Advancing Our Understanding of Mechanosensitive Microtubule Acetylation in Neurons

Margaret Earl Utgaard

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<tbody>
<tr>
<td>aTAT1</td>
<td>alpha tubulin N-acetyltransferase 1</td>
</tr>
<tr>
<td>ECM</td>
<td>extracellular matrix</td>
</tr>
<tr>
<td>EMEM</td>
<td>Minimum Essential Medium with Earle’s salts</td>
</tr>
<tr>
<td>GTP</td>
<td>guanosine-5'-triphosphate</td>
</tr>
<tr>
<td>GDP</td>
<td>guanosine diphosphate</td>
</tr>
<tr>
<td>HDAC6</td>
<td>histone deacetylase 6</td>
</tr>
<tr>
<td>iPSC</td>
<td>induced pluripotent stem cell</td>
</tr>
<tr>
<td>KIF17</td>
<td>kinesin-like protein 17</td>
</tr>
<tr>
<td>MAP2</td>
<td>microtubule associated protein 2</td>
</tr>
<tr>
<td>NEFL</td>
<td>Neuropilin light chain</td>
</tr>
<tr>
<td>NMDA</td>
<td>N-Methyl-D-aspartic acid</td>
</tr>
<tr>
<td>PBS</td>
<td>phosphate buffered saline</td>
</tr>
<tr>
<td>PTM</td>
<td>post translational modification</td>
</tr>
<tr>
<td>ROI</td>
<td>Region of Interest</td>
</tr>
<tr>
<td>RhoA</td>
<td>transforming protein RhoA/Ras homolog family member A</td>
</tr>
<tr>
<td>Sept7</td>
<td>septin 7</td>
</tr>
<tr>
<td>Sept9</td>
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<tr>
<td>shRNA</td>
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CHAPTER ONE
INTRODUCTION

Introduction

Mechanotransduction is the ability of cells to convert mechanical information into biochemical signaling. Primary components of mechanotransduction are the extracellular matrix (ECM) and the cytoskeleton, which are involved in both sensing and responding to mechanics (Humphrey et al. 2014). It is known that matrix stiffness cues contribute to neuronal development (Even-Ram et al. 2006) and that mature neurons must interact with different ECM stiffnesses, either as a member of the peripheral nervous system interacting with muscles, bones, and organs or in the central nervous system where different regions of the cortex have different stiffness (Barnes et al. 2017). The brain also changes stiffness in normal aging (Hiscox et al. 2018), injury (Yin et al. 2018), and disease states (ElSheikh et al. 2017). Therefore, one can conclude that mechanical signals and how cells respond to them are integral to understanding neuronal function.

The impact of mechanical signals is primarily mediated by the cytoskeleton (Humphrey et al. 2014), which in neurons is highly organized and compartmentalized due to the polarization of the cell (Kelliher et al. 2019). Microtubules compose a large part of the neuronal cytoskeleton and provide more stability than actin. They are found in the axon, cell body, terminal and larger branches of dendrites (Malacrida et al. 2019). Microtubule dynamics are vital for cell function, as they serve as both supportive structure and a transport system. Microtubules are constantly depolymerized and repolymerized and their selective stability is an important feature because it
allows specific microtubule structures to be remodeled without losing the overall cellular or compartment structure (Garnham and Roll-Mecak 2012) (Dent 2020). One mechanism of microtubule stabilization is acetylation of K40 in alpha-tubulin via the protein alpha tubulin N-acetyltransferase1 (aTAT1), which is thought to prevent microtubule catastrophe due to cracking (Janke and Montagnac 2017).

Microtubule acetylation has been observed in response to changes in environmental stiffness; however, there is not a consensus as to what that response is. Seetharaman et al, 2020 found that increasing stiffness of the environment caused an increase of acetylated tubulin. However, Heck, et al. 2012 found that an increase in environmental stiffness decreased acetylated tubulin (Heck et al. 2012; Seetharaman et al.). While there is a disagreement in the literature on the precise outcome, it is clear that microtubules are thought to play an important role in mechanosensation.

Septins are a group of small GTPases thought to be involved in regulation of microtubule stability. GTPase proteins bind guanosine-5'-triphosphate (GTP) and hydrolyze it to guanosine diphosphate (GDP). Their GTPase activity plays a part in septins’ dynamic abilities. Septins have the unique ability to form large complex structures like rings, bundles, and gauze cages (Neubauer and Zieger 2017). They have also been shown to interact with microtubules, and septin 9 (Sept9) specifically has been shown to have a microtubule binding domain (Kremer et al. 2005), (Spiliotis 2018), (Sadian et al. 2013). Septins have been explored in depth in yeast cells, where they were first identified, but are only beginning to be explored in mammalian systems. Septin 9 could play a role in microtubule stabilization or in time dependent reorganization through its microtubule binding domain. Understanding the interplay between
stiffness, septins, and microtubule stability could provide important insights into neuronal development, maintenance, and survival in the changing conditions of the brain.

SH-SY5Y cells are a well-known model for studying neuronal like cells as they can be kept in their more neuronal precursor like state or differentiated to be closer to mature neurons (Kovalevich and Langford 2013; Agholme et al. 2010). SH-SY5Y cells are known to express septins and have complex cytoskeletal architecture. It is not known how tubulin acetylation in these cells is effected by environmental stiffness, or how septins might effect this process.

Specific Aims and Experiments

Aim 1: Determine the effect of matrix stiffness on microtubule acetylation in SH-SY5Y cells. 
Rationale: There are post translational modifications to cytoskeletal proteins that are thought to be mechanosensitive. Microtubule acetylation is a modification thought to increase stability of the microtubule filament. Microtubule acetylation has been shown in separate studies, to respond to mechanical changes in the environment (Heck et al. 2012; Seetharaman et al.). However, the directional effect of matrix stiffness on microtubule acetylation is unknown and little is known about the effect of matrix stiffness on the microtubules of neuronal cells. Potentially, stiffer surfaces could exert more force via integrin activation and strain microtubules leading to increased cracking and increased opportunity for acetylation.

Hypothesis 1: Increasing matrix stiffness will increase microtubule acetylation. Increased acetylation will lead to increase microtubule stability through acetylation or other protein interactions.

Experiment 1: SH-SY5Y cells were plated on polyacrylamide gels of 16kPa and 500Pa as well as glass. Lysates of cells from these gels were collected for western blots to probe for alpha and acetylated tubulin.
Experiment 2: SH-SY5Y cells were plated on polyacrylamide gels of 16kPa and 500Pa as well as glass and then label alpha and acetylated tubulin using immunocytochemistry to determine if there was a measurable difference in structure. Quantitative analysis of the acetylated tubulin intensity was performed relative to total tubulin intensity and comparing the ratios across different stiffness.

Aim 2: Determine the role of matrix stiffness in Sept9 organization and the impact of Sept9 on microtubule acetylation.

Rationale: Septins, in general, have also been shown to be mechanosensitive. At least two septins Septin 7 (Sept7) and Sept9 have been shown to impact microtubules specifically. Sept9 has been shown to interact directly with microtubules, and shown to bundle and increase stability of microtubules. The role of septins in acetylation, if any, is unknown especially within the context of mechanosensation.

Hypothesis 2: Increased acetylation on stiffer surfaces will increase the amount of septin localization and higher order structures. The increase in microtubule acetylation will lead to an increase in microtubule Sept9 interactions.

Experiment 1: SH-SY5Y cells were plated on increasingly stiff gels (glass, 16kPa, and 500Pa) and immunocytochemistry was used to stain cells for Sept9 and alpha tubulin to determine colocalization with microtubules using super resolution microscopy and if that colocalization changes on different stiffness.

Experiment 2: Using small hairpin ribonucleic acid (shRNA), Sept9 was knocked down in SH-SY5Y cells. The cells with reduced Sept9 expression were then plated on gels of different stiffness and fix and stained for alpha and acetylated tubulin. Quantitative analysis was performed for the acetylated: alpha tubulin ratio as used on the normal cells.
CHAPTER TWO
LITERATURE REVIEW

The stiffness of the environment surrounding neurons can significantly impact neuron behavior and function. Stiffness is interpreted by cells in a process called mechanotransduction, where mechanical signals are converted to biochemical signals, typically via the cytoskeleton (actin, microtubules, septins, etc.). The cytoskeleton is thus thought to be responsible for coordinating mechanical signaling, in addition to its more typically considered roles of regulating the cell’s shape and coordinating transport of material across the cell. The following section covers what is known about each step in this cascade, and how it relates specifically to neuronal cells.

Stiffness as a Signaling Cue to Neurons

The cytoskeleton of neurons is tightly regulated. In addition to chemical signals, mechanical signals, like stiffness, tension, or flow, can orchestrate changes in the neuronal cytoskeleton (Barnes et al. 2017). Stiffness is rapidly becoming appreciated as a key signaling factor in the brain. Stiffness is known to be involved in development (Lv et al. 2015), injury response and repair (Song et al. 2019), and degenerative diseases in the brain (ElSheikh et al. 2017). Moreover, different regions of the brain are known to vary in stiffness (Yin et al. 2018). These varying stiffness measurements suggest that neurons in different regions of the brain are adapted to specific stiffness, and these neurons might respond differently to changes in their environmental stiffness. For example, it has been shown that dendrite branching of hippocampus neurons increase on stiffer substrates up to 3000 Pa, compared to substrates of 300-600 Pa which
are typical of healthy brain tissue (Previtera et al. 2010). Understanding the molecular mechanism by which neurons interpret and respond to the environmental stiffness remains an under-developed question in the field.

Brain stiffness can be measured using magnetic resonance elastography, which uses a phase-contrast magnetic resonance technique combined with low-frequency vibrations to estimate the rigidity of soft tissue (Dong et al. 2018). Using magnetic resonance elastography young brains were found to be much stiffer than normally aged brains, normally aged brains in this study were considered brains of individuals with no detectable disease ages 66-73 years (Yin et al. 2018; Hiscox et al. 2018). The brain changing stiffness overtime indicates that stiffness and cell mechanics may be an important cue in development. To highlight this, a foundational study by Engler and colleagues cultured induced pluripotent stem cells (iPSC) in identical media environments but placed them on different 2D stiffnesses (Engler et al. 2006). Amazingly, iPSCs on soft substrates (similar to the stiffness of the brain) started differentiating toward a neuronal lineage, while iPSCs on medium stiffness substrates expressed markers for a myogenic lineage, and iPSCs on the stiffest substrates expressed markers for an osteogenic lineage (Engler et al. 2006). This work clearly demonstrated that stiffness can play a key signaling role in delineating stem cell differentiation.

Stiffness and mechanotransduction are also thought to play an important role in development in a number of ways. One example of the role of stiffness in development is during neuronal migration, where stiffness is thought to act as a guidance cue for cells that are attempting to migrate to the correct location. Numerous studies have shown that cells, including neurons and neuronal precursors, will preferentially migrate towards stiffer substrates in a process called “durotaxis” (Shellard and Mayor 2021). Koser and colleagues, for example, show
that stiffness is important for optic tract neurons to correctly migrate and form the optic tract (Koser et al. 2016). Altering the stiffness gradient of this region in vivo, of Xenopus embryos led to a splayed phenotype, and the neurons failed to create the optic tract. Changes in substrate stiffness have also been demonstrated to impact differentiation. It has been shown that hippocampal neuritogenesis is encouraged by softer substrates that mimic the brain, and is suppressed by stiffer substrates (Tanaka et al. 2018).

Post development, mechanotransduction continues to play important roles in maintaining normal cell function. Thus, mechanotransduction is susceptible to perturbations from disease and injury such as traumatic brain injury. In traumatic brain injury, there are drastic regional decreases in brain stiffness at the site of injury accompanied by changes in stiffness in other regions of the brain (Yin et al. 2018). The impact of changing stiffnesses on the neurons themselves is an active area of research. For example, in a drosophila nerve crush injury model, when the segmental nerves of 3rd instar larvae were briefly pinched with tweezers, mechanosensitive ion channels that respond to changing mechanical forces opened to inhibit axon regeneration (Song et al. 2019). Deactivation of these channels increased axon regeneration in response to the applied pinch (Song et al. 2019). Since axon regeneration depends on the cytoskeleton, these studies imply that neuronal mechanosensation regulates signaling to the cytoskeleton to modulate neuronal regeneration.

Degenerative disease is another process in which stiffness changes in the brain and may alter neuronal function. The stiffness of adult brain regions varies greatly from 0.3kPa to 3kPa as determined by a compilation of measurements (Hall et al. 2020). In response to ageing, the mechanical properties of the brain are altered partially due to changes in the ECM (Lau et al. 2013). In Alzheimer's disease there are alterations in ECM composition particularly around
amyloid plaques and neurofibrillary tangles, which creates changes in the mechanical properties of the tissue for healthy cells in the area (Hall et al. 2020; Lau et al. 2013). These stiffness changes that occur in Alzheimer’s disease can extend beyond local environments, and include entire regions of the brain. For example, the frontal cortex of patients with Alzheimer’s disease was demonstrated to decrease significantly in stiffness (Yin et al. 2018). Interestingly, brain stiffness has also been correlated with Alzheimer’s disease severity, suggesting that neuronal function is impacted by these changes in stiffness, which are a direct output of structural changes in the cytoskeletal and the extracellular matrix. (ElSheikh et al. 2017; Murphy et al. 2019).

In summary, it is clear that neurons, like all cells, interpret stiffness as a signaling cue. Understanding their ability to respond to changes in stiffness is thus important for gaining insight into the functions of neurons. Since many of these processes involve the cytoskeleton, the next section will discuss how the cytoskeleton interprets and transmits mechanical signals.

**Mechanotransduction**

Mechanotransduction is the process by which cells convert mechanical information into biochemical signaling (Chighizola et al. 2019). Mechanical information includes tension, stiffness, bending, compression and more, and can originate from within the cells, cell-cell interactions, external applications of force, or ECM interactions (Janmey et al. 2020). The latter (cell-ECM communication) is critical in interpreting environmental stiffness and controls many aspects of neuronal cell biology (Sun et al. 2016; Chighizola et al. 2019).

The brain’s extracellular matrix is a highly unique environment with vast molecular diversity. There are many sub-divisions within the brain’s ECM: the basement membrane around blood vessels, perineuronal nets surrounding dendritic arbors of neurons, and the neural interstitial matrix (Chighizola et al. 2019). The lipids and proteins in these different ECM
compartments vary in composition and organization but, the main components are glycosaminoglycans, like hyaluronic acid and proteoglycans, and glycoproteins like laminin (Chighizola et al. 2019; Lau et al. 2013).

The interactions between the cell and ECM are mediated primarily through integrins (Kechagia et al. 2019). Integrins are a group of heterodimeric proteins that span the cell membrane and connect the ECM outside the cell to adaptor/scaffold proteins and the cytoskeleton inside the cell, thereby coupling the outer mechanical environment with the internal cytoskeleton (Schwarz and Gardel 2012). Multiple layers of proteins regulate the transmission of mechanical cues (Kanchanawong et al. 2010). Integrin-based adhesion complexes balance the elasticity of the ECM and the tension created by the cytoskeleton, which generates responsive forces (Oakes et al. 2018). The forces experienced during mechanotransduction can allosterically initiate signaling cascades with either short or long term effects (Sun et al. 2016). Many of these cascades flow through the Rho GTPase superfamily, which leads to cytoskeletal remodeling.

RhoA, transforming protein RhoA, or Ras homolog family member A, is considered a master regulator of the cytoskeleton (Burridge and Wennerberg 2004). Downstream effectors of RhoA include formins, which mediate actin polymerization, and Rho associated kinase (ROCK) which leads to myosin activation (Ridley and Hall 1992). RhoA is thought to be elevated on stiffer substrates (Kim et al. 2009; McBeath et al. 2004). Excessive RhoA activity can lead to suppression of neurogenesis in neural stem cells (Keung et al. 2011). There are potential upstream activators of RhoA which are also mechanosensitive. For example a guanine nucleotide exchange factor, GEF-H1, exhibits increased activity and release of GEF-H1 from microtubules on stiff matrices (Heck et al. 2012). Heck et. al, also found that GEF-H1 exchange activity is
required for RhoA activation (Heck et al. 2012). RhoA is involved in mediating several mechanosensing pathways in neurons.

While the cytoskeleton mediates a number of potential mechanosensing pathways, it is not the sole operator. For instance, ion channels are also capable of transducing mechanical stimuli. Mechanosensitive ion channels are important in several pathways including, touch sensation, blood pressure regulation, hearing, and osmoregulation (Zhao et al. 2016). Some ion channels are known to be both voltage gated and mechanically gated such as voltage-gated sodium channel, NaV1.7 (Raouf et al. 2012). Other ion channels are primarily mechanically gated like Piezo1, which is a cation channel with a preference for Ca2+ over other cations (Zhao et al. 2016). These channels are activated by stretch or compression which is applied by the ECM. It has been shown that activity of Piezo1 after nerve crush injury inhibits axon regeneration in drosophila (Song et al. 2019).

These examples make clear that mechanotransduction is an important process throughout neuronal development, where several large mechanical events occur, including: neural tube closure, neural crest migration, neural stem cell precursor differentiation, neuronal migration, axonal guidance, and gyration (Abuwarda and Pathak 2020). Regulation of the stiffness of a tissue modulates RhoA activity which impacts neurogenesis, and neuronal lineage commitment (Abuwarda and Pathak 2020).

**Neuronal Cytoskeleton**

The neuronal cytoskeleton is highly organized and polarized. The relationship between structure and function of the cytoskeleton enables both immature neuroblasts to migrate and polarize properly, and mature neurons to maintain morphology (Kapitein and Hoogenraad 2015). The cytoskeleton is typically considered to be composed of actin, myosin, microtubules and
intermediate filaments. However, additional protein families have more recently been included (Mostowy and Cossart 2012), such as septins, which will be discussed in detail in the following section.

The neuronal cytoskeleton varies vastly from between cellular compartments that include the soma, dendrites, axon initial segment, and the axon. Neuronal compartments are distinct in protein composition and organization of their cytoskeletal elements (Miller and Suter 2018; Zhao et al. 2017). The dendrites contain microtubules in the largest branches, and in their finer protrusions F-actin serves as the main structural component. Microtubules in the dendrites are not polarized as the dendrites are very dynamic and are constantly being remodeled (Kelliher et al. 2019). The axon initial segment is a distinct compartment that serves as a regulatory entrance to the axon. The axon is a tightly bundled long-lived protrusion, which is composed of microtubules which are all polarized in the same direction. Actin also plays a role in the axon, forming regularly spaced rings down the axon that are connected by spectrin (Xu et al. 2013).

Some of the most important roles of the neuronal cytoskeleton are neurite formation, and maintenance of stable protrusions. Neuritegenesis is not only important for development, but continues to occur after development, especially in conditions of trauma or disease (Miller and Suter 2018). Bundled, stable microtubules are crucial for the formation of neurites (Falconer et al. 1989), though other cytoskeletal proteins contribute greatly to the function of neurons, microtubules are the backbone and create the main structure of neurons (Kelliher et al. 2019).

**Microtubules**

Microtubules serve a variety of functions in mammalian cells, and are vital for numerous processes, including mitosis, migration, subcellular transport, and cellular polarity (Kelliher et al. 2019). In non-neuronal cells, they are typically highly dynamic and are constantly undergoing
catastrophe or repolymerization (Gardner et al. 2013). However, neurons must balance the need for dynamic regulation with long term stability. In the neuron, microtubules are particularly important in facilitating neurogenesis in development and maintaining stable protrusions throughout the life of the neuron (Kapitein and Hoogenraad 2015). However, microtubule dynamics are important for learning and memory (Dent 2017).

Microtubules are dynamic structures composed of dimers of alpha and beta tubulin. Alpha and beta subunits polymerize first to form dimers, and then into long protofilaments, or a single rod of polymerized alpha-beta dimers. Protofilaments interact laterally to form hollow microtubules. Microtubules of mammalian cells are made of 13 protofilaments, while non-mammalian cells can have 10-15 protofilament microtubules (Baas et al. 2016). The dimer structure of microtubules causes them to be inherently polar, with new alpha-beta dimers being added to the plus end where beta tubulin is exposed.

Both tubulin subunits bind GTP nucleotides. Alpha tubulin cannot hydrolyze or exchange GTP, while beta tubulin can hydrolyze GTP to GDP. Generally, if the plus-end beta tubulin is bound to GTP, it is capable of accepting an additional tubulin subunit, thereby allowing polymerization and extension of the microtubule. However, if the plus end of the microtubule has beta tubulin bound to GDP it will either depolymerize or stabilize to maintain at its current length (Alushin et al. 2014).

In neurons, tubulin can be classified in three main fractions: labile, stable, and cold stable. The distribution of tubulin between these fractions is primarily the result of post translational modifications (PTMs). The labile or mobile fraction can be free tubulin or polymerized microtubules that do not have key PTMs and quickly will undergo catastrophe. The
stable fraction is much less likely to undergo catastrophe due to PTMs, especially acetylation and detyrosination (Baas et al. 2016; Song et al. 2013).

The cold stable fraction of microtubules is only observed in nervous tissue, and is polyaminated and stable to most factors that would cause microtubules depolymerization, including cold temperatures. There are several PTMs that can be made to both alpha and beta tubulin. Some PTMs increase stability, such as acetylation (Xu et al. 2017). Other PTMs, such as detyrosination and polyglutamylation, do not promote stability, but often amass on already stable microtubules (Song and Brady 2015; Baas et al. 2016).

As mentioned above, acetylation is one of the dominant PTMs thought to regulate microtubules. Acetylation of tubulin primarily occurs on lysine 40 of the alpha subunit, which is intraluminal (inside the tube). Shown to accumulate on long-lived microtubules, acetylation of alpha tubulin has been shown to actually provide resistance to mechanical breakage of tubulin filaments by making the microtubules more flexible (Janke and Montagnac 2017; Xu et al. 2017). The protein responsible for the addition of the acetyl group is aTAT1. There are several theories of how aTAT1 enters the microtubule lumen. One of the most convincing theories is that aTAT1 enters through cracks in the microtubule during strain or depolymerization, thereby creating a “sensing” mechanism in which microtubules that repeatedly experience mechanical strain are modified to resist damage (Janke and Montagnac 2017).

In addition to being acetylated, microtubules can be de-acetylated. One protein involved in deacetylation is histone deacetylase 6 (HDAC6) (Li and Yang 2015). Knock out of HDAC6 in mice lead to hyper-acetylation of alpha tubulin, which indicates that HDAC6 is one of the major deacetylation proteins in mammals (Zhang et al. 2008). Deacetylation of tubulin leads to decreased stability and microtubule catastrophe (Matsuyama et al. 2002). Considering the
importance of long-lived microtubules in neurons, the consequences of acetylation or
deacetylation could be very important in this setting. This project will be looking at how
acetylation is impacted by changes in environmental stiffness to further understanding of
regulation of acetylation in neurons.

**Septin**

Septins are a family of GTP binding proteins capable of forming large polymeric
structures, like actin and tubulin, are recently being considered as part of the cytoskeleton
(Mostowy and Cossart 2012). In addition to polymerizing into large structures, septins have been
shown to recognize curvature at the micron scale, which is much larger scale of curvature than
other curvature sensing proteins like BAR domain proteins or Dynamin (Cannon et al. 2017).

Humans have 13 individual septins that are separated into four families based on
homology; Septin 2 subgroup (septins 4, 5, 1, 2), Septin 3 subgroup (septins 3, 9, 12), Septin 6
subgroup (septins 6, 11, 8, 10, 14), and Septin 7 subgroup (septin 7). While some septins are
ubiquitously expressed, others are tissue dependent. For example, septin 2, septin 6, septin 7, and
septin 9 are all ubiquitously expressed, while septin 3 is only expressed in the central nervous
system (Dolat et al. 2014).

Most septins have four main domains in their structure; a polybasic region that binds
phosphoinositides near the N-terminus, a conserved GTP-binding domain, a septin unique
domain, and most members have a coiled-coil domain near the C-terminus (Neubauer and Zieger
2017). Septins have a high affinity for each other and form both linear palindromic hexameric
and octomeric complexes of multiple septins. Hexameric septin complexes consist of tandem
triplets of subgroups 2, 6, 7 where septin 2 is repeated in the center (7-6-2-2-6-7), while octamers
include a member of septin 3 family at the termini (3-7-6-2-2-6-7-3). The binding partners within
these repeats can be interchanged for any member of the subgroup (Bukharaeva and Khuzakhmetova 2018). When septins from these hexameric and octomeric polymers, they can then assemble into higher order structures, such as filaments, rings and gauze cages (Tokhtaeva et al. 2015). These higher order septin structures are thought to be the functional form throughout cell biology, where they can act as scaffolds to control protein localization or create diffusion barriers. Individual septins have complex interactions with other septins and with other proteins. Septin interactions are mediated by the makeup of their hexamer or octamer and the locally expressed septin isoforms (Spiliotis and Nakos 2021). With regards, to microtubules septins have a complex interactions; Sept9 dimers and octamers containing Sept9 have been shown to increase microtubule stability (Bai et al. 2013; Spiliotis and Nakos 2021), while Sept7 dimers and hexamers containing Sept7 can interact with HDAC6 and cause deacetylation of microtubules, leading to increased depolymerization (Ageta-Ishihara et al. 2013).

Within neurons septins participate in numerous processes such as neuronal morphogenesis and synaptic transmission via vesicle release. For example, in hippocampal neurons, when either septin 2, 6, or 7 is overexpressed it caused increased branching and protrusion density (Tada et al. 2007). Changes in septin expression have also been implicated in several neurological disorders and diseases including Alzheimer's disease, bipolar disorder, schizophrenia, down’s syndrome, and Parkinson’s (Bukharaeva and Khuzakhmetova 2018).

**Septin 9**

A member of the septin 3 subfamily, Sept9, is of particular interest to this project because of its interaction with microtubules. Sept9 has an amino-terminal domain that is able to bind and bundle microtubules. Sept9 is the only septin paralog that has this specific domain, though other septins may be able to interact with microtubules (Kuzmić et al. 2021; Spiliotis and Nakos).
In addition to binding to microtubules, Sept9 is capable of bundling microtubules. In a purified protein experiment addition of purified Sept9 to purified tubulin caused more microtubule bundling than with tubulin alone (Bai et al. 2013). The association of Sept9 with microtubules can also impact microtubule-dependent motor dynamics.

Microtubule-associated Sept9 interacts with kinesin-like protein 17 (KIF17), in KIF17’s cargo-binding conformation via Sept9’s C-terminus. This interaction between Sept9 and KIF17 inhibits kinesin-dependent NMDA receptor subunit 2B delivery to the dendrites of hippocampal neurons (Bai et al. 2016). Mutations in the microtubule-binding domain that inhibit microtubule bundling activity also led to neuralgic amyotrophy, suggesting that bundling activity is a critical function of septin in neurons. (Seror 2017).

In addition to its microtubules binding capabilities, Sept9 is of interest to this project because it is also involved in mechanosensing pathways. It has been demonstrated in endothelial cells that on soft substrates, Sept9 was more highly expressed than on hard substrates (Yeh et al. 2012). In addition, knock down of Sept9, using shRNA, increased RhoA activity on soft substrates but not hard substrates (Yeh et al. 2012). There are several Sept9 isoforms that may be regulated differently depending on tissue expression or mechanical signal (Lam and Calvo 2019). How septins are involved with mechanosensing in neurons has not been thoroughly investigated and will be investigated in this project.
CHAPTER THREE

RESULTS

Aim 1: Determine the effect of matrix stiffness on microtubule acetylation in SH-SY5Y cells.

To examine whether or not changes in environmental stiffness have an impact on tubulin acetylation in SH-SY5Y cells, western blot lysates were collected from undifferentiated SH-SY5Y cells plated on either glass or polyacrylamide gels, either soft or medium stiffness, coated with laminin. Glass coverslips were first coated with poly-L-lysine and subsequently incubated with 50ug/ml laminin. Polyacrylamide gels were first functionalized with the bifunctional crosslinker sulfo-SANPAH and then incubated with 1mg/ml laminin. A higher concentration of laminin was needed to coat the polyacrylamide gels due to the poor crosslinking of the sulfo-SANPAH. Previous studies have shown that at these concentrations of laminin the amount of protein on the surface should be roughly equivalent between the glass and polyacrylamide substrates (Aratyn-Schaus et al. 2010). Each coverslip was collected individually and run as separate samples in western blots (Fig. 1A).

Data are presented in the form of a box plot, which shows the distribution of a data set in four quartiles. The line in the middle of the box is the median of the data set, the box itself is the middle 50% of scores, the lines extending from the top and bottom of the box are the top and bottom 25% of scores. The values being depicted are of the ratio of mean intensity of acetylated tubulin and the mean intensity of alpha tubulin, referred to from here on as the tubulin ratio. Using a linear model for these data, mean tubulin ratio values were predicted, and significance was determined. Western blots of lysates from undifferentiated cells plated on glass did not
indicate a significantly higher tubulin ratio compared to cells plated on 16kPa or 500Pa (Fig. 1B). There was also no statistical difference in the tubulin ratio between cells plated on 15kPa and 500Pa (Fig. 1B). Detailed methodology of each experiment is provided in the methods section (Chapter 6), the methods section.

Though there was no significant difference between the tubulin ratios in the western blots, there appeared to be a trend of increased acetylation on the stiffer surfaces. Immunostaining was used to investigate the tubulin ratio in undifferentiated SH-SY5Y cells, in order to further explore the acetylation response of SH-SY5Y cells on substrates of different stiffness.

Cells were plated on the same three stiffness conditions as before, which included glass, 500Pa, and 16kPa. Cells were fixed using a protocol that was developed to preserve the cytoskeleton that combines paraformaldehyde, glutaraldehyde, and a buffer that helps stabilize the skeleton. The cells were then stained using primary antibodies for alpha and acetylated tubulin and imaged on a Zieiss LSM Airyscan 880. It was immediately apparent that the morphology of cells plated on the stiffer surfaces was different, as the cells were larger and had more protrusions (Fig. 2A). A linear model that relates the whole cell tubulin ratio with stiffness predicted that cells plated on glass would have an average tubulin ratio of 0.6, which is higher than the ratio for cells plated on gels of 16kPa (***P<0.0001) and 500Pa (**P<0.001) (Fig. 2B). There was no difference between cells plated on the different stiffness gels (P>0.05, Fig. 2B).

Interestingly, when analysis was restricted to protrusions of undifferentiated cells the tubulin ratio of cells plated on glass, predicted to have a mean of 0.3, was significantly higher than cells plated on 16kPa. Protrusion analysis of cells plated on 16kPa are predicted to have a decrease in the ratio of 0.2 compared to cells plated on glass (***P<0.0001) (Fig. 2C). Cells plated on 16kPa and 500Pa were not found to be different (Fig. 2C).
Figure 1. Bulk Lysates of Undifferentiated Cells Demonstrate No Significant Differences in the Ratio of Acetylated to Alpha Tubulin on Different Substrates.

A. Representative western blot image of alpha tubulin and acetylated tubulin from undifferentiated SH-SY5Y cells. Samples were plated on either glass or polyacrylamide gels with a stiffness of 16kPa or 500Pa. B. Quantification tubulin ratio, glass (n=9), 16kPa (n=9), and 500Pa (n=8). Data are represented in a box plot, showing the distribution for each stiffness condition. The box represents the data spread from the 25 to the 75th percentile, the line in the middle of the box is the median value. The whiskers represent the spread of the lowest and highest quartiles.
Figure 2. Analysis of Fluorescent Intensity in Undifferentiated SH-SY5Y Cells Shows Cells Plated on Glass Have the Highest Ratio of Acetylated to Alpha Tubulin.

A. Representative immunofluorescent images of undifferentiated SH-SY5Y cells on glass, 16kPa, or 500Pa gels, at 63x (oil). In the merged image in the first column, alpha tubulin is green and acetylated tubulin is magenta. B. The whole cell analysis is the mean intensity of each channel of all the cells in the image. The graph is the ratio of mean intensities of each channel of all the cells in the image. The graph is a ratio of mean intensities from these images, (Glass n=29), (16kPa n=24), and (500Pa n=21). C. Protrusion analysis was done by hand drawing maps of protrusions and measuring the mean intensity in each channel. The tubulin ratio is depicted in the graph (glass n= 39), (16kPa n= 89), and (500Pa n=58). ***P<0.0001 **P<0.001. B, C are box plots which presents the data distribution for each stiffness condition. The box represent the
data spread from the 25th to the 75th percentile, the line in the middle of the box is the median value. The whiskers are the lowest and highest 25 quartiles.

There substantial changes in morphology and expression of several proteins in SH-SY5Y cells after differentiation (Agholme et al. 2010; Dwane et al. 2013; Mendsaikhan et al. 2018). Therefore, the question explored was whether acetylation response to stiffness was the same in differentiated SH-SY5Y as in the undifferentiated cells. SH-SY5Y cells were differentiated with a seven day protocol by a gradual decrease in serum, and treatment with retinoic acid (Serdar et al. 2018; Agholme et al. 2010; Dwane et al. 2013). After differentiation, the cells developed much longer and more complex protrusions and overall gained a more neuronal appearance (Fig. 3A). Neurofilament light chain (NEFL) and microtubule associated protein 2 (MAP2) were used on western blot lysates collected at specific points during a differentiation to explore whether or not the differentiation caused protein expression changes in SH-SY5Y cells. NEFL and MAP2 are known markers for adult neurons (Cockova et al. 2019).

After differentiating the SH-SY5Y cells the same immunofluorescence approach to analyze the tubulin ratio was used. In contrast to the undifferentiated cells, the differentiated cells exhibited a significant increase in acetylated tubulin to alpha tubulin on the soft substrates (Fig. 4).

In whole cells analysis, the tubulin ratio of cells on 500Pa gels had predicted true mean of (1.55), which was approximately 3-fold higher than that of cells plated on either glass or 16kPa gels (0.5 **P<0.001). No statistical difference was seen between differentiated cells plated on glass and 16kPa (Fig. 4B).
Figure 3. Differentiation of SH-SY5Y Cells Causes Changes in Morphology and Increases in Expression of Neuronal Markers.
A. Phase contrast images of SH-SY5Y cells at 20x magnification, on day 1 (A) and day 7 (B) of differentiation. Yellow box denotes zoomed in area shown in the panels to the right. C. Western blot of NEFL, and MAP, from cells collected at the indicated time points during differentiation.
Figure 4. Differentiating SH-SY5Y Cells Changes the Mechanosensitive Acetylation Response, Showing Cells Plated on Soft Gels Have the Highest Ratio of Acetylated to Alpha Tubulin.

A. Representative immunofluorescent images of differentiated SH-SY5Y cells on glass, 16kPa or 500Pa gels, at 63x (oil). In the merged image in the first column, alpha tubulin is green and acetylated tubulin is magenta. B. The whole cell analysis is the mean intensity of each channel of
all the cells in the image, the graph is the ratio of mean intensities from these images. (glass n=4), (16kPa n=6), (500Pa n= 6). C. Protrusion analysis of the same images. The tubulin ratio is depicted in the graph (glass n=35), (16kPa n=48), (500Pa n= 48). **P<0.0001 ***P<0.001 B, C. The box plots show the data distribution for each stiffness condition. B, C are box plots which presents the data distribution for each stiffness condition. The box represent the data spread from the 25th to the 75th percentile, the line in the middle of the box is the median value. The whiskers are the lowest and highest 25 quartiles.

When analyzing only the protrusions of differentiated cells, the tubulin ratio of cells plated on 500Pa gels was still significantly higher than cells plated on glass and 16kPa (**P<0.001) (Fig. 4C) with a predicted mean tubulin ratio of 1.21. This was the same trend seen in the whole cell analysis. The magnitude of the effect, however, was reduced with the ratio only approximately 2-fold higher versus the 3-fold increase seen across the entire cell. As in the whole cell analysis, the tubulin ratio in protrusions in cells plated on glass and 16kPa was not significantly different (P>0.05) (Fig. 4C). These results illustrate that differentiated SHY5Y cells are sensitive to their substrate stiffness.

**Aim 2:** Determine the role of matrix stiffness in Sept9 organization and the impact of Sept9 on microtubule acetylation.

It was hypothesized that septins played a role in mediating microtubule acetylation in response to substrate stiffness because of their known association with microtubules. To investigate this, cells were immunostained for Sept9, actin and tubulin in differentiated SHY5Y cells and differentiated SH-SY5Y cells in which Sept9 expression was knocked down, Sept9 knock down cells. Using differentiated SHY5Y cells, immunostaing of Sept9, actin and microtubules was done (Fig. 5). Qualitatively, it appeared that Sept9 preferred strong regions of curvature (Fig. 5B), consistent with previously published results ([Bridges et al. 2016; Cannon et al. 2019](Bridges_etal_2016_Cannon_etal_2019)). Interestingly, Sept9 appeared to colocalize with F-actin more frequently than with acetylated tubulin. There were regions, however, where the acetylated tubulin and Sept9
overlapped, particularly where the acetylated tubulin appeared in longer protrusions (Fig. 5C). This colocalization could potentially indicate a role either in stabilizing tubulin or it could be acting as a signaling scaffold for other events (Fig. 5C).

Figure 5. In Fixed Images Sept9 Colocalizes with Actin More Than Microtubules
A. Images obtained on a 3i Spinning Disk with 100x oil, Merged image of all three channels with Sept9 in magenta, acetylated tubulin in cyan, and phalloidin (F-actin) in yellow. Box 1 and Box 2 in the merged image correspond to magnified regions shown in B and C, respectively. Individual channels split and depicted in grayscale. B. Box1 depicts Sept9 localizing to regions of curvature. C. Box2, depicts a long acetylate microtubule protrusion with potential Sept9 colocalization.

To understand the role that sept9 may be playing in the stability or acetylation events of tubulin in response to matrix stiffness, an shRNA was used to knock down Sept9. Differentiation of wild type cells (Fig 6.) was carried out in parallel to knock down cells, and plated on glass,
16kPa, or 500Pa gels. Cells were differentiated using the same protocol as described above (Fig. 26 4).

First, the data sets were analyzed individually as just wild type or just knockdown. When analyzed alone the differentiated wild type cells, consistent with the results described in the previous section the whole cell fluorescence tubulin ratio of cells plated on 500Pa gels was higher than cells plated on glass (**P<0.001), with a predicted mean ratio value of 0.7 (Fig. 6). However, unlike previous data, the acetylated to alpha tubulin ratio of cells on 16kPa gels was also increased and not significantly different than cells plated on 500Pa gels (P>0.05) (Fig. 6B). The tubulin ratio of cells on glass was significantly lower than cells on both 16kPa and 500Pa (**P<0.001), expected mean tubulin ratio of 0.25. When only the protrusions of differentiated cells were analyzed, results were consistent with the previous results, in which tubulin ratio was significantly higher on the softest gel. The estimated tubulin ratio for cells plated on 500Pa was 1.05, similar to previous differentiated cells’ mean ratio values in protrusions (Fig. 6C).

In contrast to wild type differentiated cells, when analyzed as a separate data set differentiated cells where Sept9 had been knocked down by shRNA construct 19070 the whole cell intensity fluorescence tubulin ratio of cells plated on glass was not statistically different than cells plated on gels of 16kPa or 500Pa (P>0.05) (Fig. 8B), both predicted mean tubulin ratio values were around 0.5. However, the ratio for cells plated on 500Pa, predicted to be 0.5, was still significantly higher than cells plated on 16kPa (*P<0.01) (Fig. 8B). There was no significant difference between 500Pa and glass (P>0.05). Similar results were found when only the protrusions were analyzed, suggesting that the Sept9 knock down had a universal effect (Fig. 8C).
Figure 6. Wild Type Repeat of Differentiation Shows Increase in Acetylation on Soft Substrates.

A. Representative immunofluorescent images of differentiated SH-SY5Y cells on glass, 16kPa or 500Pa gels, at 63x (oil). In the merged image in the first column, alpha tubulin is green and acetylated tubulin is magenta. B. Whole cell analysis of differentiated cells, graphic representation of ratio of mean intensities. (Glass n=16), (16kPa n=16), (500Pa n=15). C. Protrusion analysis of the same images. The tubulin ratio is depicted in the graph (glass n=38), (16kPa n=41), (500Pa n=43). ***P<0.0001 ** P<0.001 *P<0.01 B, C. Are box plots which presents the data distribution for each stiffness condition. The boxes is the data spread from 25-75th percentile, the line in the middle of the box is the median value. The whiskers are the lowest and highest 25 percentiles.
Figure 7. Western Blot Confirmation of Septin 9 Knock Down by shRNA in SH-SY5Y Cells.
A. Western blot of non-muscle myosin IIB. TRCN is the identification number given by Sigma-Aldrich to each of the Sept9 shRNA constructs. Control cells were not treated with lenti viral media, lane two are cells treated with shRNA construct 19068, lanes 3 and 4 respectively were treated with shRNA construct 19070 and 19071. TRCN 19070 was the chosen construct used for sept 9 knockdown cells. B. Western blot of alpha tubulin for the same samples used in A. C. Western blot of Sept9 using the same samples as A. TRCN 19070 was the only construct that appeared to have a strong decrease in expression of Sept9.
Figure 8. Sept9 knock down Decreases Ability of SH-SY5Y to Have a Stiffness Dependent Acetylated to Alpha Tubulin Response.

A. Representative immunofluorescent images of Sept9 knock down SH-SY5Y cells that have been differentiated on glass, 16kPa or 500Pa gels, imaged at 63x (oil). In the merged image in the first column, alpha tubulin is green and acetylated tubulin is magenta. B. Whole cell analysis of differentiated cells, graphic representation of ratio of mean intensities. (Glass n=15), (16kPa n=12), (500Pa n=12) C. Protrusion analysis of the same images. The tubulin ratio is depicted in the graph (glass n=15), (16kPa n=17), (500Pa n=16). *P<0.01 B, C. Are box plots which presents the data distribution for each stiffness condition. The boxes is the data spread from 25-75th percentile, the line in the middle of the box is the median value. The whiskers are the lowest
and highest 25 percentiles.

After analyzing each data set, either wild type or knock down cells, as separate experiments, to be able to compare one experiment to another using a more complex linear regression model which is described in the methods sections of this paper. This linear model included an interaction term between cell-type, which describes whether or not the cells are wild type or knock down, and stiffness. This interaction term means that when controlling for cell type and controlling for stiffness as individual factors, there is a significant relationship between cell type and stiffness.

In the whole cell analysis using the linear model that compared wild type cells to knock down cells, the difference between glass and either gel, 16kPa or 500Pa, is statistically significant. The predicted difference in the tubulin ratio in wild type cells plated 16kPa gels was 0.22 (**P<0.0001) higher than knock down cells tubulin ratio 16kPa. For wild type cells plated on 500Pa, it is predicted that the tubulin ratio will be 0.17 (**P<0.001) points higher than knock down cells plated on 500Pa. In whole cell analysis, there was not a statistical difference between wild type cells and knockdown cells when comparing 16kPa to 500Pa.

In the protrusion analysis comparing wild type to knock down cells, there was again a statistical difference between glass and 500Pa gels, but not between glass and 16kPa gels. Wild type cells plated on 500Pa did have a significantly higher tubulin ratio (0.62 ***P<0.0001) points higher than the ratio of knock down cells plated 500Pa gels.
CHAPTER FOUR
DISCUSSION

The current literature is conflicted regarding acetylation of tubulin as a player in mechanosensitivity. In theory, it makes sense that it would be. Microtubules are long, stiff tubes that are subject to mechanical loads, especially in neurons. Acetylation alters the microtubule’s mechanical properties. It is logical then that cells would utilize this modulatory PTM to sense or respond to mechanical loads. However, previous analysis of cells using western blots suggested that tubulin acetylation decreased as a function of stiffness (Heck et al. 2012), while other analysis relying on immunofluorescence microscopy suggested the opposite, that tubulin acetylation increases with increased stiffness (Seetharaman et al. 2020). Collectively, this data is consistent with these previously published reports that the acetylation ratio can be mechanosensitive, and provides some insight into how the method of collecting this data can impact the observed results. In addition, it was found that the differentiation status of the cells played an important role.

Our original hypothesis was that increased matrix stiffness would lead to increased acetylation of microtubules. This was motivated by data showing that cells on stiffer substrates have enhanced integrin activation and downstream signaling (Lv et al. 2015), leading to increased acetylation (Wickström et al. 2010). However, difference was observed in tubulin acetylation ratio via western blot analysis in undifferentiated cells regardless of substrate stiffness (Fig. 1). Immunofluorescence analysis, however, did reveal a relative increase in acetylated tubulin for undifferentiated cells on glass compared to either gel stiffness (Fig. 2).
A difference was not detected between undifferentiated cells on the different gels (Fig. 2B). It was anticipated that these two assays, western blot and immunofluorescence, would parallel one another and reinforce observed trends in acetylation, but these initial data suggested that acetylation might be sensitive to fixation/lysis conditions and the assay used to detect tubulin acetylation is important, future experiments might tease apart these possibilities.

To better understand why differences in acetylation were not observed that were similar to the published literature, the general morphology of neuronal cells was considered. The fact that even undifferentiated neuronal cells, like neuroblastoma SH-SY5Y cells, make microtubule-dense protrusions to explore their surroundings was also taken into account. Since SH-SY5Y cells form relatively long-lived protrusions, dependent on stable microtubules, it was hypothesized that collecting data from the soma might obscure differences in the protrusions. Therefore, an immunofluorescence analysis focusing solely on the protrusions done (Fig. 2C). Unlike the whole cell analysis, in the undifferentiated cells the protrusions did not display a significant difference in acetylation ratio between the three stiffnesses in acetylation.

Overall, in the undifferentiated cells the data on mechanosensitive tubulin acetylation remains unclear. Significant differences was only detected when considering the whole cell immunofluorescence data (Fig. 2B). For all other data, no statistical difference was observed between undifferentiated cells on glass or gels of any stiffness. These results are thus only somewhat consistent with the results of Seetharaman’s paper which found that primary rat astrocytes had increased acetylation on stiffer substrates (Seetharaman et al., 2020).

There are a few subtle differences between the approaches that could account for the differences. First and foremost, this could be a cell type dependent response, as Seetharaman’s group used primary astrocytes and this study used the neuroblastoma stable cell-line SH-SY5Y.
Second, laminin was used as the ECM protein instead of collagen, as laminin is the standard substrate used in many neuronal studies. Third, the “stiff” substrate was only 16kPa compared to their stiffest which was 48 kPa. Previous research has suggested that the change in stiffness sensing by cells happens around 8 kPa and that it is insensitive to different ECM ligands (Oakes et al. 2018), therefore, it is thought a more likely cause of the lack of correlation between stiffness and acetylation is due to cell type. Initially it was hoped that primary neuronal cultures could be used, to more closely replicate and build off the previous literature while also using more “neuronal” cells. However, due to both Covid restrictions and animal facility troubles, instead differentiation of SH-SY5Y cells was chosen to investigate whether or not differentiation could have an impact on the ratio of acetylation.

During and after differentiation, SH-SY5Y cells develop longer stable protrusions than undifferentiated cells, which theoretically might rely more on acetylation for increasing microtubule stability and the life of these protrusions. Interestingly, the differentiated cells had the highest tubulin ratio on the softest gel, the opposite of this project’s original hypothesis (Fig 4). This trend was true using both the whole cell analysis and the protrusion analysis. Suggesting that the microtubules in protrusions in the differentiated cells could be more sensitive to mechanical loads than their counterparts in the undifferentiated cells. These assays are carried out over multiple days, providing enough time that these changes could be due to either transcriptional/translational changes or localized cytoplasmic signaling. The later was chosen as the focus of testing, specifically investigating a relatively unexplored member of the cytoskeleton family: septins. Septins became the focus because they have a complex relationship with microtubules, their PTM regulation, and stability (Spiliotis 2018). Sept9 has been shown to
interact with microtubules directly and increase their stability (Bai et al. 2013). However, other septins, like Sept7, modulate microtubule PTMs via interaction with HDAC6, which leads to microtubule acetylation and increased catastrophe (Ageta-Ishihara et al. 2013).

These features together make Sept9 an intriguing potential binding partner and regulator of microtubule acetylation. To investigate this interaction and its potential involvement in mechanosensitive pathways, immunofluorescence was used to observe Sept9 co-localization. In preliminary fixed images, Sept9 appeared to localize strongly to regions of membrane curvature (Fig. 6), which is a known property of septins (Bridges et al. 2016). Despite known microtubule interactions, Sept9, in fixed images, appeared to primarily co-localize with the actin cytoskeleton and the cell cortex. This Sept9 localization did not readily support a strong subcellular interaction between Sept9 and microtubules. There were, however, some regions of acetylated tubulin with extreme curvature where Sept9 did appear to be localized (Fig. 6). While significant direct colocalization of Sept9 and microtubules was not observed, it remains possible that these proteins are interacting dynamically and transiently. If this were the case, live-cell imaging with fluorescently-tagged proteins would be more appropriate than image analysis of fixed cells to discern these interactions.

As an alternative approach to investigate potential Sept9 roles in regulating microtubule acetylation, Sept9 was chose to knock-down using shRNA. This assay was performed in differentiated cells, since previous assays saw the strongest mechanosensitive tubulin acetylation response in this condition. Unfortunately, in this round of differentiation, the SH-SY5Y morphology was not as pronounced as in previous differentiation experiments (Fig. 5), begging the question of whether the cells had differentiated successfully. It was hypothesized that this was due to a change in the brand of laminin used to coat the surface of both the glass and the
gels. The brand was changed due to a backorder of laminin which would not have allowed completion of the experiment in time. This laminin also appears to have gelled prematurely into clumps, thus not evenly coating the surface. Either changing brands or early gelling of laminin could explain why the cells did not look the same as previous differentiation experiments and demonstrates how critical consistency is in experimental replication and how neuronal differentiation is partially dependent on ECM-mediated signaling pathways. Perhaps future experiments using micropatterned fluorescently-tagged laminin could take advantage of this observation to directly compare differentiation and tubulin acetylation of undifferentiated and differentiated cells in the same dish.

Despite this difference in morphology, the wild type cells still exhibited a significant increase in acetylation ratio on soft substrates compared to glass (Fig. 5). There was not, however, a difference between the soft and stiff gels. The protrusion analysis again showed more sensitivity between stiffness than the whole cell analysis (Fig. 5C), with a significantly higher tubulin ratio seen on the softest gel compared to the stiff gel and glass. The tubulin ratio being significantly different between 16kPa and 500Pa in only the protrusion analysis again points towards the protrusions of differentiated cells being more sensitive to changes in the mechanical environment. This may be because the differentiated SH-SY5Y cells are more like adult neurons and put more energy into maintaining stable protrusions than migrating like neuronal precursors.

While the wild-type cells did not appear to fully differentiate in this experiment, the Sept9 knock down cells were even less differentiated (Fig. 8). Originally it was predicted that Sept9 could aid in microtubule stability because of its microtubule binding domain, and potentially, Sept9 could aid in a mechanosensitive pathway of tubulin acetylation. After differentiation, the Sept9 knock down cells in fixed images qualitatively had very little branching
and shorter protrusions when compared to the wild type cells (Fig. 7A). This could indicate that Sept9 plays an important role in the branching and maintenance of protrusions in differentiating cells. Considering septin localization to points of curvature, like the base of branches and protrusions, a role in this process is logical. The fixed image whole cell analysis of differentiated Sept9 knock down cells showed that cells on soft gels had slightly higher acetylation than those on stiff gels but were not different from glass (Fig. 8B). The protrusion analysis also showed there was no difference between any of the stiffnesses. These results indicate that loss of Sept9 is having a significant impact on the morphology and structure of the cell compared to wild type cells (Fig. 8C). It is, however, unclear whether knock down of Sept9 is directly mediating a change in microtubule acetylation, or whether it is merely a consequence of the change in morphology. Future experiments might explore this by using temporal control of Sept9 knock down. One could allow cells to differentiate, induce Sept9 knock down, and observe if the microtubule acetylation or the cell morphology is affected first.

There are many potential pathways that Sept9 could be acting on in order to affect microtubule stability. It could be involved in a signal cascade that leads to acetylation of tubulin through interaction with Rho GTPases. Another possibility is that Sept9 is involved in the prevention of deacetylation by binding microtubules and blocking proteins that would deacetylated alpha tubulin, such as HDAC6. Further experiments using point mutations in Sept9 that specifically inhibit interactions with microtubules, HDAC6, and other putative binding partners, coupled with live-cell imaging will be needed to explore these many intriguing options.
CHAPTER FIVE

CONCLUSIONS

Taken together, these results indicate that acetylation of alpha tubulin is mechanosensitive, as acetylation does change depending on environmental stiffness. This response appears to be finely regulated, and dependent on cell type and differentiation status. This preliminary data suggests that Sept9 is an intriguing candidate to mediate this process, as knock-down of this protein abrogated the difference between soft and stiff substrates. Future experiments will be needed to determine whether Sept9 is playing a direct or indirect role in regulation of microtubule acetylation.
CHAPTER SIX

METHODS

Polyacrylamide Gels

To manipulate the stiffness of the extracellular environment polyacrylamide gels were used to examine the effects of changing stiffness on cytoskeletal organization. Glass coverslips were activated using 2% 3-aminopropyltrimethoxysilane in isopropanol, and 1% glutaraldehyde solution in deionized H2O. Rain-x was put on a glass slide and rubbed into the surface using a Kimwipe. The glass slide was then rinsed with water to create a hydrophobic surface. A master mix of each stiffness was prepared by mixing 2% bis-acrylamide and 40% acrylamide with water. The master mix is then added to the gel solution with water and fluorescent beads. Tetramethyl ethylenediamine is added as a catalyst and lastly 10% ammonium persulfate is added to start the polymerization. 6.5μl of gel solution is placed on a glass slide and an activated coverslip is placed on top to spread out the gel. The gel is allowed to polymerize for 40 min and is then placed into deionized water for at least 1hr. 300μl of Sulfo-SANPAH is added to the surface of the gel at a concentration of 50mg/ml. The gel with Sulfo-SANPAH was then placed in an ultraviolet oven for 5 min. The gel was rinsed in deionized water until clear. The gel was coated with 1mg/ml laminin for 1hr at 37°C. Cells were then seeded on the gel.

Cell Culture

SH-SY5Y cells were obtained from American Type Culture Collection, passage 1. Medias used were, Minimum Essential Medium with Earle’s Salts, L-glutamine, 92mg/ml D-Valine were, Minimum Essential Medium with Earle’s Salts (EMEM), L-glutamine, and 92mg/
ml D-Valine from Cassion Labs. Ham’s F-12 1x modified with L-glutamine from Corning. Cells cultured in 1:1, EMEM:Ham’s F12 supplemented with 10% fetal bovine serum, and Antibiotic Antimycotic. Cells were split when they reached 70-80% confluence. Cells were lifted with TrypLE Express from Gibco. Cells were spun down for 4min at 0.9 relative centrifugal force before re-suspending and re-plating. Cells were not used over passage 15 due to decreases in neuronal morphology and known loss of neuronal characteristics with passage. HEK-293-FT cell were passed when they reached 80% confluent and were never plated below 50% confluent. HEK-293-FT cells were cultured in Dulbecco's Modified Eagle Medium Corning™ Dulbecco's Modified Eagle Medium, with L-Glutamine, 4.5g/L Glucose and Sodium Pyruvate, supplemented with 10% FBS and anti-anti.

**SH-SY5Y Differentiation**

To observe morphology closer to that of primary neurons, SH-SY5Y cells were differentiated using a protocol similar to previous studies (Agholme et al. 2010; Serdar et al. 2018; Dwane et al. 2013; Shipley et al. 2016). Cells were plated on glass coverslips or polyacrylamide gels coated with 1mg/ml laminin. Cells were plated at approximately 60% confluence (dependent on surface area) using regular growth media. After 24 hours, the media was replaced with media 1, consisting of EMEM: Ham’s F-12, with 2% FBS, and 10uM trans-Retinoic Acid (EMD Millipore, re-suspended fresh in 95% EtOH). Cells are incubated in media 1 for 72 hrs, with a single media swap after 48hrs. After 72 hrs in media 1, cells are transitioned to media 2, consisting of EMEM:F-12, with 1% FBS, and 10uM trans-Retinoic Acid. The cells are incubated in media 2a for 72 hrs, with a single media swap at 48 hrs. Cells are ready to use on day 7.
Lenti-Viral Transfection

HEK-293-FT cells were plated at approximately 80% confluent. Media was changed on HEK-293 FT cells 30-60 min before transfection and replaced with normal media. The SignaGen LipoD293 transfection protocol for lentivirus was followed for the transfection of HEK cells. Lentiviral packaging plasmids included psPax.2 and pMD2.G. The ratio of DNA used was 3:3:1 for the plasmids psPax, DNA of interest, pMD2.G. DNA of interest was Sept9 shRNA construct form Sigma-Aldrich MISSION shRNA for Sept9 TRCN:0000119070.

DNA and LipoD293 Transfection Reagent from SignaGen, were allowed to incubate for 15min. The complexes were then added to the dish of HEK cells, and treated for 24hrs. The media was then removed and placed into bleach. Media on the HEK cells was replaced with. 24hrs after the media is changed the first time the media is collected and stored at -80c. Lenti meida is collected and stored 48hrs and 72hrs after transfection. The day before treatment of SH-SY5Y cells with lenti virus, plate SH-SY5Y cells so that they are 80% confluent. The lenti viral media collected from the HEK cells must be thawed and is then mixed 1:1 with normal SH-SY5Y media. Polybrene from Sigma-Aldrich, was added to media mixture at a concentration of 8ug/ml and put on SH-SY5Y cells. Media was removed from SH-SY5Y cells and put into bleach and replaced with SH-SY5Y selection media 24hrs post lenti treatment. Selection was done using 1.5ug/ml puromycin added to SH-SY5Y normal media. For fluorescent markers there is no drug selection process.

Fix and Stain Buffers

Cytoskeletal buffer was made with double deionized water, 80mM 1,4-Piperazinediethanesulfonic acid sodium salt or PIPES, 1mM $MgCl_2$, 5mM EGTA, ethylene glycol-bis(β-aminoethyl ether)-N,N′,N′,N′-tetraacetic acid, 0.05mM EDTA,
Ethylene diaminetetraacetic acid, 4% PEG-8000, Polyethylene glycol. The cytoskeletal buffer PH 41 was brought to 6.8 using KOH, potassium hydroxide. The Fix-perm solution was made using a cytoskeletal buffer with 3.7% formaldehyde, 0.075% glutaraldehyde, and 1% TritonX-100. Fixative solution was prepared with cytoskeletal buffer with 3.7% formaldehyde, 0.075% glutaraldehyde. The permeabilization solution was made with cytoskeletal buffer with 1% TritonX-100. Blocking buffer was made using phosphate buffered saline (PBS), 2% bovine serum albumin, and 0.02% Tween-20.

Fix and Stain Protocol

Fix-perm solution was pre-warmed to 37°C and was added to the coverslip and set on the shaker at low speed for 2 min at room temperature. The fix-perm solution was removed and then replaced with fixative solution for 15 min at room temperature. Fixative solution was removed and the sample was washed one time with PBS. Permeabilization buffer was then added, the sample was shaken slowly for 30 min at room temperature.

To remove free aldehyde groups, the permeabilization buffer is then removed and replaced with sodium borohydride, NaBH4, using a concentration of 1mg/ml, for 5 min. The sample was then washed with PBS. Sodium borohydride quench was repeated two more times 5 min each with PBS washes. After the last wash, PBS is removed, and replaced with blocking buffer at room temperature for 5 min. The sample was then inverted into 100ul of primary antibody in blocking buffer overnight at 4°C. Alpha tubulin was stained for using Abcam Anti-Tubulin antibody [YOL1/34] - Microtubule Marker (ab6161) at 1:1000. Acetylated tubulin was stained using Sigma-Aldrich Monoclonal Anti-Acetylated Tubulin antibody produced in mouse clone 6-11B-1 used at 1:200. Sample was rinsed with PBS after overnight incubation and placed in secondary antibody diluted in blocking buffer for 1hr at room temperature.
The secondary antibodies used included Abcam, Goat Anti-Rat IgG H&L (Alexa Fluor® 488) (ab150157) at 1:400, and Goat anti-Mouse IgG (H+L) Cross-Adsorbed Secondary Antibody, Alexa Fluor 647 at 1:200. After 1 hr, the sample was washed with PBS and mounted onto a glass slide using 20ul of PBS and sealed with nail polish. Slides were cured for 15min before imaging.

**Western Blot**

Western blot lysates were collected across 4 stiffness in only undifferentiated cells as a preliminary experiment to determine if protein expression was different across stiffness. Lysates were taken from individual coverslips. Each coverslip was removed from the cell culture dish and placed into a petri dish. The coverslip is washed twice using PBS. 250ul of 2x lameli buffer with BME was added to each coverslip and allowed to incubate for 3min. The coverslip is then scrapped with a rounded cell scraper.

Coverslips with gels were scraped using the rounded side of the cell scraper. Sample were boiled at 55°C for 3mins, then removed and placed on ice for 7min. Lysates were sonicated at 30kHz, for 12sec and then stored at -20°C. Bio-Rad precast 10well 4-20% gels were used for all western blots. The ladder used was Precision Plus Protein Dual Color Standards from Bio-Rad, 5ul in the first well. 40ul of sample was loaded into each well after the ladder. Gels were run at 86 volts for 30min and then the voltage was turned up to 120V. Gels were then removed from the case and activated on the chemi doc using the stain-free, protein gel setting.

Transfer is done using the GenScript eBlot™ L1 Fast Wet Transfer System for Mini Gels, using the long run protocol. Polyvinylidene fluoride or polyvinylidene difluoride membrane activated in isopropanol and then placed in eBlot membrane equilibration buffer for 1min. The transfer sandwich is then assembled using the proprietary sponge, membrane, gel. The
The cassette is then placed into the EBLOT and run on the long setting. The membrane is then removed and stained using ponceau. The membrane was washed in PBS with 0.1% Tween, to remove ponceau stain.

The membrane was blocked in 5% milk in PBS-Tween for 1 hr at RT. The blot was incubated in Primary antibodies overnight at 4°C. Acetylated tubulin was stained using Sigma-Aldrich Monoclonal Anti-Acetylated Tubulin antibody produced in mouse clone 6-11B-1 used at 1:2000. Alpha tubulin was stained using Proteintech polyclonal anti-alpha tubulin antibody at 1:3000. NEFL was stained using ABclonal Neurofilament L Rabbit pAb at 1:2000, and MAP2 was stained for using ABclonal MAP2 Rabbit pAb A2572. Septin 9 was stained for using Abclonal Septin 9 Rabbit pAb (A8657) 1:2000. Non-muscle myosin IIB was stained for with Santa-Cruz Biotech Anti-MYH10 Antibody (A-3): sc-376942. All blots were then washed in PBS-Tween three times for 10min. The blot was incubated in a secondary antibody for 1hr at room temperature. Secondary antibodies used are horseradish peroxidase conjugated secondary antibodies at a concentration of 1:2000.

**Imaging**

Images for quantification of tubulin ratio were taken on a Zeiss LSM 880. The lasers used for collection were Argon and HeNe633 lasers for the 488 channel and the 647 channel. Lasers were set using the range indicator display. The lasers were set for glass condition and maintained at the same power for all conditions. Using the 63x oil objective, frame size was 1548x1548 and sampling was set to optimal. The Speed was set to max (0.50), and averaging was set to 2. Z-stacks were taken over a range of 1.66um with the optimal step size of 0.185um. The Airyscan processing program was then applied to each image.
Image analysis

An ImageJ macro was written to automate the analysis. Images were opened and a sum-projection was made from the z-stack. The sum-projection is then thresholded and the thresholded image is then turned into a mask of all the cells. The region that has a value of 1, the cells, is turned into a region of interest (ROI). The ROI is applied to each channel to measure mean intensity within the given region.

Figure 9. Code for Whole Cell Image Analysis
A. The code was written in javascript for use with ImageJ to obtain the average intensity for each channel of an image with-in an ROI.
Figure 10. Whole Cell Analysis
A. A representative input image that the macro will open and make into a z-sum projection. B. The ROI of all the cells in the image that is generated by the macro. C. What the ROI looks like applied to each of the channels. D. The results output of mean intensity for each channel.

Protrusion analysis was performed by hand. Input images were made into a sum-projection made from the z-stack. Using the segmented line tool, individual ROIs were drawn for each protrusion. The ROIs were applied to each channel and the mean intensity was then measured for the individual protrusions. Measurements were saved in a CSV file separated by image and stiffness.
Figure 11. Protrusion Analysis
A. Input image which is turned into a z-sum projection. B. Using the segmented line tool in image J. Lines were drawn with a width of 15 pixels overlaying the protrusions and generated individual ROIs for each protrusion. C. The ROIs are then applied to each channel. D. Results for each ROI in each channel.

Statistical Analysis

The results of each channel’s mean intensities from the image analysis are compiled into a CSV file, and the ratio of acetyl to alpha tubulin is determined.

\[
\text{Tubulin ratio} = \left( \frac{\text{Mean intensity Acetyl}}{\text{Mean Intensity Alpha}} \right)
\]

Because analysis of variance tests, or ANOVAs are now often considered anachronistic, a linear regression model was used to interpret these data sets (Bullock 1990). A linear regression model is a better tool for this data set because it can discover a relationship between two variables and detect the directionality of the relationship between them, while an ANOVA is used mainly to find the average mean or the midpoint in between variables.

Using the program Rstudio, a linear model is able to predict the true mean of a data set, which is the mean of the data set if there were infinite data points. In the single variable linear
model, shown below, gamma is the value of the tubulin ratio. Gamma is the predicted mean for the tubulin ratio when $\beta_0$ is the intercept, $B_1$ is the value if $X_1$ is equal to one which is true if stiffness is 500Pa. $B_2$ is the value to add to the intercept if $X_2$ is equal to one which is true if stiffness is 16kPa. If $X_1$ and $X_2$ are both equal to zero, then the intercept value, $B_0$, is the predicted mean for the referent stiffness value in this example glass.

$$\hat{y} = \hat{\beta}_0 + \hat{\beta}_1(X_1) + \hat{\beta}_2(X_2).$$

This same model was used for both the differentiated and undifferentiated cell data sets.

Assumptions of normality, distribution, and outliers (using cook’s distance), were met by all datasets. The linear regression generates a predicted intercept, or mean value, for the data based on all the other values. The 95% confidence interval provides an interval that one can be 95% sure the true mean falls in. If the linear model’s predicted value falls in this 95% confidence interval and the interval does not include zero, it can be assumed that it is a good estimate of the true mean value of the data. Statistical significance was determined by p-values of each level within the variable “stiffness” in the model.

In order to analyze the knockdown cells in comparison with the wild type cells, a linear model was again used. However, in order to compare the wild type to knock down cells the linear model must include more elements in the equation. Cell-type which describes whether the data is either knockdown or wildtype. Stiffness was also included, and an interaction term of cell-type*stiffness in the model. This interaction between cell type and stiffness was statically significant when included in the model so it was kept in the analysis. The equation for the whole-cell data is:

$$\gamma = 0.37 + 0.08(KnockDown) + 0.25(16kPa) + 0.32(500Pa) - 0.30(KnockDown * 16kPa) - 0.25(KnockDown * 500Pa)$$

The equation for the protrusion analysis is similar:
\[ \gamma = 0.34 - 0.001(Knock\ Down) + 0.27(16kPa) + 0.71(500Pa) - 0.28(Knock\ Down \ast 16kPa) \\
- 0.62(Knock\ Down \ast 500Pa) \]

Using this equation with the interaction term allows determination of statistical difference in the tubulin ratio based not only on stiffness but on cell type as well. It also explains that there is indeed an interaction between cell type and stiffness.


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VITA

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