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Abstract

Previously, we reported elevated levels of the neuronspecific tropomyosin receptor kinase B (TrkB) transcript, TrkB- sarc homology containing (Shc) in the hippocampus of Alzheimer's disease (AD) brains. In this study, we determined how TrkB-Shc transcripts are increased in AD. Utilizing a TrkB minigene transiently transfected into SHSY5Y cells, we found increased exon 19 inclusion in TrkB minigene transcripts (to generate TrkB-Shc) following cellular exposure to amyloid beta 1–42 (Ab42). As this suggested altered TrkB pre-mRNA splicing in AD, we conducted an in silico screening for putative splice regulatory protein-binding sites in the intron/exon splice regulatory regions of exons 18 and 19 of the TrkB gene and then assessed their gene expression profiles using a microarray database of control/AD post mortem human hippocampal brain tissue. We found significant changes in serine/arginine protein 20 (Srp20) gene expression in AD cases and confirmed this using a second cohort of control/AD. In vitro, we found increased Srp20 mRNA levels in SHSY5Y cells treated with Ab42 fibrils. Moreover, Srp20 over-expression was found to increase exon 19 inclusion in TrkB minigene transcripts and ratio of endogenous TrkB-Shc:TrkB-TK+ mRNA expression. Conversely, Srp20 expression knockdown produced the opposite effects. Our findings suggest that dysregulation of factors regulating TrkB pre-mRNA splicing may contribute to gene expression changes that occur in AD.

Keywords

pre, mrna, splicing, generate, shc, transcripts, implications, regulates, alzheimer, disease, srp20, trkb

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Srp20 regulates TrkB pre-mRNA splicing to generate TrkB-Shc transcripts with implications for Alzheimer's disease

Running Title: Srp20 regulates TrkB-Shc mRNA production

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Keywords: exon splicing, BDNF, amyloid beta, TrkB, Alzheimer's Disease, Srp20

Abbreviations: amyloid beta 1-42 ($A\beta_{42}$), Alzheimer's Disease (AD), brain-derived neurotrophic factor (BDNF), heterogeneous nuclear ribonucleoprotein K (hnrnpK), quantitative real-time PCR (qPCR), sarc homology binding domain (Shc), serine/arginine protein 20 (Srp20), tropomyosin receptor kinase B (TrkB)

Abstract

Previously, we reported elevated levels of the neuron-specific TrkB transcript, TrkB-Shc in the hippocampus of Alzheimer's disease (AD) brains. In this study, we determined how TrkB-Shc transcripts are increased in AD. Utilizing a TrkB minigene transiently transfected into SHSY5Y cells, we found increased exon 19 inclusion in TrkB minigene transcripts (to generate TrkB-Shc) following cellular exposure to amyloid beta 1-42 ($A\beta_{42}$). As this suggested altered TrkB pre-mRNA splicing in AD, we conducted an in silico screening for putative splice regulatory protein-binding sites in the intron/exon splice regulatory regions of exons 18 and 19 of the TrkB gene and then assessed their gene expression profiles using a microarray database of control/AD postmortem human hippocampal brain tissue. We found significant changes in Srp20 gene expression in AD cases and confirmed this using a second cohort of control/AD. In vitro, we found increased Srp20 mRNA levels in SHSY5Y cells treated with $A\beta_{42}$ fibrils. Moreover, Srp20 overexpression was found to increase exon 19 inclusion in TrkB minigene transcripts and ratio of endogenous TrkB-Shc:TrkB-TK+ mRNA expression. Conversely, Srp20 expression knockdown produced the opposite effects. Our findings suggest that dysregulation of factors regulating TrkB pre-mRNA splicing may contribute to gene expression changes that occur in AD.

Introduction

Multiple lines of evidence exist suggesting that dysfunction in brain-derived neurotrophic factor (BDNF) signaling may contribute to and promote neurodegeneration in Alzheimer's disease (AD) (Phillips et al. 1991; Connor et al. 1997; Allen et al. 1999; Ferrer et al. 1999). At present, the mechanisms underlying the deficit in BDNF signaling in AD have not been defined. In this study, we focus on the principal component of the BDNF signaling pathway, the tropomyosin receptor kinase B (TrkB) receptor. TrkB is a receptor tyrosine kinase that resides in the cell plasma membrane. Binding to extracellular BDNF leads to dimerization of monomeric TrkB receptors and transphosphorylation of key tyrosine residues in the C-terminal domain that couple it to downstream signaling pathways that promote neuronal survival, growth, differentiation, and plasticity including mitogen-activated protein kinase kinase, phosphatidyl inositol 3 kinase, and phospholipase C-gamma (Biffo et al. 1995; Eide et al. 1996; Ninkina et al. 1996; Snapyan et al. 2009).

In the human brain, three major isoforms of TrkB are expressed: the full-length (TrkB-TK+) and two C-terminal truncated TrkB receptors (TrkB-TK- and TrkB-Shc) (Fig. 1). Receptor dimer combinations that involve any of the truncated TrkB receptors are capable of binding to BDNF but they cannot activate downstream signaling pathways. Thus, alterations in the expression ratio of full-length (TrkB-TK+) to truncated (TrkB-TK- and TrkB-Shc) TrkB receptors expressed (Eide et al. 1996; Arevalo and Wu 2006), such as increased TrkB-TK- and/or TrkB-Shc, will have a profound negative impact on BDNF/TrkB-TK+ signaling. Indeed, both TrkB-TK- and TrkB-Shc, have been

demonstrated to inhibit BDNF-stimulated TrkB-TK+ signaling (Eide et al. 1996; Ninkina et al. 1996; Stoilov et al. 2002; Wong and Garner 2012; Wong et al. 2012).

The three TrkB isoforms are alternative transcripts generated by alternative exon splicing of the TrkB pre-mRNA (Stoilov et al. 2002). Splicing of pre-mRNAs is mediated by the spliceosome complex in conjunction with splicing regulatory proteins (Neubauer et al. 1998) that target enhancer and silencer sequences in the introns and exons (exonic splicing enhancers (ESE) and exonic splicing silencers (ESS) (Schonrock et al. 2010)) of genes to regulate exon splicing (Cartegni et al. 2003; Zheng 2004). In mammals, the splicing regulatory proteins belong to two superfamilies, the serine/arginine (SR) proteins and the heterogeneous nuclear ribonucleoproteins (hnrnp) (Graveley 2000; Dreyfuss et al. 2002). Inclusion of specific exons within a transcript is determined by the relative levels of SR proteins and hnrnp expressed (Kamma et al. 1995; Hanamura et al. 1998). Considering the importance of BDNF-stimulated TrkB-TK+ signaling for neuronal survival and function, it is unclear why the truncated TrkB receptors are generated. The regulation of TrkB alternative transcript expression begins at the level of pre-mRNA splicing (alternative exon splicing) where each specific transcript variant is first generated. The difference between each TrkB transcript variant lies in the C-terminal domain. Through alternative splicing, the C-terminal domain of TrkB-TK+ is encoded by exons 15, 17-18, 20-24, which allows the expression of a full-length tyrosine kinase-containing receptor. The C-terminus of TrkB-TK- and TrkB-Shc is encoded by exons 15-16 and exons 15, 17-19, respectively. Both exons 16 and 19 contain a stop codon and thus lead to the translation of unique C-terminal domains lacking the tyrosine kinase domain

(Klein et al. 1990). However, TrkB-Shc retains a sarc homology-binding domain (Shc) which is encoded by exon 18 (Stoilov et al. 2002).

Recently, we characterized the expression levels of TrkB-TK+, TrkB-TK-, and TrkB-Shc in various regions of control and AD brains and found a selective elevation in TrkB-Shc transcript levels in the AD hippocampus (Wong et al. 2012). Further, using a neuronal cell-line model, we also showed that overexpression of TrkB-Shc can function as a dominant negative receptor by inhibiting TrkB-TK+ phosphorylation (similar to previous reports (Stoilov et al. 2002)) but interestingly, can selectively inhibit downstream second messenger signaling activity via ERK1/2 (Wong et al. 2012). TrkB-Shc is a brain- and neuron-specific transcript unlike TrkB-TK+ and TrkB-TK- which have demonstrated expression in glia (Ohira and Hayashi 2003; Ohira et al. 2005b; Ohira et al. 2005a). Considering that there is progressive neuron and brain volume loss in AD, it is important to establish what role TrkB-Shc plays in AD development and progression. The selective elevation in TrkB-Shc transcript levels in the AD hippocampus combined with reported alterations in exon splicing in neurodegenerative disease (Gao et al. 2007) imply that mechanisms involved in the regulation of alternative exon splicing are dysregulated in AD. At present, the mechanisms involved in the regulation of TrkB pre-mRNA splicing and alternative transcript production have not been described. In this current study, we delineate how increases in TrkB-Shc alternative transcripts occur in AD by defining the factors involved in regulating TrkB pre-mRNA splicing.

Methods

Human brain tissue

The human hippocampal and cerebella brain tissues utilized in this study comprised n=6 controls and n=6 AD cases and were supplied following approvals from the Sydney Brain Bank and New South Wales Tissue Resource Centre, Sydney, Australia. A second brain cohort was used to assess changes in gene expression in the temporal cortex. This brain cohort comprised n=8 controls and n=9 AD cases (generously provided by Professor Glenda Halliday). All brain donors were participants in ethically approved longitudinal research programs, and the data collected and brain collection procedures approved for research purposes by Institutional Human Ethics Committees. AD cases fulfilled clinical and pathological NIA-Reagan criteria, as described previously (NIA 1997; Gregory et al. 2006; Kim et al. 2010), and had Braak neuritic stages V and VI. Demographic data for the cohorts are detailed in Table 1 and Table S1. Control and AD cases were matched for age and gender where possible. The diagnostic groups did not differ according to age, brain pH, or postmortem interval (PMI) (all $p \geq 0.12$). Samples were taken from the same anatomical regions of the brain, as previously described (Kagedal et al. 2010; Kim et al. 2010; Wong et al. 2012).

Cell culture and treatments

The neuronal cell-line, SHSY5Y were obtained from the American Type Culture Collection and grown at 37°C in a 5% CO_2 atmosphere. For continuous culture, cells were cultured in RPMI media containing 10% (v/v) FBS supplemented with 2 mM

glutamax. For experiments, SHSY5Y cells were differentiated for 9 days using all-trans retinoic acid (ATRA:10 μM) which was added to the culture medium. During differentiation, the differentiation media (growth media + ATRA) was refreshed every 3 days. For transfections, differentiated SHSY5Y cells were transfected using OptiMEM medium and Lipofectamine 2000 (Life Technologies). For treatments, serum-free RPMI media supplemented with N2 was used. For $A\beta_{42}$ experiments, cells were treated for 6 h and harvested. No cell-toxicity was observed in any of the treatment conditions at the concentration employed.

Plasmid construction

The TrkB minigene plasmid was cloned by GeneArt (Life Technologies) using insert sequences provided by us: exons 18 to 20 flanked by 1kb of TrkB intronic or intervening sequences (Fig. 2A and B). pcDNA3.1-Srp20.1 was cloned using methods previously described (Wong et al. 2008). Briefly, DNA fragments encoding Srp20 were amplified from SHSY5Y cDNA using the following primer sets: Srp20.1-BamHIF: CCCAAAGGATCCATGCATCGT; Srp20.1-EcoRIR: GGGTTTGAATTCCTATTTCCTT. cDNAs were then digested with BamHI and EcoRI restriction endonuclease and ligated into the pcDNA3.1 vector using T4 DNA ligase (Fermentas). Positive clones were verified by sequencing.

In vivo exon splicing assay

Differentiated SHSY5Y cells were either: transiently transfected with the TrkB minigene for 24 h and treated with $A\beta_{42}$, co-transfected with the TrkB minigene and pcDNA3.1-

Srp20.1 for 24 h, or transfected with siSrp20 for 72 h (Fig. 2A and B). Cells were harvested for total RNA and reverse transcribed into cDNA using established/published protocols (see below) (Wong et al. 2012). TrkB splice transcripts generated from the minigene were detected by semi-quantitative PCR using exon 18 forward primers and minigene specific BGH reverse primers (pan primers), exon 18/20 forward primers and BGH reverse primers (TrkB-TK+ transcript specific primers), and exon 18/19 forward and exon 19 intronic reverse primers (TrkB-Shc transcript specific primers) (Table 2). PCR cycling conditions were: 94°C for 2 min, 40 cycles of 94°C for 30 seconds, 58°C for 10 seconds, and 72°C for 80 seconds (or 30 seconds for PCR products <500 bp); and one cycle of 72°C for 3 min. PCR products were visualized by agarose gel electrophoresis. Identities of the PCR products have been verified by sequencing. Bands were quantitated by densitometry using Image J (version 1.37v) (National Institutes of Health USA). Changes in alternative splicing was determined by calculating the ratio of TrkB-Shc:TrkB-TK+. TrkB-TK+ was used as an internal normalization control for TrkB-Shc expression levels. This was possible as both PCR products were generated using the same PCR primers.

RNA extraction, cDNA synthesis, and quantitative real-time PCR (qPCR)

Total RNA was extracted from tissues (20 mg) and cells using Trizol Reagent according to the manufacturer's instructions (Life Technologies). Concentrations of total RNA were measured using a Nanodrop 2000c Spectrophotometer (ThermoScientific). Reverse Transcriptase-PCR was performed according to the manufacturer's protocol for the SuperScript III First Strand cDNA Synthesis Kit (Life Technologies) using oligodT or

minigene-specific BGH primers. QPCR was performed using Sso Fast Evagreen Supermix (BioRad) on a Roche LightCycler 480 with a 96-well format (Roche). Primer pairs used for gene expression analyses include: TrkB-Shc, TrkB-TK+, Srp20, Srp20.1, and β-actin (Table 2). Primers were designed to span an intron/exon boundary to avoid amplification of possible genomic DNA. PCR cycling conditions were: 95°C for 1 min, 45 cycles of 95°C for 30 seconds, primer specific annealing temperature for 30 seconds (Table 2), and 72°C for 30 seconds. Control reactions with no template were included which produced no signal. Melt curve analysis and agarose gel electrophoresis were performed to confirm production of a single product. Changes in gene expression levels were determined by normalizing mRNA levels of the gene of interest to the mRNA level of the housekeeping gene, β-actin using the $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen 2001). β-Actin did not vary with treatment or between control and AD cases in any brain region examined (all $p>0.06$). Details on housekeeping gene selection have been published (Wong et al. 2012).

$A\beta_{42}$ preparation

Full details on the preparation (see Supplemental Methods) and characterization of the $A\beta_{42}$ monomers, oligomers, and fibril preparations are published (Wong et al. 2012). For experiments, 1 μM was used as the final concentration.

Western blotting

Cells were harvested for total protein using RIPA buffer supplemented with protease (Sigma) and phosphatase (Pierce) inhibitor cocktails. Protein concentrations were

determined by the bicinchoninic acid method. Twenty micrograms of cell lysates were mixed with 5 × SDS loading buffer (containing β-mercaptoethanol), boiled at 95ºC for 5 min, and separated on 10% SDS-PAGE gels. Proteins were transferred onto nitrocellulose membranes and blocked using 5% (w/v) non-fat milk, 0.1% (v/v) Tween-20 in TBS (TBST) at room temperature for 1 h. Membranes were incubated with primary antibodies: Srp20 (1:5000) (Millipore, cat# MABE116) and β-actin (1:10000) (Sigma, cat# A5060). Membranes were washed 3 x 10 min with TBST and incubated with mouse peroxidase-conjugated affinity purified secondary antibody for 1 h (Dako). After further washing, bound antibodies were incubated with enhanced chemiluminescence reagent (Millipore) and visualized by autoradiography. Bands were quantitated by densitometry using Image J (version 1.37v) (National Institutes of Health USA). The brightness/contrast of images was adjusted using Adobe Photoshop CS (version 8).

Statistical analysis

Outliers were determined and removed from subsequent analyses using the Grubb's test or if data points were greater or less than two standard deviations from the mean. One control and one AD case were removed from Srp20 qPCR analyses of the hippocampus. No other outliers were detected in controls or AD cases for Srp20 in the cerebellum and hnrnpK in the hippocampus and cerebellum. One control and one AD case were removed from Srp20 qPCR analyses of the temporal cortex. Statistical analyses were conducted using STATISTICA 7 (StatSoft Inc., 2000, STATISTICA for Windows). Tests for normality were conducted. As all data in this study was normally distributed. Two-tailed unpaired t-tests were used to assess significance in gene expression changes. Pearson's

Product Moment correlations were used to determine if any relationship existed between Srp20 and hnrnpK mRNA expression and demographic variables. A p-value less than 0.05 (two-tailed) was considered statistically significant.

Results

$A\beta_{42}$ increases TrkB-Shc alternative transcript levels by increasing exon 19 inclusion during alternative splicing

Previously, we observed a significant increase in endogenous TrkB-Shc alternative transcript levels in the hippocampus of AD brains (Wong et al. 2012). Moreover, we demonstrated that TrkB-Shc alternative transcript levels in the neuronal cell-line SHSY5Y could be increased by treatment with preparations of $A\beta_{42}$ fibrils (Wong et al. 2012). To determine if alternative exon splicing of the TrkB pre-mRNA is altered in AD, particularly by $A\beta_{42}$ treatment increasing TrkB-Shc alternative transcript production, we conducted in vivo exon splicing assays utilizing a TrkB minigene construct (Fig. 2A and B). TrkB-Shc alternative transcripts are generated from exon 19 inclusion rather than alternate splicing of exon 18 to exon 20 (for the generation of TrkB-TK+). Using differentiated SHSY5Y cells as a cell-line model for differentiated neurons, cells were transiently transfected with the TrkB minigene for 24 h and treated with $A\beta_{42}$ monomers, oligomers, and fibrils for 6 h. Figure 3A shows a representative image of the PCR products generated from the TrkB minigene using a pan primer set following alternative splicing. PCR products containing exons 18/19/20 correspond to TrkB-Shc (1250 bp) and exons 18/20 correspond to TrkB-TK+ (221 bp). From this, we measured the alternative splicing effect by quantifying the ratio of TrkB-Shc:TrkB-TK+ (Fig. 3A and B). In this manner, expression of TrkB-Shc transcripts could be internally normalized by expression of TrkB-TK+ transcripts. We observed a significant increase in the ratio of TrkB-Shc:TrkB-TK+ mRNA expression when cells were exposed to $A\beta_{42}$ fibrils (t=2.44,

df=12, p=0.03) (Fig. 3B). No significant differences in TrkB-Shc:TrkB-TK+ expression ratios were detected when cells were exposed to $A\beta_{42}$ monomers and oligomers (all p>0.11).

To validate the in vivo splicing assay, we used two different primer sets to target transcripts corresponding to TrkB-Shc (236 bp) and transcripts corresponding to TrkB-TK+ (197 bp) separately. Compared to the pan primer set, the PCR product sizes for the two transcripts are similar in length. We found that the ratio of TrkB-Shc:TrkB-TK+ calculated from these similar PCR fragment lengths confirm our results found using the pan primers. We found that $A\beta_{42}$ fibrils significantly increased the ratio of TrkB-Shc:TrkB-TK+ (t=5.33, df=12, p=0.0002) (Fig. S1). No significant differences in TrkB-Shc:TrkB-TK+ expression ratios were detected when cells were exposed to $A\beta_{42}$ monomers and oligomers (all p>0.20).

In silico screening of splice regulatory proteins predicted to bind to the TrkB pre-mRNA

At present, the mechanisms underlying TrkB alternative transcript splicing are not known as the splice regulatory factors responsible for modulating TrkB pre-mRNA splicing have yet to be identified. Using the RNA-splicing protein binding prediction algorithm of SpliceAid (http://www.introni.it/splicing.html), we conducted an in silico screen of the intron/exon splice junctions and surrounding nucleotide bases of exons 18 and 19 to predict splice regulatory protein binding sites. From this, we identified multiple putative splice regulatory proteins/binding sites that could potentially modulate TrkB pre-mRNA splicing to generate TrkB-Shc transcripts (Fig. 4). To determine which candidate splice

regulatory protein(s) identified can modulate TrkB-Shc transcript production *and* is altered in the AD hippocampus, we also screened microarray gene expression data from a control/AD hippocampal brain cohort (from Blalock et al (2004)) publicly available from the NCBI GEO database [Affymetrix GeneChip (HG-U133A); accession GDS810]. This consisted of gene expression data of the hippocampus from control and varying cases of AD severity. As our brain cohort consisted only of control and severe AD cases in Braak stages V and VI, we focused on the gene expression changes for the corresponding groups. From this, we found significant reductions in mRNA expression of two splice regulatory proteins between control and severe AD cases, the SR protein, Srp20 ($t=-3.32$, $df=12$, $p=0.006$), and heterogeneous nuclear ribonucleoprotein, hnrnpK ($t=-2.28$, $df=13$, $p=0.04$) (Fig. S2). No significant differences were observed between incipient and moderate AD cases compared to controls for both Srp20 and hnrnpK mRNA expression. Srp20 and hnrnpK mRNA expression were not significantly correlated with age (Srp20: $r=0.19$, $p=0.50$; hnrnpK: $r=0.04$, $p=0.86$). PMI and brain pH data were not available for analysis.

Srp20 expression is increased in the hippocampus of AD brains and in neuronal cell-lines treated with $A\beta_{42}$ fibrils

We next determined whether mRNA levels of Srp20 and hnrnpK are also correspondingly altered in our control/AD brain cohort. Using hippocampal and cerebella tissue from n=6 controls and n=6 AD cases, we found a significant increase in Srp20 mRNA levels in the AD hippocampus compared to controls ($t=3.44$, $df=8$, $p=0.009$) (Fig. 5A), but no significant change in the cerebellum ($t=1.83$, $df=10$, $p=0.1$) (Fig. 5B).

Interestingly, we found no significant change in hnrnpK mRNA levels between control and AD cases in either brain region (all $p>0.83$). In AD, the cerebellum is the least affected brain region with regards to AD pathological changes e.g. Aβ accumulation and neurotoxicity in AD patients and animal models. Thus, we the cerebellum was used as a control brain region. Srp20 and hnrnpK mRNA levels did not correlate with any of the cohort demographics (hippocampus: age: all $p>0.85$; pH: all $p>0.26$; PMI: all $p>0.76$; Cerebellum: age: all $p>0.63$; pH: all $p>0.1$; PMI: all $p>0.56$).

We then determined experimentally if Srp20 and hnrnpK mRNA levels could be modulated by $A\beta_{42}$ exposure. Using differentiated SHSY5Y cells treated with $A\beta_{42}$ monomers, oligomers, and fibrils, we found that Srp20 mRNA levels were significantly increased when cells were exposed to $A\beta_{42}$ fibrils ($t=3.84$, $df=15$, $p=0.002$) (Fig. 6). Exposure of cells to $A\beta_{42}$ monomers or oligomers had no significant effect on Srp20 mRNA levels (all $p>0.07$). No significant changes were observed in hnrnpK mRNA levels when cells were exposed to all three species of $A\beta_{42}$ (all $p>0.1$) (Figure not shown).

As our results suggested that Srp20 expression is elevated in AD and is affected by AD pathology e.g. $A\beta_{42}$, we next determined if changes in Srp20 expression may be specifically occurring in the AD hippocampus by assessing Srp20 mRNA levels in another brain region also affected in AD, the temporal cortex. We found a significant increase in Srp20 mRNA levels in AD cases compared to controls ($t=2.38$, $df=14$, $p=0.03$) (Fig. S3). Srp20 mRNA levels did not correlate with any of the cohort demographics (age: $r=-0.35$, $p>0.81$; PMI: $r=0.08$, $p=0.77$).

Srp20 can regulate TrkB pre-mRNA splicing to generate TrkB-Shc alternative transcripts

Spr20 overexpression

Following our identification of Srp20 as a possible splice regulatory protein that can modulate TrkB pre-mRNA splicing to favor production of TrkB-Shc transcripts, we then confirmed this by overexpression of Srp20 in differentiated SHSY5Y cells transfected with the TrkB minigene. Figure 7A shows a representative image of the PCR products generated from the TrkB minigene following alternative splicing. Qualitatively, PCR products containing exons 18/19/20 (corresponding to TrkB-Shc) were increased compared to the non-transfected condition whereas PCR products containing exons 18/20 (corresponding to TrkB-TK+) were unchanged with Srp20 overexpression. When we quantitated the ratio of TrkB-Shc:TrkB-TK+ transcript expression, we found a significant increase when Srp20 was overexpressed ($t=11.4$, $df=4$, $p=0.0003$) (Fig. 7B).

To determine if overexpression of Srp20 can increase the ratio of endogenous TrkB-Shc:TrkB-TK+ transcript expression, we transiently transfected incremental amounts of Srp20 plasmids in differentiated SHSY5Y cells to titrate the level of Srp20 expressed. With increasing amounts of Srp20 transfected, we observed corresponding increases in the mRNA expressed (ANOVA: $F=24.2$, $df=3, 8$, $p=0.0002$; Fisher LSD: control vs. 1000 ng $p=0.00006$) (Fig. 7C). When we assessed cellular Srp20 protein levels following Srp20 overexpression and at 1000 ng, we found a significant increase in protein levels (145% increase; $t=4.15$, $df=6$, $p=0.006$) (Fig. 7D). When we calculated the ratio of endogenous TrkB-Shc:TrkB-TK+ mRNA expression, we found significant increases in expression levels corresponding to increasing Srp20 expression (ANOVA:

F=5.59, df=3, 8, p=0.02; Fisher LSD: control vs. 250 ng p=0.03; control vs. 500 ng p=0.03; control vs. 1000 ng p=0.004) (Fig. 7E).

Srp20 knockdown

Having observed an increase in TrkB-Shc alternative transcript generation when Srp20 is overexpressed, we then conducted the reciprocal experiments by knocking down Srp20 protein levels. Differentiated SHSY5Y cells were transiently transfected with siRNA targeted to Srp20 and knockdown was conducted for 72 h. We obtained significant knockdown in Srp20 protein levels after 72 h with no significant effect on β-actin protein levels (34% reduction; t=-3.31, df=12, p=0.006) (Fig. 8A). When we assessed the effect of Srp20 knockdown on TrkB pre-mRNA splicing using the TrkB minigene, qualitatively, we found that PCR products containing exons 18/19/20 (corresponding to TrkB-Shc) were decreased compared to the non-transfected condition whereas PCR products containing exons 18/20 (corresponding to TrkB-TK+) were unchanged with Srp20 kncokdown (Fig. 8B). When we quantitated the ratio of TrkB-Shc:TrkB-TK+ transcripts expressed from the minigene, we found a significant decrease when Srp20 was knocked-down (t=-2.50, df=14, p=0.03) (Fig. 8C). Similarly, when we measured the ratio of TrkB-Shc:TrkB-TK+ endogenous mRNA expression, we found a significant decrease in expression levels with Srp20 knockdown compared to the control condition (t=-4.37, df=4, p=0.01) (Fig. 8D).

Discussion

Previously, we found a selective up-regulation in the neuron-specific TrkB alternative splice transcript, TrkB-Shc, in the hippocampus of AD brains and in a neuronal cell-line (SHSY5Y) treated with $A\beta_{42}$ fibrils (Wong et al. 2012). As no significant changes were detected for the TrkB-TK+ and TrkB-TK- transcripts in the AD hippocampus, our findings suggested that processes governing alternative exon splicing of the TrkB pre-mRNA may be dysregulated in AD (Wong et al. 2012). Alterations in the splicing elements and/or splice regulatory proteins can lead to aberrant splicing (Faustino and Cooper 2003; Tazi et al. 2009). To test this hypothesis, we conducted an in vivo exon splicing assay using a TrkB minigene. In comparison to measuring endogenous transcript changes, the exon splicing assay and TrkB minigene has the advantage of allowing selective assessment of pre-mRNA processing and minimizing confounding effects such as miRNA-mediated regulation of mRNA. We found significant increases in exon 19 inclusion in transcripts corresponding to TrkB-Shc as well as the ratio of TrkB-Shc:TrkB-TK+ mRNA expression when cells were exposed to $A\beta_{42}$ fibrils, confirming our previous findings where endogenous TrkB-Shc mRNA levels were increased when exposed to $A\beta_{42}$ fibrils (Wong et al. 2012).

At present, the mechanisms regulating TrkB alternative transcript production are not known. To establish if TrkB pre-mRNA splicing is dysregulated to favor increased TrkB-Shc alternative transcript levels in AD, we took two approaches. First, we conducted an in silico screen of the splice regulatory regions of the TrkB gene in the intron/exon junctions around exons 18 and 19 to identify candidate splice regulatory proteins capable of binding to and regulating TrkB pre-mRNA splicing. Next, we

examined gene expression levels of candidate splice regulatory proteins identified using two cohorts of control versus AD hippocampi (one from Blalock et al (2004) (NCBI GeoData Sets) (microarray) and the other being our own cohort (qPCR) (Kagedal et al. 2010; Kim et al. 2010; Wong et al. 2012) as well as in SHSY5Y cells treated with $A\beta_{42}$. From the microarray data, we identified Srp20 to be a candidate regulator of TrkB pre-mRNA splicing capable of modulating TrkB-Shc alternative transcript production and then confirmed that Srp20 expression was altered in the AD hippocampus and with $A\beta_{42}$ treatment in SHSY5Y cells. The increase in Srp20 expression levels in the hippocampus (and not cerebellum) of our control/AD cohort and with $A\beta_{42}$ treatment are consistent with our previous findings for TrkB-Shc mRNA levels (Wong et al. 2012). However, it should be noted that the directional change in Srp20 mRNA levels in our hippocampal brain cohort (increase) was opposite to that observed in the microarray dataset from Blalock et al (2004) (decrease). This discrepancy between our findings is likely due to cohort differences. In our cohort, all control cases were classed in Braak stage O (no detectable AD pathology) (Table 1), whereas in Blalock et al. (2004), the control cases utilized were classed in Braak stage II, suggesting that control cases displayed some AD pathology and plaque accumulation. From this, it may be likely that in Blalock et al.'s (2004) study, the control cases with some AD pathology already displayed increased Srp20 mRNA levels, thus raising baseline expression levels of Srp20. Conversely, the control cases utilized in our current study are likely to have lower baseline Srp20 mRNA levels. An elevation in baseline Srp20 expression likely explains the non-significant increase in Srp20 mRNA levels in the incipient cases and decrease in Srp20 expression in the severe AD cases in Blalock et al.'s (2004) study. By the same reasoning, a lower

baseline in Srp20 expression in our cohort also explains why elevated levels of Srp20 expression was observed in the AD cases as well as the small magnitude of increase. It is likely that Srp20 expression levels are increased early in AD pathology and as AD pathology progresses, cell loss may explain the reduction in Srp20 expression over the disease course and in the severe AD stage in Blalock et al.'s study. In support of this, we also found a significant elevation in Srp20 mRNA levels in the temporal cortex. In AD, the temporal cortex is affected following the hippocampus. Thus, elevations in Srp20 expression in the temporal cortex is consistent with an earlier or less severe stage of AD pathology in that brain region.

Another important point to note is that while we found a significant increase in Srp20 expression in the temporal cortex, in our previous study (Wong et al. 2012), we found no significant change in TrkB-Shc mRNA levels in this brain region. This discordance between Srp20 and TrkB-Shc mRNA expression levels in the AD temporal cortex suggests that additional regulatory processes are involved in regulating TrkB pre-mRNA splicing and TrkB-Shc alternative transcript expression which may be brain region specific.

Srp20

Previously, exon selection by Srp20 during alternative splicing was found to be influenced by the presence of suboptimal or weak exon splice acceptor and donor sites (Jumaa and Nielsen 1997). The splice acceptor site of Srp20 exon 4 contains a weak polypyrimidine tract and the last two nucleotides of exon 4 at the splice donor site do not match the AG consensus. Mutagenesis of the nucleotides in the exon 4 splice acceptor

site to generate a strong polypyrimidine tract was found to enable constitutive inclusion of exon 4 by eliminating the need for Srp20 in the pre-mRNA splicing process (Jumaa and Nielsen 1997). Moreover, the Drosophila homologue of Srp20, RBP1, has also been shown to activate a polypyrimidine tract in the 3' splice acceptor site in dsx (Heinrichs and Baker 1995). In the TrkB pre-mRNA, we found a putative splice acceptor site in exon 19 (Fig. S4), suggesting that splicing of exon 19 can be regulated by Srp20. Indeed, we found that cellular overexpression of Srp20 increased exon 19 inclusion in alternative splicing to increase the ratio of exon 18/19/20:exon 18/20 PCR products (corresponding to the ratio of TrkB-Shc:TrkB-TK+ transcripts) in our in vivo splicing assay using the TrkB minigene. Moreover, when we measured endogenous alternative splicing in SHSY5Y cells overexpressing Srp20 or with Srp20 protein knocked down, we found corresponding changes in the ratio of TrkB-Shc:TrkB-TK+ expression. Modulation of Srp20 expression had little to no effect on alternative splicing of exon 18 to 20 to generate TrkB-TK+ transcripts in the in vivo exon splicing assay. This would suggest that Srp20 may be a specific splice regulatory factor for TrkB-Shc transcript production.

Pre-mRNA splicing dysregulation and disease

Dysregulation in mRNA splicing has been implicated in a range of human diseases (Faustino and Cooper 2003; Tazi et al. 2009). The most compelling evidence to link dysregulation of exon splicing to neurodegeneration comes from findings in patients with frontotemporal dementia with Parkinsonism. Altered splicing of adult-specific tau exon 10 leads to abnormal ratios of tau isoform expression (Jiang et al. 2000; Kalbfuss et al. 2001; Gao et al. 2007). The inclusion/exclusion of exon 10 is regulated by the binding of

SR proteins including 9G8 and Srp75 as well as hnrnpG and hnrnpE2 (Gao et al. 2007; Wang et al. 2011). Point mutations in the splice regulatory region that affect splice factor binding or alterations in splice factor regulation (e.g. by phosphorylation) can all modulate tau exon 10 splicing (Gao et al. 2007; Ding et al. 2011; Wang et al. 2011). Our current finding that Srp20 can modulate the inclusion/exclusion of exon 19 during TrkB pre-mRNA splicing to modulate TrkB-Shc transcript production and that its expression is elevated in AD suggests that dysregulated exon splicing may contribute to the neurotrophin signaling dysfunction observed in AD. However, it should be noted that dysregulation in exon splicing is one of multiple molecular changes that occur in AD that is capable of disrupting BDNF/TrkB-TK+ signaling and contribute to neurodegeneration.

In summary, we demonstrate that TrkB pre-mRNA splicing can be regulated by the SR protein Srp20, and that elevated expression levels of Srp20 in AD may contribute to the selective increase in TrkB-Shc transcript levels we previously reported in AD (Wong et al. 2012) (Fig. 9). Elevated levels of Srp20 increase exon 19 inclusion during splicing of the TrkB pre-mRNA to increase TrkB-Shc transcript expression. This increase in TrkB-Shc expression may then interfere with BDNF/TrkB-TK+ signaling by acting as a neurotrophin sink or by acting as a dominant negative receptor by forming inactive heterodimers unable to initiate second messenger signaling to activate cell survival pathways (Biffo et al. 1995; Eide et al. 1996; Ninkina et al. 1996; Fryer et al. 1997) (Fig. 9). Our data provide further evidence that dysregulation of BDNF/TrkB-TK+ signaling in neurons in AD occurs at the level of pre-mRNA splicing. Our findings also provide novel insights into the splicing regulation of the TrkB gene and present a possible target for

correcting the deficit in neuronal neurotrophic support caused by changes in the ratio of "functional" versus "non-functional" TrkB alternative transcripts expressed.

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Conflict of Interest

There are no conflicts of interest

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Figure Legends

Table 1. Cohort demographics

Table 2. PCR and qPCR primer sequences

Fig. 1. TrkB alternative transcripts and protein isoforms

Upper panel: exons are represented by numbered rectangles, solid lines joining exons depict exon splicing. Colored exons correspond to unique protein domains in lower panel. White exons represent non-coding sequences. Nomenclature and gene organization derived from (Luberg et al. 2010) and (Wong et al. 2011) and (Wong et al. 2012). Lower panel: BDNF can bind to all dimer combinations of TrkB isoforms. However, only the homodimers of TrkB-TK+ can undergo transphosphorylation upon BDNF binding and initiate second messenger signaling via AKT, ERK1/2, and PLCγ. Figure utilizes modified ProteinLounge graphics created using the Pathway Builder Tool (www.proteinlounge.com).

Fig. 2. TrkB minigene and in vivo splicing assay

(A) A TrkB minigene was constructed using pcDNA3.1 as a vector and an insert comprising exons 18 to 20 (colored boxes) flanked by 1kb of TrkB intronic or intervening sequences (IVS) (solid lines). The 1kb IVS18 comprises 0.5kb of intronic sequence from [a]position 87482347 to 87482847 and 0.5kb of intronic sequence from [b]position 87486703 to 87487203. The 1kb IVS19 comprises 0.5kb of intronic sequence

from [c]position 87486732 to 87487232 and 0.5kb of intronic sequence from [d]position 87549076 to 87549576. (B) The TrkB minigene plasmid construct was transfected into cells for 24 h where the minigene insert can be expressed and undergo alternative splicing. Alternative splicing (dashed lines) of the TrkB minigene gives rise to two transcripts containing exons 18/19/20 representing TrkB-Shc and exons 18/20 representing TrkB-TK+. Cells were Trizol extracted for total RNA and used for cDNA synthesis. The cDNA synthesized was used for semi-quantitative or quantitative real-time PCR using minigene specific primers as indicated by the red lines. PCR products were visualized by agarose gel electrophoresis and quantified by densitometry. L1=100 bp ladder; L2 represents PCR products containing exons 18/19/20 correspond to TrkB-Shc (1250 bp) and exons 18/20 correspond to TrkB-TK+ (221 bp). Note that TrkB-Shc PCR products are large as exon 19 does not contain a splice donor site and thus transcripts generated from the minigene will include all nucleotide bases up until the BGH pA region.

Fig. 3. Effect of different structural forms of Aβ_{42} on alternative exon splicing of a TrkB minigene expressed in differentiated SHSY5Y cells

SHSY5Y cells were differentiated for 9 days and transfected with a TrkB minigene for 24 h. Cells were then incubated in the absence (control) (white) or presence of Aβ_{42} monomers (AβM) (grey), oligomers (AβO) (black), and fibrils (AβF) (hatched) for 6 h and harvested. (A) Alternative splicing was detected by PCR amplification using mini-gene specific primers and visualized by agarose gel electrophoresis. PCR products containing exons 18/19/20 corresponds to TrkB-Shc transcripts. PCR products containing

exons 18/20 correspond to TrkB-TK+ transcripts. Bands were quantified by densitometry. (B) Relative ratio of TrkB-Shc:TrkB-TK+ mRNA expression. Data is averaged from n=3 separate experiments and expressed as mean + SEM relative to the control condition, which was set to 1. $A\beta_{42}$ fibril *p=0.03.

Fig. 4. Splice regulatory proteins predicted to bind to the TrkB gene

Schematic of putative splice regulatory proteins predicted to bind to the TrkB gene in the splice regulatory regions between exons 18 and 19. The binding sites were identified using SpliceAid (http://www.introni.it/splicing.html). A positive score indicates that the target sequences facilitate binding of a splice regulatory factor to ESE (exonic splicing enhancer) and ISS (intronic splicing silencer) motifs. A negative score indicates that the target sequences facilitate binding of a splice regulatory factor to ESS (exonic splicing silencer) and ISE (intronic splicing enhancer) motifs. The variable width of the bars and height respectively indicates the number of nucleotides in the binding site and its binding affinity (score axis).

Fig. 5. Srp20 and hnrnpK mRNA expression in AD

Expression of Srp20 (circles) and hnrnpK (squares) mRNA levels in the (A) hippocampus and (B) cerebellum of control (white) and AD (black) brains were measured by qPCR. **p=0.009.

Fig. 6. Effect of different structural forms of $A\beta_{42}$ on Srp20 mRNA expression in differentiated SHSY5Y cells

SHSY5Y cells were differentiated for 9 days and incubated in the absence (control) (white) or presence of $A\beta_{42}$ monomers (AβM) (grey), oligomers (AβO) (black), and fibrils (AβF) (hatched) for 6 h and harvested. Expression of Srp20 mRNA levels was then measured by qPCR. **p=0.002. Data is from n=3 separate experiments conducted in triplicate.

Fig. 7. Effect of Srp20 overexpression on alternative exon splicing of a TrkB minigene and endogenous TrkB alternative transcript levels in differentiated SHSY5Y cells

SHSY5Y cells were differentiated for 9 days and co-transfected with a TrkB minigene and (A, B, and D) 1000 ng of Srp20 or (C and E) incremental amounts of an Srp20 overexpression plasmid (as indicated in the figure) for 24 h and then harvested. (A) Alternative splicing was detected by PCR amplification using minigene-specific primers and visualized by agarose gel electrophoresis. PCR products containing exons 18/19/20 correspond to TrkB-Shc transcripts. PCR products containing exons 18/20 correspond to TrkB-TK+ transcripts. Bands were quantified by densitometry. (B) Relative ratio of TrkB-Shc:TrkB-TK+ mRNA expressed from the TrkB minigene. (C) Expression of Srp20 was measured by qPCR. (D) Srp20 protein expression following Srp20 overexpression as measured by western blotting. (E) Relative ratio of TrkB-Shc:TrkB-TK+ endogenous mRNA expression as measured by qPCR. Data is expressed as mean + SEM relative to the control condition, which was set to 1. ****p=0.00006; ***p=0.0003; **p=0.004 (TrkB-Shc:TrkB-TK+ 1000 ng); *p=0.048 (Srp20); *p=0.03 (TrkB-

Shc:TrkB-TK+ 250 ng and 500 ng). (A-C, E) Representative of n=3 separate experiments; (D) Representative of n=2 separate experiments conducted in triplicate.

Fig. 8. Effect of Srp20 knockdown on endogenous TrkB alternative transcript levels in differentiated SHSY5Y cells

SHSY5Y cells were differentiated for 9 days and transfected with an siRNA for Srp20 for 72 h and harvested. (A) Representative western blot image of Srp20 and β-actin with and without Srp20 knockdown. (B) Alternative splicing was detected by PCR amplification using minigene-specific primers and visualized by agarose gel electrophoresis. PCR products containing exons 18/19/20 correspond to TrkB-Shc transcripts. PCR products containing exons 18/20 correspond to TrkB-TK+ transcripts. Bands were quantified by densitometry. (C) Relative ratio of TrkB-Shc:TrkB-TK+ mRNA expressed from the TrkB minigene. (D) Relative ratio of TrkB-Shc:TrkB-TK+ endogenous mRNA expression as measured by qPCR. Data is expressed as mean + SEM relative to the control condition, which was set to 1. *p=0.01. Representative of n=3-4 separate experiments.

Fig. 9. Summary diagram

Upper panel: In the normal, non-diseased state, binding of BDNF to TrkB leads to autophosphorylation of TrkB-TK+ homodimers (but not other TrkB dimer combinations). This leads to activation of downstream second messenger signaling pathways including PLCγ (phospholipase C-gamma), MEK (mitogen-activated protein kinase kinase) and PI3K (phosphatidyl inositol 3 kinase), which are critical for neuronal viability and function. TrkB-TK+ homodimers can also be autophosphorylated in the absence of

BDNF, although activation of downstream signaling pathways are less intense. The arrow thickness indicates the magnitude of effect. Lower panel: In AD, there is Aβ plaque accumulation. Neurons are exposed to mixed species of Aβ, including $A\beta_{42}$ fibrils and oligomers at various stages of aggregation. BDNF protein expression has been reported to be reduced in AD (indicated by red arrow) and TrkB-Shc levels have been reported by us (Wong et al. 2012) to be increased in AD (hippocampus, CA1) (indicated by green arrow) and in response to $A\beta_{42}$ fibril exposure. In this current study, we found that modulation of Srp20 expression can influence TrkB-Shc levels e.g. increased Srp20 expression leads to increased TrkB-Shc transcript levels and vice versa. Srp20 expression levels are increased in AD and in response to $A\beta_{42}$ fibril exposure. However, the mechanism by which Srp20 expression is increased in AD and in response to $A\beta_{42}$ is currently unknown. The increase in TrkB-Shc is predicted to increase heterodimer combinations of TrkB-TK+/TrkB-Shc and homodimer combinations of TrkB-Shc, which would lead to an overall reduction in downstream signaling. Thus, the combination of reduced BDNF expression and increased TrkB-Shc expression in the AD hippocampus would likely result in an overall decrease in BDNF/TrkB-TK+ signaling. Figure utilizes modified ProteinLounge graphics created using the Pathway Builder Tool (www.proteinlounge.com).