Proteomic and Functional Analysis of Neurochondrin: A Potential Therapeutic Target for Spinal Muscular Atrophy and Other Neurodegenerative Diseases.

Lucy Knight Robinson

University of
St Andrews

University of St Andrews, School of Biology

Supervised by Dr. Judith Sleeman

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Abstract

Spinal Muscular Atrophy (SMA) is an inherited neurodegenerative disease caused by deletion of the survival motor neuron 1 (SMN1) gene, resulting in a decrease in the expression of survival of motor neuron (SMN) protein. It has been shown that neurochondrin (NCDN) interacts with SMN, indicating that SMN deficiency, as in SMA, may impact the localization and/or function of NCDN (Thompson et al., 2018). Individually, both proteins are implicated in neurite outgrowth. With the objective of identifying a mechanistic role for NCDN and/or SMN in the process, NCDN and SMN quantitative proteomes were compared to identify and investigate NCDN and/or SMN interacting proteins known to be associated with neurite outgrowth, with NCDN/SMN Co-interacting Protein (NSCIP) selected for further investigation. NSCIP was constitutively expressed in a cell line with normal NCDN expression (SH-SH5Y) and a cell line where NCDN was under-expressed (shNCDN), and neurite lengths evaluated at 24- and 48-hour intervals and 24-, 48-, and 72-hour intervals, respectively. Expression of NSCIP had a significant effect on neurite outgrowth for both the SH-SH5Y and shNCDN cells, with longer neurites observed in NSCIP-expressing cells, indicating that enhanced NSCIP expression can partially compensate for NCDN loss. NSCIP is suggested to have promise as a therapeutic target for SMA and other neurodegenerative diseases.

1 Introduction

Spinal muscular atrophy (SMA) is an inherited neurodegenerative disease, most commonly caused by homozygous deletion of the survival motor neuron 1 (SMN1) gene (Mercuri et al., 2020) resulting in reduction in functional survival motor neuron (SMN) protein quantity. In humans, the survival motor neuron 2 (SMN2) gene can partially compensate for the loss of *SMN1*, producing a small amount of full-length SMN protein. However, *SMN2* contains a mutation in an exon splicing enhancer located in the centre of exon 7. As such, exon 7 is skipped, resulting in truncation of most SMN protein produced. In this form, SMN is less stable, rapidly degraded, and hence, *SMN2* is unable to fully compensate for *SMN1* (Lorson and Androphy, 2000; Thompson et al., 2018). Extensive research aimed at increasing SMN quantity by targeting either *SMN1* or *SMN2* has resulted in three approved therapies: Spinraza (NHS access since 2019), Zolgensma (NHS access since 2021), and Evrysdi (NHS access since 2022) (smauk.org.uk, 2022). However, it has been demonstrated that SMN overexpression

may, over time, be toxic to neurons (Van Alstyne et al., 2021). As a consequence, the delicate balance known as the "Goldilocks effect" - the requirement to identify the perfect amount, neither too much nor too little- poses a potential challenge in the future as the long-term efficacy, safety, and optimal dosage of SMN-based therapies have not yet been thoroughly established (Sleeman, 2021). As a result, finding SMN-independent targets is essential.

In its most common form, type 1, SMA is characterised by onset at younger than 6 months with a life expectancy of less than 2 years, though there are prenatal and adult/late-onset forms (Russman, 2007). The SMN protein is multifunctional, with roles in DNA repair, cytoskeletal regulation, molecular trafficking, and studies have shown that SMN deficiency reduces neurite outgrowth (Singh et al., 2017). In the PC12 and NSC-34 cell lines, depletion of SMN resulted in reduced neurite outgrowth, and in an induced pluripotent stem cell-derived neuronal culture, of a type 1 SMA patient, neurite outgrowth was also reduced (Bora-Tatar and Erdem-Yurter, 2014; Li et al., 2015).

Neurite outgrowth is the process by which neurons produce new projections, developing into a single axon and numerous dendrites, in response to environmental guidance cues such as slits, semaphorins, and ephrins (Miller and Suter, 2018). Despite understanding the main structures associated with neurite outgrowth, the exact mechanism remains elusive, with involvement of individual proteins being elucidated but not conclusively linked to one another. Neurochondrin (NCDN) is an essential neural protein with several proposed roles in the central nervous system. While mechanistically unclear, NCDN is suggested to regulate signal transduction (Francke et al., 2006), vesicle trafficking (Thompson et al., 2018), synaptic plasticity (Dateki et al., 2005), and neurite outgrowth (Dateki et al., 2004). SMN and NCDN have been shown to co-localise in mobile cytoplasmic vesicles of neurites in SH-SY5Y cells. Therefore, it has been suggested that SMN depletion, as in SMA, may affect the localisation and/or function of NCDN (Thompson et al., 2018). Given that both proteins are implicated in neurite outgrowth and have been shown to interact, it is of interest to determine if there is a mechanism by which both proteins co-dependently initiate or promote the process.

Protein-analysis technologies have historically trailed behind DNA-based technologies, and so proteomics has only recently hit its stride. This is owing, in large, to advances in mass spectrometric techniques (Mann, 2006). One such technique is stable-isotope labelling by amino acids in cell culture (SILAC) which has frequently been used to study protein

phosphorylation, signal transduction, and to determine protein-protein interactions (Chandramouli and Qian, 2009). In triplex SILAC, proteins of interest are cultured in a medium containing light, medium, and heavy amino acids, thus metabolically labelling them, rendering them discernible by mass spectrometry. Based on the intensities of the light, medium, and heavy peptides, the relative abundance of the proteins can be determined (Zhang and Neubert, 2009). Ratios reflecting relative protein abundance between two fractions (heavy/light ratio or instance) indicate protein expression in one fraction compared to another, allowing for quantification and comparison of proteomes.

This study aims to compare SMN and NCDN SILAC derived proteomes to identify and investigate interacting proteins known to be relevant in neurite outgrowth, with the intent of uncovering a mechanistic role for NCDN and/or SMN in the process.

2 Results

SILAC proteomics initially showed proteins interacting with NCDN and SMN. Two NCDN-interacting proteins[1] were selected for further investigation from the NCDN proteome. Given that only preliminary experiments were conducted for these proteins, the results have been omitted from this report. NCDN/SMN Co-interacting Protein (NSCIP)[2] was selected as it was shown to interact with both NCDN and SMN.

In 2018, NSCIP and NCDN were explored in an independent proteomics screen aimed at identifying proteins that may interact with SMN by examining the interactions between two Sm proteins (spliceosomal RNA-binding proteins), SmN and SmB (Thompson, 2017). In this thesis, NSCIP was not further investigated due a lack of colocalization with SmN and SmB. However, given its additional appearance in our current screen in relation to NCDN and SMN, it was selected as a target for further investigation.

NSCIP has been implicated in neuritogenesis with interacting protein G-protein β2 subunit (GNB2) where constitutive expression of NSCIP induced neuritogenesis by activating the Ras-MAPK pathway in PC12 cells (REDACTED, 2006). Thus, we were interested to explore

[1] As research is unpublished, exact protein names cannot be disclosed at this time.
[2] As research is unpublished, the official protein name cannot be disclosed at this time.

whether neurite outgrowth could be promoted by overexpressing NSCIP in the SH-SY5Y cell line, in cells with normal NCDN expression (SH-SY5Y), and cells with reduced NCDN expression (shNCDN). Average neurite lengths were evaluated at 24- and 48-hour intervals and 24-, 48-, and 72-hour intervals respectively (Figure 1) for cells overexpressing NSCIP, and cells with normal NSCIP expression. Measurements for each respective cell line were compared.

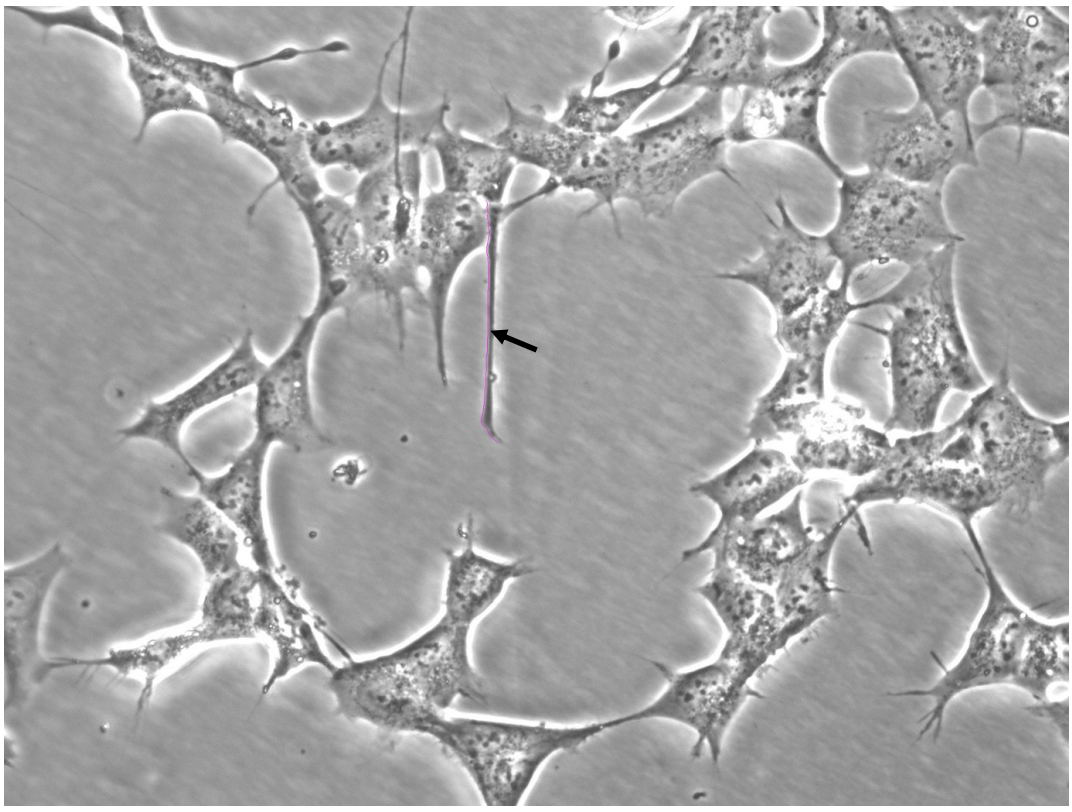

Figure 1: Representative Image Showing How Neurites Were Measured. *Image of SH-SY5Y cells 48 hours after NSCIP transfection. Images were obtained using the EVOS M5000 cell imaging system at a magnification of 40X. Based on green fluorescent protein (GFP) imaging, cells transfected with NSCIP were identified. An example of a neurite overexpressing NSCIP being measured is shown by the arrow, the pink line showing where the measurement was taken from/to. Raw images were imported into Fiji (ImageJ) and neurites were measured with the NeuronJ plugin.*

Longer neurites were observed in SH-SY5Y cells overexpressing NSCIP compared to cells with normal NSCIP expression across all timepoints (Figure 2).

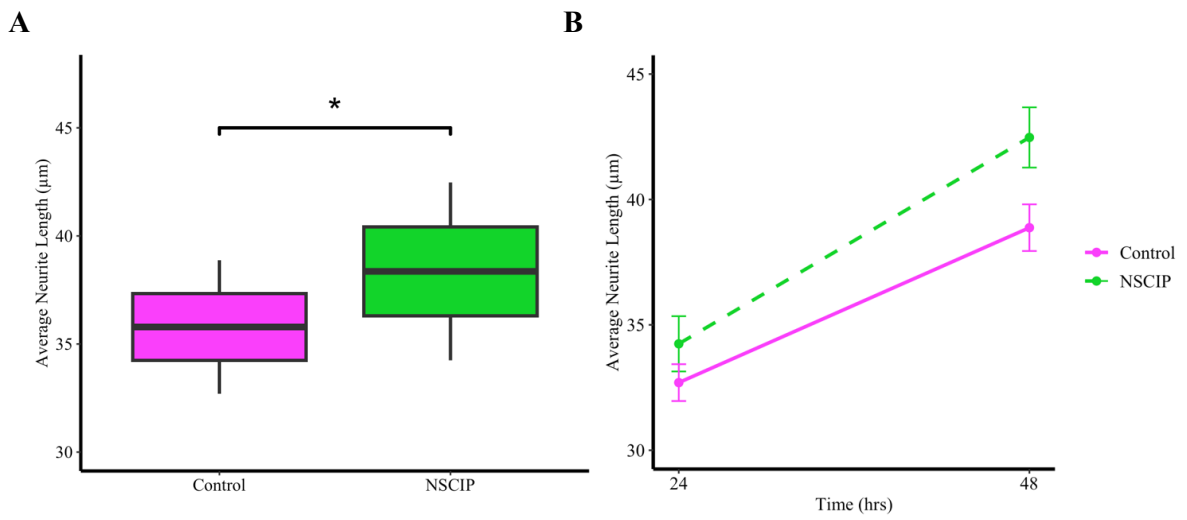

Figure 2: Increased Neurite Outgrowth in SH-SY5Y Cells through NSCIP Overexpression. *Average neurite length was recorded at 24 hours and 48 hours for cells transfected with NSCIP (NSCIP, green) and for cells not transfected with NSCIP (Control, magenta). Neurites from 100 cells were counted per timepoint per experimental repeat (three biological replicates performed). A: A between-subjects factorial ANOVA, showed a significant main effect of NSCIP over-expression, $F(1, 1511) = 6.287$, $p = 0.012$ on neurite outgrowth across all timepoints. B: There was not a significant interaction between time and NSCIP overexpression, $F(1, 1511) = 0.960$, $p = 0.327$. Error bars are ±SEM (µM): 24-hours NSCIP, 1.102; 48-hours NSCIP, 0.735; 24-hours control, 1.201; 48-hours control, 0.93.*

Longer neurites were observed in shNCDN cells overexpressing NSCIP compared to cells with normal NSCIP expression (Figure 3).

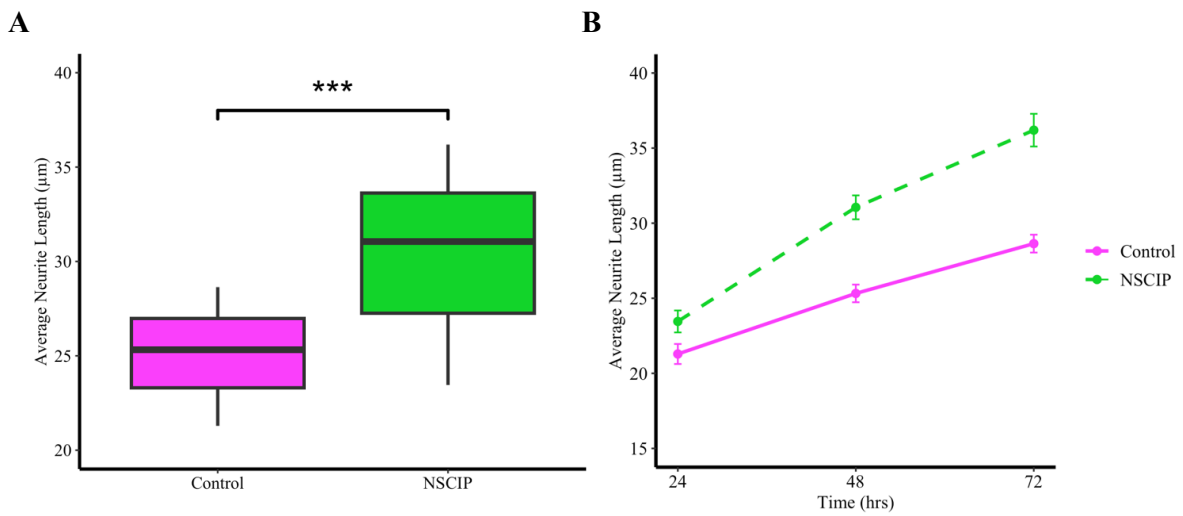

Figure 3: Increased Neurite Outgrowth in shNCDN Cells through NSCIP Overexpression. *Average neurite length was recorded at 24 hours, 48 hours, and 72 hours for cells transfected with NSCIP (NSCIP, green) and for cells not transfected with NSCIP (Control, magenta). Neurites from 100 cells were counted per timepoint per experimental repeat (three biological replicates performed. A: A between-subjects factorial ANOVA, showed a significant main effect of NSCIP overexpression, $F(1, 2177) = 65.851$, $p < 0.001$ on neurite outgrowth across all timepoints. B: There was not a significant interaction between time and NSCIP overexpression, $F(2, 2177) = 1.392$, $p = 0.249$. Error bars are ±SEM (µM): 24-hours NSCIP, 0.731; 48-hours NSCIP, 0.797; 72-hours NSCIP, 1.088; 24-hours control, 0.666; 48-hours control, 0.587; 72-hours control, 0.595.*

While transfection with NSCIP was not able to fully rescue the neurite length loss observed with NCDN depletion, partial compensation was observed with an increase in neurite length observed in shNCDN cells transfected with NSCIP compared to shNCDN cells not transfected with NSCIP and a control SH-SY5Y cell line (Figure 4).

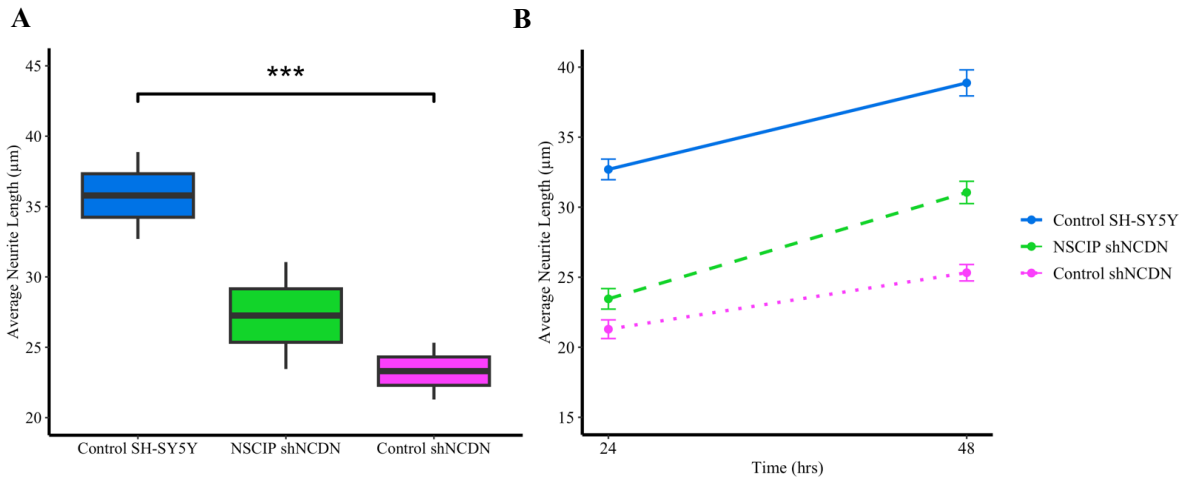

Figure 4: Overexpression of NSCIP Promotes Neurite Outgrowth and Partially Compensates for the Loss of NCDN. *Average neurite length was recorded at 24 hours and 48 hours for shNCDN cells transfected with NSCIP (NSCIP shNCDN, green) and for shNCDN cells not transfected with NSCIP (Control shNCDN, magenta). This was compared to SH-SY5Y cells not transfected with NSCIP (Control SH-SY5Y, blue). Neurites from 100 cells were counted per timepoint per experimental repeat (three biological replicates performed). A: A between-subjects factorial ANOVA, showed a significant main effect of NSCIP overexpression, $F(2, 2378) = 169.875$, $p < 0.001$ on neurite outgrowth across all timepoints. B: There was not a significant interaction between time and NSCIP overexpression, $F(2, 2378) = 2.629$, $p = 0.072$. Error bars are ±SEM (µM): 24-hours NSCIP shNCDN, 0.731; 48-hours NSCIP shNCDN, 0.797; 24-hours control shNCDN, 0.666; 48-hours control shNCDN, 0.587; 24-hours control SH-SY5Y, 0.735; 48-hours control SH-SY5Y, 0.93.*

3 Discussion

NSCIP is a poorly characterised protein with suggested roles in cytokinesis (REDACTED, 2011), neuronal differentiation (REDACTED, 2006) and Golgi membrane dynamics (REDCATED, 2017). Its relevance in neurodegenerative processes stems, in part, from the pentanucleotide repeat, ATTCT, of the NSCIP gene, which has been associated with spinocerebellar ataxia (REDCATED, 2006).

Neurite length was significantly greater in cells overexpressing NSCIP compared to cells with normal NSCIP expression for both the shNCDN and SH-SY5Y cell lines. Given that NSCIP promotes neurite outgrowth with a knockdown of NCDN, we suggest either: 1) NCDN and

NSCIP promote neurite outgrowth via distinct pathways, and therefore function of NSCIP is not regulated by NCDN; or 2) NCDN and NSCIP promote neurite outgrowth in a shared pathway, where increased NSCIP expression is able to partially compensate for loss of NCDN. Given that increased NSCIP expression did not fully rescue the effects observed by reduced NCDN expression, based on the former model where separate pathways are in operation, NSCIP does not promote neurite outgrowth as strongly as NCDN. Based on the latter model, NSCIP and NCDN participate in the same pathway with similar roles. As neurite outgrowth is halted with absence of NCDN this suggests a significant function for NCDN in the process. Increased NSCIP expression allows for this function to continue but not as efficiently as if NCDN were present, hence the partial but not complete rescue. As normal NCDN levels are present in the SH-SY5Y cells, this compensation is not required and subsequently, neurite outgrowth is not promoted as greatly as in shNCDN cells. Given this result in conjunction with the outgrowth observed in the shNCDN cells, we suggest the latter model is more plausible. It would be of interest to see whether this changes with addition of brain-derived neurotrophic factor (BDNF) and/or retinoic acid (RA), reagents commonly used to promote cell differentiation, where in both cell lines, a higher level of neurite outgrowth and interaction effect would be expected. It would also be interesting to investigate whether neurite outgrowth could be further promoted in a cell line with overexpression of both NSCIP and NCDN, or if there is a limiting factor to the growth.

Furthermore, in all repeats, transfection efficiency and longevity were greater in the shNCDN cells compared to the SH-SY5Y cells – SH-SH5Y neurite lengths were only measured for 48 hours rather than 72 hours for this reason. It is unclear whether this is a methodological issue, though poor efficiency was consistently observed across all three repeats in only the SH-SH5Y cell line. Stable cell lines with NSCIP overexpression and/or knockdown may be more suitable in future experiments to examine this further.

Prior experiments within the lab conducted by Thompson, 2017 showed cytoplasmic localisation of NSCIP, concentrated in the perinuclear region in SH-SY5Y cells. This is consistent with results found by REDACTED, 2006 in COS7 cells. Additionally, while not quantifiable (i.e., ratios reflecting relative protein abundance could not be obtained), the NSCIP interacting protein GNB2 was detected within the SILAC screen. Given that GNB2 has been shown to interact with NSCIP rather than NCDN, the non-quantifiable result would be expected. Consequently, it is possible that there may be a role for NCDN in the pathway

proposed by REDACTED, 2006, whereby the Ras-MAP kinase-Elk-1 cascade is activated to promote neurite outgrowth. Further insights may be provided by determining whether MAP kinase and/or GNB2 activity is altered by reduction or increase of NCDN to determine if there is a functional link in neurite outgrowth, where no change would be expected if NCDN does not promote neurite outgrowth through an interaction with NSCIP via the pathway proposed by REDACTED, 2006.

An interaction between NCDN and both SMN and NSCIP has been demonstrated in this study; previously, SMN and NSCIP had no known common interacting partners. SMN and NSCIP have independently been implicated in regulating Golgi dynamics, which if disrupted could impact neurite outgrowth through altered protein trafficking, intracellular signalling, and cytoskeletal interactions. From type I and II SMA patient-derived fibroblasts, it was found that Golgi apparatus morphology is abnormal (demonstrated a diffuse distribution), in SMN depleted conditions. From this, it was determined that coat protein complex I (COPI)-dependent intracellular trafficking is altered with SMN knockdown (Custer et al., 2019). Interestingly, a similar diffuse Golgi structure was observed in HeLa cells transfected with siNSCIP, suggesting that the Golgi dispersal phenotype is regulated by NSCIP (REDACTED, 2015). Although the exact cellular structures involved remain unclear, SMN has been implicated in the trafficking of mature mRNAs, with the aforementioned structures described as vesicular or granular. Additionally, SMN decrease has been associated with endosomal defects, suggesting significance for vesicular transport (Thompson et al., 2018). Given that it has been proposed that NSCIP may be at the interface of the Golgi membrane and cytoskeleton, reinforced by the C-terminus armadillo repeat of NSCIP which is suggested to interact with cytoskeletal structures (REDACTED, 2017), it would be of interest to determine whether there is a mechanistic link between NSCIP and SMN in vesicular transport. Furthermore, since NCDN has been suggested to be involved in the transport of trafficking vesicles (Thompson et al., 2018), there may be a mechanism by which the three proteins interact.

In conclusion, the potential significance of NSCIP as a therapeutic target for SMA and other neurodegenerative diseases holds promise. However, additional research is required to further examine the dynamic interplay between NSCIP, NCDN, and SMN in order to construct a clearer and more cohesive picture.

4 Materials and Methods[3]

4.1 Cell Lines and Culture

Stable SH-SY5Y cell lines with constitutive NCDN under-expression, shNCDN, and normal NCDN expression, SH-SY5Y were used. Cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) containing 10% foetal bovine serum (FBS) and 1% penicillin/streptomycin at 37°C and 5% CO_2. For transfection of NSCIP plasmid into SH-SY5Y and shNCDN cells, effectene (Qiagen, Hilden, Germany) was used according to the manufacturer's instructions.

4.2 Proteomics

From ongoing research in Dr. Sleeman's lab, a quantitative proteomic data set was obtained using SILAC comparing the interactomes of stable cell lines constitutively over-expressing NCDN and SMN. Raw data files were processed using MaxQuant before bioinformatic analysis.

4.3 Microscopy and Image Analysis

4.3.1 EVOS

Following transfection, SH-SY5Y and shNCDN cells were measured at 24- and 48-hours and 24-, 48- and 72-hours, respectively. Images were obtained using the EVOS M5000 cell imaging system at a magnification of 40X, imported into Fiji (ImageJ) and neurites were measured with the NeuronJ plugin. To acquire the realised neurite length, data was exported into Excel and pixel length converted to μm using calculations optimised within the lab. Neurite lengths from 100 cells per condition across three biological repeats for each cell line were combined to obtain an average neurite length (μm).

4.4 Statistical Analysis

[3] As research is unpublished, detailed methodological procedures cannot be disclosed at this time.

Statistical analysis was conducted with R (version 4.2.3) with neurite length as the dependant variable and condition and time as independent variables. In all statistical tests, the Levene and Shapiro-Wilk assumptions were violated and so data was logarithmically transformed to obtain a corrected *P*-value. Error bars are ± standard error of mean (SEM). Graphs were all produced using R (version 4.2.3), ggplot2 software.

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[4] As research is unpublished, some references have been redacted and cannot be disclosed at this time.

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