provided by AstraZeneca (Macclesfield). Stock solutions were generated using dimethyl sulfoxide (Sigma Chemical Company) and stored at 20°C. Before use, a 10mM stock solution was made using phosphate-buffered saline (PBS) and 30% acetic acid.

DNA Vector Constructs

The constitutively active and over-expressed wild type Fyn constructs used in this study were generously provided by Dr. Chandra (MD Anderson). shRNA for Fyn was used in knockdown experiments (Origene).

Alamar Blue proliferation assays

Neuroblastoma cells were plated in a 96-well plate (~15,000/well) in triplicate and allowed to adhere overnight. BDNF/drug combinations were added on day 0 and incubated for 72 hours at 37°C. Then 10µL of Alamar blue dye (Invitrogen) was added to each well on day 3 were incubated at 37°C for at least 2 hours. Plates were analyzed by a Spectramax Gemini EM spectrophotometer (Molecular Devices) at 544nm and 590nm. Change in

proliferation was calculated by dividing the relative fluorescence value of the treated group from the untreated group.

Taqman Quantitative Reverse Transcriptase-Polymerase Chain Reaction

Neuroblastoma cells were grown to near-confluence on 10-cm tissue culture plates. Cells were harvested and RNA was purified using Qiagen RNeasy Mini-kits following (Qiagen) and quantified using a Nanodrop 1000 spectrophotometer (Nanodrop). Then cDNA was synthesized using the Omniscript reverse transcription kit (Qiagen). Taqman quantitative reverse transcriptase-polymerase chain reaction (RT-PCR) using specific primers for TrkA, TrkB, TrkC (NTRK1, NTRK2, and NTRK3) and p75NTR (Applied Biosystems) was performed in triplicate using a BioRad iCycler iQ real-time PCR detection system. Relative levels of each sample were normalized to glyceraldehyde 3-phosphate dehydrogenase (GAPDH).

Western Blot

Neuroblastoma cells were plated on 10-cm dishes and grown until approximately 70% confluent. The night before treatment, cells were washed with PBS and serum free media was added. For BDNF only experiments, 20 or 100ng/mL of BDNF was added to media 10 minutes before lysing. For AZ23/BDNF experiments, cells were treated with 25nM or 50nM of AZ23 for 50 minutes, followed by 0 – 100ng/mL of BDNF for an additional 10 minutes. For the BDNF/Triciribine experiment, cells were treated with 10mM of Triciribine for 50 minutes, followed by 10ng/mL of BDNF for an additional 10 minutes. The media was then aspirated and a combination of PhosphoSafe Extraction Reagent

(Novagen) with 10% protease inhibitor was used to lyse cells directly. Cells were rocked in 4 degree cold room for 30 minutes. Protein concentrations were determined using BCA assay kit (Pierce). Protein lysate (50µg) was analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to 0.22µM polyvinylidene fluoride (PVDF) membrane. Membranes were blocked with 5% milk in TBST and incubated with the indicated primary antibodies, diluted according to manufacturer's recommendations. Phosphorylated TrkB – LifeSpan Biosciences LS-C50093

Total TrkB – R&D Systems – AF397

Phosphorylated PI3K – Cell Signaling #4228S

Phosphorylated AKT – Cell Signaling #9271S

Phosphorylated BAD – Cell Signaling #9291S

Phosphorylated mTOR – Cell Signaling #2971

Phosphorylated p70S6K – Cell Signaling #9205

Phosphorylated protein S6 – Cell Signaling #2211S

Phosphorylated MEK ½ - Cell Signaling #9102

Total MEK ½ - Cell Signaling #9126

Phosphorylated ERK ½ (MAPK) - Cell Signaling #9154S

Phosphorylated Rb - Cell Signaling #2181

CDK4 - Cell Signaling #2906

Cyclin D - Cell Signaling #2922

Cdc25A – Cell Signaling #3652

Phosphorylated CDK2 – Cell Signaling #2561S

Cyclin E – Santa Cruz 247

Beta Actin – Sigma A5441

Probes were detected with appropriate horseradish peroxidase (HRP)-conjugated secondary antibodies (GE Healthcare) and developed with the ECL Western Blotting Analysis System (Amersham) and a Kodak film developer (Eastman Kodak).

Flow Cytometry – Cell surface staining

SMS-KCN and SY5Y cells were grown in 6-well dishes until about 70% confluency. The cells were then extracted, washed with PBS, and appropriately stained for. For each cell line, there was an unstained control, PE secondary only control, and total extracellular TrkB primary antibody (R&D systems, AF397) with PE secondary antibody. A relative TrkB cell surface expression was found by dividing the mean fluorescent PE intensities for the TrkB stained samples from the PE secondary stained alone.

Flow Cytometry – Cell cycle

IMR-32 cells were treated with varying doses of AZ23 (1 – 7mM) for 1 hour in a 6-well plate before being washed in PBS. Cells were then fixed in 70% ethanol overnight at -20°C. Samples were washed with PBS and then stained with 0.1 mg/ml PI in 0.6% Triton-X in PBS for at least 30 minutes in the dark. Cell cycle was then analyzed by FACSCalibur (BD Biosciences).

Flow Cytometry – GFP

A panel of neuroblastoma cells were transfected with a constitutively active or wild-type construct using either Lipofectamine 2000 (Invitrogen) or Fugene 6 (Roche)

transfection kits. Relative GFP expression was measured compared to a migR1 control by FACSCalibur (BD Biosciences).

Flow Cytometry – Intracellular Staining

NGP cells were transfected with a constitutively active or wild-type Fyn construct using Lipofectamine 2000 (Invitrogen). Maximal GFP expression was achieved after 48 hours of incubation at 37°C. Cells were then permeabilized and fixed using Cytofix/Cytoperm (BD Biosciences). In order to maintain permeabilization of the cells throughout the staining process, a mixture of 0.1% saponin (Sigma) and 1% FBS in PBS was used for washes and antibody diluents. Phosphorylated Src family kinases antibody, pSrc (Cell Signaling #2101S), phosphorylated Fyn (Novus Biologicals, NB100-92535), or PCNA (Cell Signaling #2586) were used in conjunction with a PE-conjugated secondary antibody according to the manufacturer's dilution recommendations. Relative mean fluorescence intensity for GFP vs. PE expression for each primary antibody was compared to a migR1 control, as analyzed by FACSCalibur (BD Biosciences).

Alamar blue proliferation assay – shRNA to Fyn

NGP cells were transfected with the Fyn shRNA or a scrambled control (Origene) using Lipofectamine 2000 in a 6-well plate. A portion of the cells were used to analyze GFP expression by FACSCalibur. The other portion of cells (15,000 cells/well) were plated on a 96-well plate in triplicate and stimulated with a range of BDNF concentrations (0 – 10ng/mL) for 72 hours. Then 10 μ L of Alamar blue dye was added and the plate was read in the same way as previously described.

Alamar blue proliferation assay – anoikis resistance

SMS-KCN cells were seeded into a 96-well plate (15,000 cells/well) in triplicate with wells coated with poly-2-hydroxyethyl methacrylate (POLY-HEMA) to prevent the attachment of cells to the bottom of the well or a normal bottom that allows attachment. Cells were treated +/- BDNF stimulation (20ng/mL) for 72 hours. Then 10 μ L of Alamar blue dye was added and the plate was read in the same way as previously described.

Alamar blue proliferation assay – anoikis resistance time course

SMS-KCN and SY5Y cells were seeded into a 96-well plate (15,000 cells/well) in triplicate and treated with 20ng/mL of BDNF from 18-104 hours. 10 μ L of Alamar blue dye was added and the plate was read in the same way as previously described.

Chapter 9

Appendix

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Presentations:

TrkB in Neuroblastoma: Following Pathways to Better Therapies, American Society of Pediatric Hematology/Oncology (ASPHO), 2009

Abstracts:

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