THE ROLE OF SQSTM1/P62 IN AXONAL DEVELOPMENT AND DEGENERATION IN AN ALS-FTLD CONTEXT

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Abstract

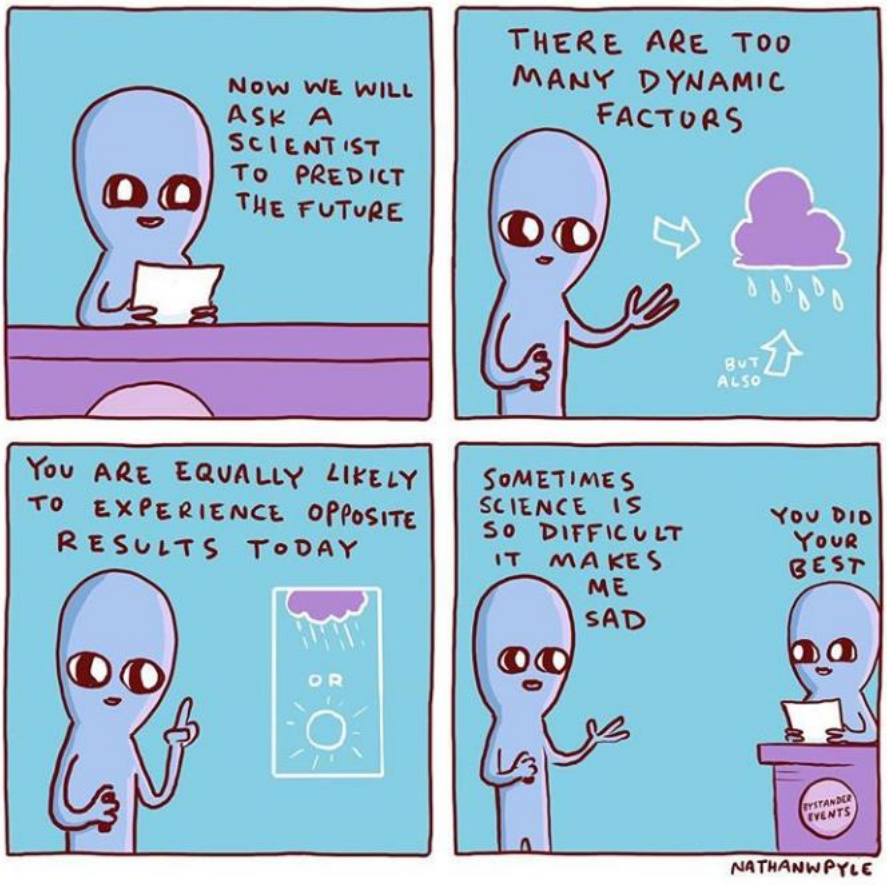
Amyotrophic lateral sclerosis and associated frontotemporal lobe dementia (ALS-FTLD) is a complex, fatal degenerative disease encompassing selective degeneration of upper and lower motor neurons, as well as neurons of the frontal and temporal brain regions, resulting in neuromuscular failure and dementia-like cognitive defects. In ALS-FTLD, disease progression is often rapid, with no effective cures currently available.

Recent studies have highlighted the contribution of dysfunctional autophagy to the development of neurodegenerative pathologies. In particular, the multi-functional autophagy receptor protein SQSTM1/P62 has been identified as a component of protein aggregates typically present in ALS-FTLD affected motor neurons. While P62 pathology has already been implicated in a wide variety of diseases, from cancer and metabolic diseases, to muscular-skeletal disorders, the $P62^{L341V}$ mutant has recently been identified as a hereditary ALS-FTLD mutation. Additionally, recent screens of FDA approved neuroleptics have highlighted the therapeutic potential of mTOR-independent autophagy activators like pimozide for ALS-related conditions. This study looks at the role that dysfunctional P62 mutations and autophagy regulation have in the axon – a highly specialised neuronal sub-unit, crucial to neuronal connectivity and thus function.

Pharmacological and transient transfection techniques were carried out in mouse primary cortical neurons. Cultures were subjected to autophagy modulating

compounds, bafilomycin A1, rapamycin and pimozide, as well as P62 siRNA constructs, and EGFP-mCherry-P62 wild-type and loss-of-function mutant constructs. Images were obtained by Zeiss 200M microscope and analysed with FIJI and Prism 8.

In brief, this work has shown that the expression of the dysfunctional $P62^{L341V}$ mutant can produce differential outcomes, dependent on the stage of neuronal development. Mutant P62 expression in developing cortical neurons results in a phenotype of increased axonal outgrowth, replicated by pharmacological autophagy inhibition. In more mature cultures, this results in increased axonal degeneration. Regardless of axon maturity, our experiments show that mTOR-independent autophagy activation by pimozide can effectively rescue such phenotypes in these models. This contributes to the growing body of evidence linking autophagy dysfunction to ALS-FTLD pathology and neurodegenerative conditions, and deepens the understanding of the impacts of autophagy dysfunction on the axon.



Strange Planet, *by Nathan W. Pyle*

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To any scientists bothering to read this horcrux of mine, please remember that good science is done when you can bring your whole self to work, fuelled by a full life beyond it and carried through by a healthy body and mind. Good science should never have to come at the expense of your health and life beyond the lab.

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1. General Introduction

1.1 Neuronal connectivity and the axon

The ability of neuronal cells to develop a functional network of axons and dendrites is essential to the ability of the nervous system to function as a fast and effective information relay system. Typically, neurons will form a single axon which allows for the conduction of information as an action potential where it is integrated into the cell body and subsequently relayed to dendritic processes. Additionally, the axon allows for the transport of essential molecules and organelles between the hub of the nucleus in the cell body and the axon tip. The development and maintenance of this asymmetrical structure is vital to the integrity of brain networks, which in turn is essential for the adequate function of the adult nervous system (T. L. Lewis, Courchet, & Polleux, 2013).

Axons are very susceptible structures, highly prone to dystrophy and dysfunction when neurons are exposed to physical or cytotoxic stressors (Son, Hee Shim, Kim, Ha, & Young Han, 2012). As such, in cases of the neurodegenerative disease, many symptoms first arise due to the loss of these functional connections, long before neuronal cell death (Bae & Kim, 2017). The correct functioning of the neuronal network is vital to the organism as a whole, but to fully appreciate the issues that can arise in cases of neuronal loss or degeneration, we must first understand how neurons develop their classic polarised morphology, and how such intricate cellular structures are maintained.

1.1.1 Neuronal polarity

The mammalian nervous system is comprised of two main functional units; the central nervous system (CNS), consisting of the brain and spinal cord, and the peripheral nervous system (PNS), which extends throughout the rest of the body. While as systems, they serve different purposes, they are highly related and inter-connected by a common, functional base unit - the neuron.

Mature, fully differentiated neurons essentially have two functionally distinct domains: the somato-dendritic compartment, comprising the cell body and dendritic processes, and the axonal compartment, consisting solely of the axon process. Each of these domains can be characterised not only by their morphology, but also by their unique protein content and differences in cytoskeletal structure (T. L. Lewis et al., 2013). While the development of this polarised structure is highly complex, the process has been very well characterised both *in-vivo* and *in-vitro* for cortical neurons.

In-vivo, neuronal cells migrate across large distances while simultaneously adopting their polarised morphology. During brain development (see fig. 1), cortical neurons are generated by neural stem cells at embryonic day 11-17 (E11-E14), after which they undergo polarisation as they migrate from their point of origin in the neocortical ventricular zone (VZ) of the neocortex outwards to the their final locations in the cortical plate (CP) (Anderson, 2002; Kon, Cossard, & Jossin, 2017). During this migration, neurons will undergo a multipolar transition in the subventricular zone (SVZ) where multiple neurites emerge from the cell body until

one major process, growing in the direction of migration, becomes defined as the leading process (LP). At this point, the neuron undergoes a process of radial translocation, migrating towards the CP, leaving behind a trailing process (TP) which will become the axon. As the cell migrates, the TP/axon rapidly elongates towards the intermediate zone (IZ) while the leading process gives rise to the apical dendrite as it enters the CP. This then initiates dendritic arborisation into the marginal zone (MZ), and subsequent axonal arborisation, which continues postnatally (Polleux & Snider, 2010).

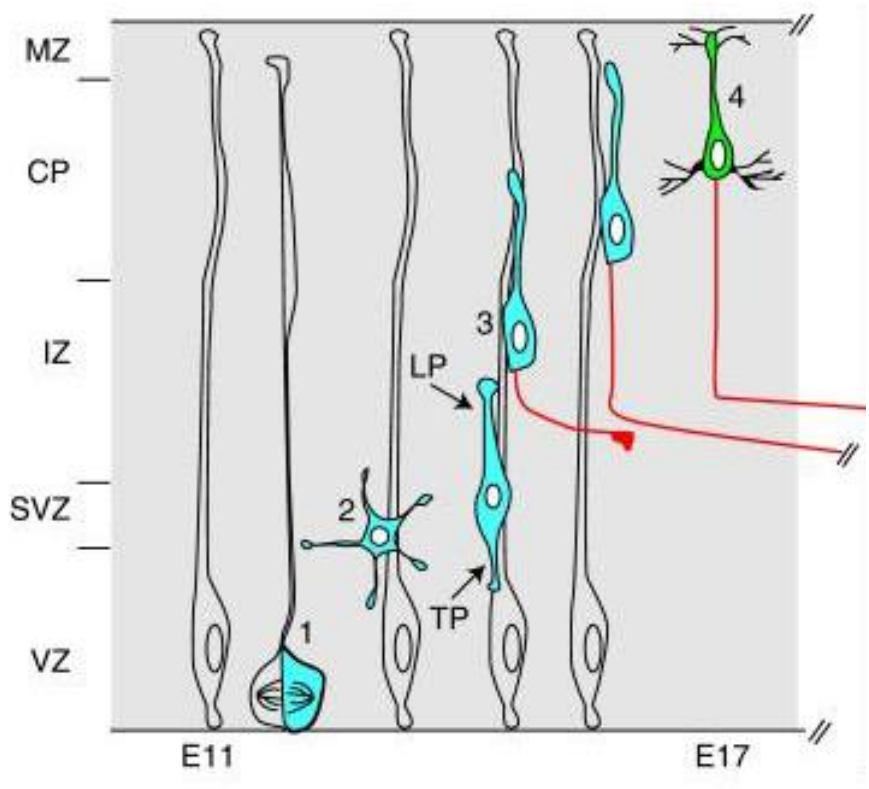


Figure 1. Cortical neuron development in-vivo. During brain development *in-vivo* neurons are generated in the ventricular zone (VZ) (1). The now post-mitotic neuron (blue) begins migrating, and as it does, begins to produce multiple neurites in the subventricular zone (SVZ) (2). From these emerging neurites, a leading (LP) and (TP and red) trailing process are defined, which will become the apical dendrite and axon, respectively (3). As the neuron continues to migrate towards the cortical plate (CP), the axon (red) rapidly elongates across the intermediate zone (IZ). Once the cell

(green) body reaches its terminal location in the CP, it initiates dendritic branching into the marginal zone (MZ) (4). Adapted from Polleux & Snider, 2010.

The use of dissociated cortical neurons in in-vitro culture has been an invaluable tool in understanding neuronal polarisation and development. Their use in such *in-vitro* models has led to a well-defined understanding of how primary cortical neurons develop in culture, illustrated in Figure 2 (Dotti, Sullivan, & Banker, 1988). In brief, from DIV (days-*in-vitro*) 0-2, newly dissociated, post-mitotic and previously polarised *in-utero* cortical neurons appear roughly spherical before they begin to extend web-like lamellipodial and finger-like filopodial structures. These quickly form multiple, immature neurites until the molecular fate of a single neurite destines it to become the axon. From DIV 2-4, this new axon undergoes rapid outgrowth from the axon initiating segment at base of the cell body, breaking cellular symmetry. As it grows, the axon is guided by a structure at its tip called the growth cone, which, guided by extracellular cues, leads it towards a pre-synaptic target. While axon outgrowth continues from DIV 4-15, it begins the process of arborisation, developing new axonal branches, and allowing a single neuron to reach multiple targets. In this time, dendrite outgrowth also increases. Finally, from around DIV 15-25, the now highly branched axons begin to terminate in newly formed synapses, while dendrites develop fine dendritic spines (Dickson et al., 2002; Hand et al., 2005; Takano, Funahashi, & Kaibuchi, 2019).

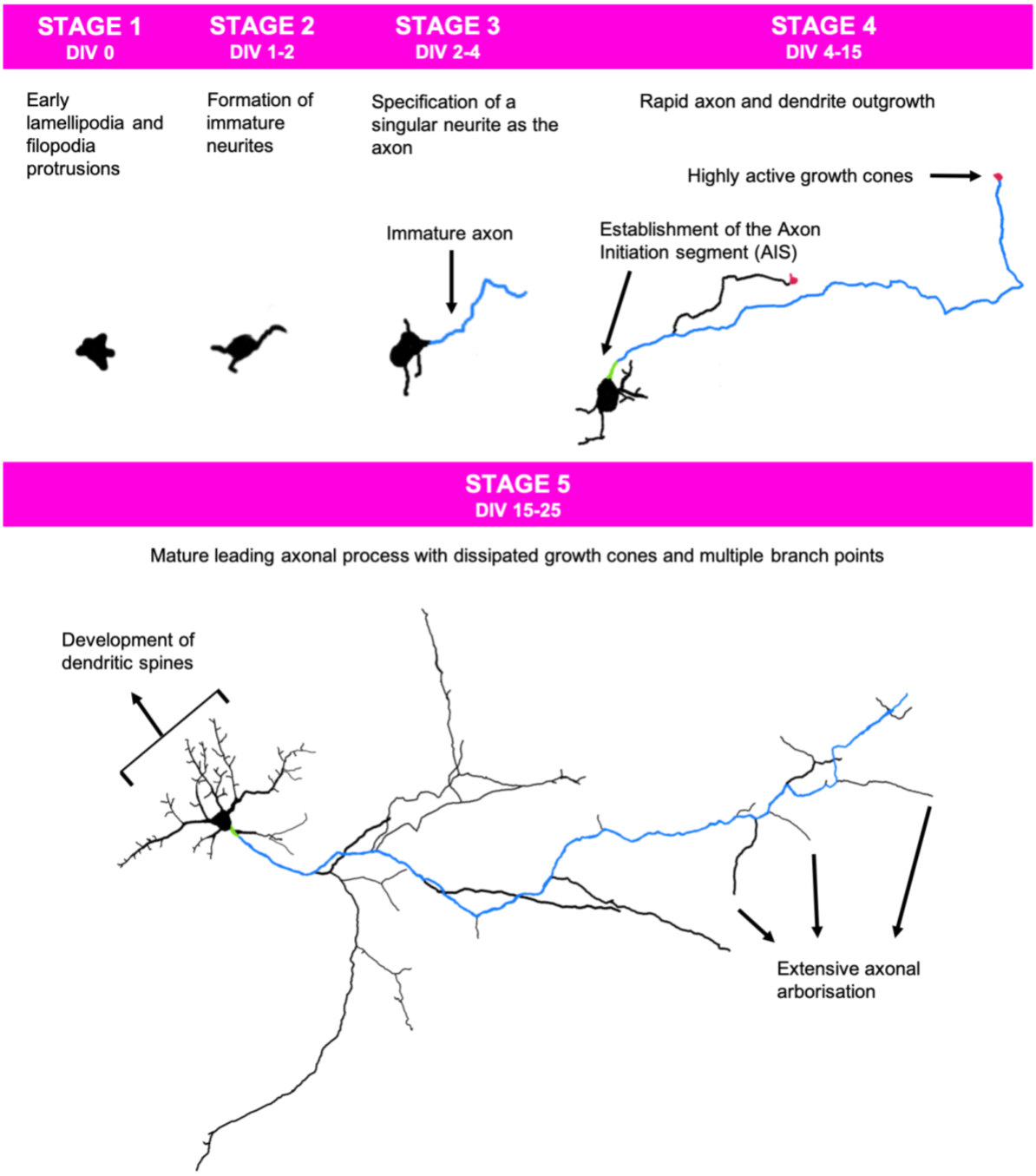


Figure 2. Stages of cortical neuron development *in-vitro*. The development of dissociated, postmitotic cortical neuron cultures occurs in specific transitional stages. (Stages 1 and 2) Initially, newly plated neurons extend lamellipodial and filopodial extensions before forming multiple, immature neurites. (Stage 3) Localised signalling results in polarisation of the cell and the establishment of the axon. (Stage 4) The axon initial segment (AIS) is established. Rapid outgrowth of processes occurs. Axon elongation towards post-synaptic targets is guided by the growth cone. (Stage 5) Between 15 and 25 days-*in-vitro* (DIV), neurons approach terminal differentiation, featuring dendritic spine morphogenesis and extensive axonal branching.

On a molecular level, despite the enormous advances in recent decades, the exact mechanisms at play during neuronal development are still not entirely elucidated, but exact mechanistic details likely differ between cell types (Neukirchen & Bradke, 2011). However, common factors affecting which initial neurite in a developing neuron will become the axon is known to rely on a fine interplay between localised extracellular cues, intracellular signalling cascades, and perhaps even the subcellular organisation of cytoplasmic components (T. L. Lewis et al., 2013).

Several different signalling molecules have been identified in the establishment of axon-dendrite polarity, all of which form part of a network of signalling pathways. Lipid kinase phosphatidylinositol 3-kinase (PI3K), glycogen synthase kinase β (GSK3 β) and Kinesin-1 have all previously been identified as such signalling molecules (Barnes & Polleux, 2009)It has been shown that reduced activity of one or more of these results in the development of several, or no axons, depending on where and how the signalling pathway is altered (Arimura & Kaibuchi, 2007; Barnes & Polleux, 2009). More recently, another intracellular cue of interest is JNK-interacting protein-1 (JIP1). It has been shown in mouse primary cortical neurons that in the early stages of neuron development, JIP1 localises to the neurite that will go on to become an axon. Further into the developmental process, JIP1 then co-localises with the protein kinase AKT in the axon growth cone where they interact to promote further axon growth (Dajas-Bailador, Bantounas, Jones, & Whitmarsh, 2014). Structural proteins also seem to be influential in determining which neurite becomes an axon during polarisation. Actin polymerisation in the peripheral regions of early neurites seems to prevent axon formation. Conversely, high levels of microtubule polymerisation at

the tip of early neurites helps promote axon development and coupled with slow transport of tubulin, provides 'pushing force' essential in axon growth (Kunda, Paglini, Quiroga, Kosik, & Cá Ceres, 2001).

1.1.2 Mechanisms of axon growth

Structural elements of the neuron's cytoskeleton play a pivotal role in establishing neuron polarity and axon growth, as well as defining the function of the axon as a unique cellular compartment in the polarised neuron. The axonal cytoskeleton is comprised of uniformly structured microtubules (consisting of α- or β-tubulin subunits) stabilised mainly by Tau protein and microtubule-associated protein 1b (MPA1b) (although many other types of microtubule-associated protein exist), actin filaments, and various intermediate filaments (also known as neurofilaments) (Kevenaar & Hoogenraad, 2015; Lowery & Van Vactor, 2009). Microtubules in the axon are arranged with their polymerising (plus) ends facing away from the soma, facilitating the movement of organelles such as mitochondria and other cargoes along the axon. Such transport is facilitated by motor proteins such as dyneins and kinesins, where most dyneins travel towards microtubule minus ends, and most kinesins towards plus ends (Zheng et al., 2008). Conversely, dendritic processes contain a different arrangement of microtubules, with plus-end microtubules facing both towards and away from the soma (Yin, Takei, Kido, & Hirokawa, 2011).

Actin exists in various sub-forms, but is mostly known as F-actin, which forms a mesh of minus-end (non-polymerising) out fibres at the base (proximal end) of the axon, creating a diffusion barrier that prevents the passive movement of any somato-

dendritic specific proteins into the axon (A. Song et al., 2009). Actin is also integral to axon path finding and growth. Fast growing actin filaments in the tip of growing axons (the growth cone) create directional steering forces as they polymerise, and a general retrograde flow of whole filaments from the tip provides a 'pushing' force for axon extension (Dickson et al., 2002; Kevenaar & Hoogenraad, 2015).

Once the fate of early neurites has been assigned and the axon begins to grow away from the soma, the role of the leading, distal end of the axon known as the growth cone becomes especially important. The growth cone (see fig. 3) is a highly dynamic, specialised extension of the growing axon that is able to accurately guide axonal growth toward specific targets by responding to intrinsic and extracellular stimuli. Finger-like projections called filopodia, composed of uniform arrangements of rapid, outwardly polymerising F-actin 'explore' the local environment and direct growth towards postsynaptic targets. This directed growth is influenced by the presence of pro-adhesion molecules like laminin or fibronectin; anti-adhesion molecules like slits and ephrins (Dickson et al., 2002; Evans et al., 2007), as well as chemotropic gradients of signalling molecules like neurotropic factors and neurotransmitters (Lowery & Van Vactor, 2009).

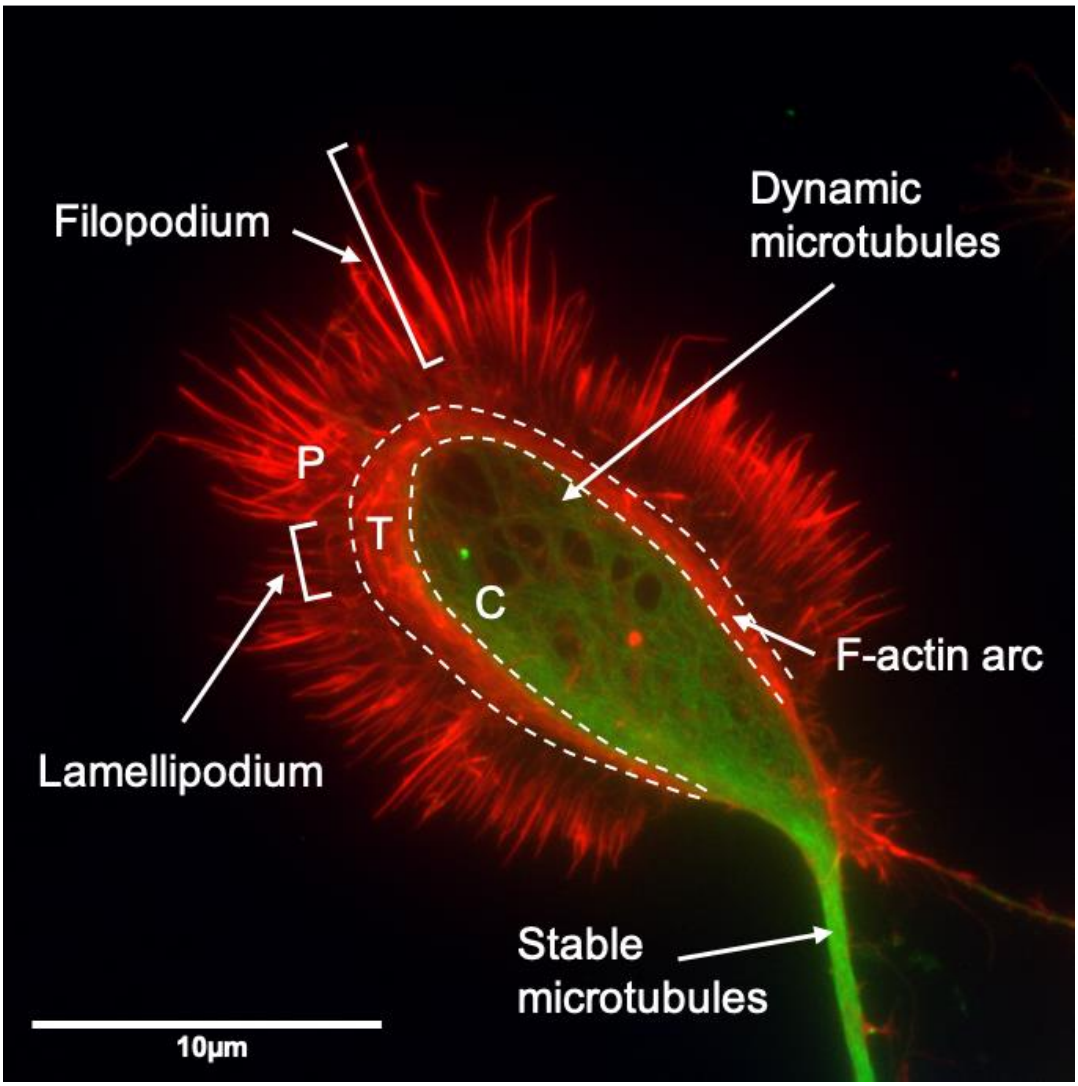


Figure 3. Growth cone structure. The growth cone can be divided into 3 sections, based on its cytoskeletal structure. The outermost is the Peripheral Domain (P, red) and comprises a network of F-actin bundles forming the finger-like filopodia projections which are connected by a mesh-like structure of F-actin called lamellipodia. The Transitional Zone (T, red) is formed of actyomyosin contractile structures, forming an F-actin arc. The Central domain (C, green) houses microtubule bundles along with other vesicles and organelles transported from the soma.

Terminal branching of the axon is characteristic in the late stages of axonal development. Branching allows a single axon to reach multiple postsynaptic targets and is controlled by two mechanisms; splitting of the growth cone, and interstitial branching (Lowery & Van Vactor, 2009). Bifurcation of the growth cone is a process linked to axon guidance, where one axon may be guided towards two separate

targets, and has been seen *in-vivo* in several neuron subtypes in *Drosophila melanogaster*, including motor neurons (Matheson & Levine, 1999). Interstitial branching is thought to serve a slightly different purpose to branching by growth cone splitting. In this case, collateral branches emerge from points lower down in the axon, below the growth cone in order to extend the overall connective range of the axon (Portera-Cailliau, Weimer, De Paola, Caroni, & Svoboda, 2005)

1.1.3 Selective protein turnover in regulation and maintenance of the axon

Axons and axon growth cones are afforded a high degree of functional independence from the soma by the presence of translation machinery and targeted mRNAs (H. Jung, Yoon, & Holt, 2012). This is extremely important to axon function as in the instances of some large motor neurons, the axon can contain up to 99% of the total cytoplasmic volume of the cell, with growth cones needing to guide the outgrowth of the axons across large distances to highly specific end-points (Milde, Gilley, Coleman, Mosely, & Andrews, 2013). The localization of specific mRNAs allows the growth cone to respond rapidly to external stimuli like netrins, slits, semaphorins, and ephrins during axon extension and pathfinding (Yoon, Zivraj, & Holt, 2013).

Local protein synthesis provides an advantage for neurons by allowing for rapid and specific changes in the axonal & growth cone proteome (H. Jung et al., 2012).

A large component of the axon's propensity for local translation is due to stores of translationally silent mRNAs which have specific un-translated region (UTR) sequences localizing them within the axon and growth cone. This enables the rapid *de-novo* protein synthesis that allows the growth cone to maintain transcriptionally active throughout the process of neuronal polarization (Eunjin Kim & Jung, 2015).

As axonal development requires such a high degree molecular dynamism, it stands to reason that unimpaired mechanisms of protein turnover are key to maintaining a high degree of function during this process. In fact, in experiments where primary ganglion neurons were treated with the proteasomal inhibitor, lactacystin, axons failed to develop entirely (Klimaschewski, Hausott, Ingorokva, & Pfaller, 2006). However, protein turnover is not just a concern for axonal development. As postmitotic cells with vital function, protein turnover activity is essential to the maintenance of axonal integrity and neuronal survival as a whole, throughout the cell's life. The integrity of these essential survival processes is thought to be impacted in cases of neurodegeneration, where the failure of one or more of such processes can lead to cytoskeletal breakdown, and ultimately axonal degeneration (J. T. Wang, Medress, & Barres, 2012). One such process that plays a vital role in neuronal homeostasis, which we will be covering in-depth in this thesis, is the ubiquitous protein turnover mechanism, autophagy.

1.2 Autophagy

1.2.1 Autophagy and cellular homeostasis

Autophagy is a conserved, catabolic process, essential in protein homeostasis and involves lysosome-dependent degradation. It is especially important in the turnover of long-lived proteins, protein aggregates, and is the only mechanism for the degradation of defective organelles (Cheng, Zhou, Lin, Cai, & Sheng, 2015). While distinctive in its functions, autophagy is intrinsically linked to the ubiquitin-

proteasome system (UPS), which conversely processes short-lived intracellular proteins (A.T. Nijholt, De Kimpe, L. Elfrink, J.M. Hoozemans, & Scheper, 2011).

Autophagy is a highly conserved, essential and continuous protein recycling system that works as a housekeeping system that prevents the build-up of potentially cytotoxic components that could damage the cell. This function is explicitly vital in long-lived cells such as neurons which are unable to shed the load of cellular waste through cell division, unlike other cell types (Majcher, Goode, James, & Layfield, 2015; Son et al., 2012). Primarily regulated by a set of Atg genes, autophagy also acts as a cell survival mechanism, being triggered in instances of stress by injury or nutrient deprivation, where the process is used to turnover non-essential proteins and organelles to use for the provision of energy, or to remove cytotoxic waste products (Nixon, 2006). The forms of autophagy currently described are known as micro autophagy, chaperone-mediated autophagy, and macro autophagy. Micro autophagy involves direct intake of surrounding cytoplasm into a lysosome by invagination of the membrane, whereas chaperone-mediated autophagy involves the chaperone protein Hsp70 and its co-chaperones facilitating the unfolding of proteins cargoes, in order for them to undergo protein-mediated translocation across the lysosomal membrane. Macro (or selective) autophagy involves the sequestration of protein cargoes autophagy receptors into an autophagosome, which later fuses with lysosomes (Nikoletopoulou, Papandreou, & Tavernarakis, 2015). For the purposes of this thesis, we will only focus on the process of macro autophagy, which will hereby be referred to as autophagy for the sake of simplicity, unless otherwise stated.

The process of (macro) autophagy can be broken down into four stages; initiation, elongation, maturation, and finally degradation (Mis, Brajkovic, Frattini, Di Fonzo, & Corti, 2016). Initiation begins with the protein (UNC-51)-like kinase1 (ULK1), which forms a complex with autophagy protein 13 (Atg13) and focal adhesion kinase family interacting protein of 200 kD (FIP200). This complex allows for ULK1 to localise to the phagophore assembly site (PAS) where it activates its kinase activity which aids in the biogenesis of the phagophore (Ganley et al., 2009), although the exact origin of the phagophore membrane is still contested (Nikoletopoulou et al., 2015). Next, elongation of the phagophore membrane is moderated by the interplay of two, ubiquitin-like conjugation systems. The Atg12-Atg5-Atg16L complex firstly localises to the outer membrane of the phagophore, while microtubule-associated protein 1 light chain 3 (LC3) is converted from its cytosolic form as LC3-I, to LC3-II by Atg3 and Atg7 which facilitate its conjugation with phosphatidylethanolamine (PE), forming PE-LC3, commonly referred to as LC3-II. LC3-II then specifically associates with phagophore membranes. Together, these two systems facilitate the tethering together of phagophore segments to allow for elongation of the membrane (Mis et al., 2016; Nikoletopoulou et al., 2015). Maturation then occurs as a wide variety of adapter proteins, such P62/SQSTM1, NBR1, NDP52, optineurin and ubiquilin-2 interact with ubiquitinated protein cargoes and phagophore bound LC3-II to complete the autophagosome, thus sequestering the cargo. These adapter proteins also allow for a degree of selectivity in the autophagy process. (Majcher et al., 2015; Shaid, Brandts, Serve, & Dikic, 2013). We will explore the specific functions of the P62/SQSTM1 adapter protein in more depth in the next section. Finally,

autophagosomes are transported via dynein-dynactin machinery to locales of endosomes or lysosomes, where they fuse with them, forming amphisomes or autolysosomes, respectively (Ravikumar et al., 2005).

1.2.2 P62/SQSTM1

Sequestosome-1 (SQSMT1 or P62), hereafter referred to as P62, is a ubiquitin binding scaffold protein involved in a wide variety of cellular processes. Not only is it involved in the sequestration of ubiquitinated proteins in autophagy, but other post-translational modifications allow it to function in induction of the signalling processes of the antioxidant response, inflammation, apoptosis and endosomal trafficking (Sánchez-Martín & Komatsu, 2018). Specifically in relation to autophagy, the Phox and Bem1p (PB1), LC3-interacting region (LIR) and ubiquitin associated domains (UBA) of P62, detailed in figure 4, allow it to act as a selective autophagy adaptor, interacting with a range of autophagy machinery proteins while simultaneously recognising cargoes 'targeted' for degradation (Katsuragi, Ichimura, & Komatsu, 2015). As well as playing a key role in connecting cargos for degradation with the autophagosome, P62 is itself subsequently degraded by the autophagy process, thus making it an important marker commonly used when assessing the levels of autophagic activity (Klionsky et al., 2016). As the focus of this thesis is of the role of P62 in the context of autophagy, we will be elaborating specifically on the signalling process undergone by P62 in order for it to perform its function as a selective autophagy adapter (summarised in figure 5).

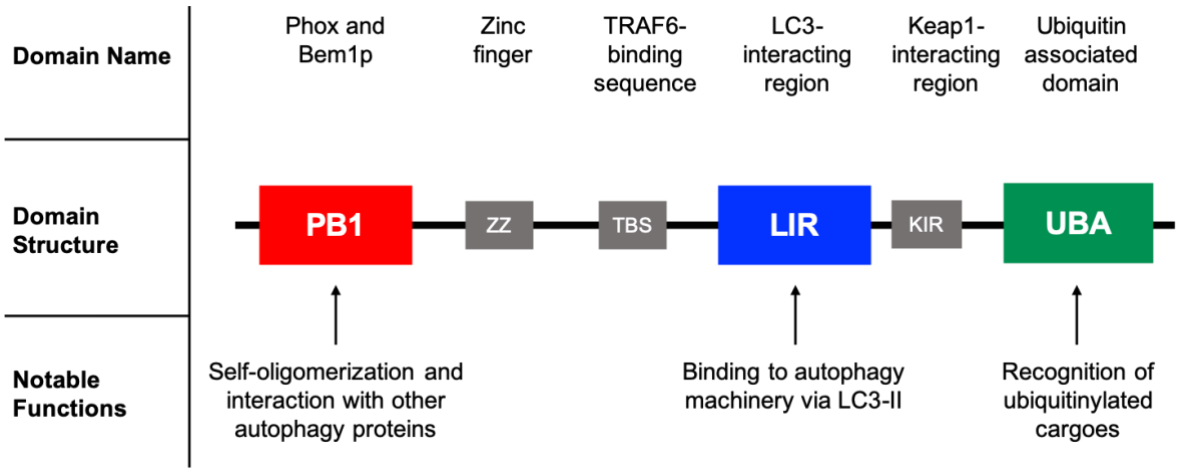


Figure 4. Functional domains of the P62 protein. The functions of the domains that are most applicable to its function in the context of this thesis (PB1, LIR and UBA), have been highlighted.

Before autophagy is induced, homodimerisation of P62 via its UBA domain keeps it in its autophagy-inactive state in the cytosol, when its activity is not required. This homodimerisation occupies the UBA domain, preventing the recognition of polyubiquitinated cargos by P62 and thus keeping this particular function, inactive (Isogai et al., 2011; J. Long et al., 2010). In order to activate P62, a series of phosphorylation, differing depending on initial signalling stimuli, convert P62 back to an active monomer and then increase its binding affinity for ubiquitin. Firstly, phosphorylation by ULK1 (at serine residue 407) breaks the dimerization, and subsequent phosphorylation, by either ULK1, casein kinase 2 (CK2) or TANK-binding kinase 1 (TBK1) (at serine residue 403) increases the affinity of P62 for polyubiquitin chains (Matsumoto, Wada, Okuno, Kurosawa, & Nukina, 2011; Pilli et al., 2012).

Next, P62 self-oligomerises via its PB1 domain, forming helical filaments that simultaneously promote the packaging of ubiquitinated cargoes, as well elongation of the growing autophagosome membrane. Initially, long P62 filaments are formed,

primarily aiding autophagosome membrane nucleation via LIR site-mediated interaction with the active, phagophore membrane-bound form of LC3, LC3-II. Then, after the recognition of ubiquitin, these longer, compact chains convert to shorter, less compact chains, which ameliorate the packaging of the polyubiquitinated cargoes into the autophagosome. The now complete autophagosome is then transported along microtubules by dynein and dynactin to lysosome sites where accessory proteins such as ATPases, ESRCT (endosomal sorting complexes required for transport), Rab proteins, and SNARE proteins (soluble N-ethylmaleimide-sensitive factor-activating protein receptor) promote the fusion of the autophagosome with lysosomes to form an autolysosome. This results in the degradation of P62 structures (as well as membrane bound LC3) in addition to their cargoes (Bjørkøy et al., 2005; Ciuffa et al., 2015; Ritz et al., 2011). As LC3-II remains bound to the autophagosome membrane, it too is often used as a marker for determining levels of autophagic activity. (Kabeya et al., 2000).

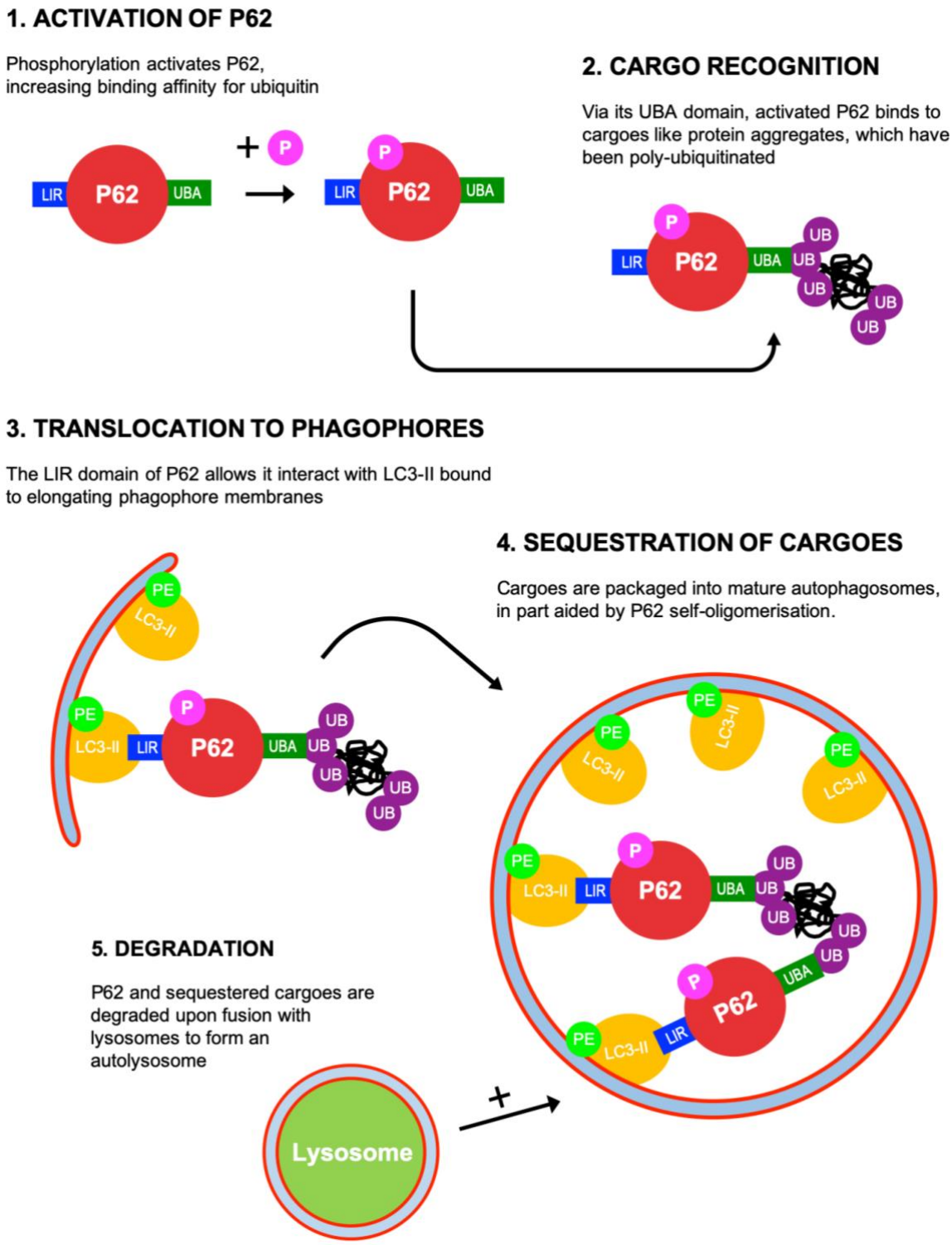


Figure 5. Cargo sequestration by P62. P62 plays a critical role as an adaptor protein in the process of selective autophagy. (1-2) Sequential phosphorylation of P62 'activates' it, enabling it to bind poly-ubiquitinated cargoes like protein aggregates. (3) The P62-ubiquitinated cargo complexes are then sequestered by phagophore membranes by P62's interaction with LC3-II, (4) before being sequestered into a mature autophagosome. (5) Finally, the autophagosome is translocated to acidic lysosomes with which it fuses, degrading its internal contents.

1.2.3 Autophagy signalling mechanisms

Autophagy occurs continuously at low levels, throughout all cells, varying slightly in its basal activity from cell-type to cell-type. A variety of cellular stresses can require an increase in autophagic activity, and as such there are multiple intracellular signalling mechanisms by which autophagy can be induced and modulated (Alegre et al., 2018). Due to the ubiquitous nature of autophagy, there is an extremely wide variety of potential signalling pathways that can regulate autophagic activity. In very general terms, these can be separated into either mTOR-dependent or mTOR-independent pathways. In this section, we will discuss those pathways with particular relevance to the work undertaken in this thesis.

1.2.3.1 mTOR-dependent autophagy regulation

As mentioned in section 1.2.1, there is a wide range of Atg and related genes which govern the various stages of autophagosome formation, cargo selection and degradation. Multiple signalling pathways exist which can affect their function, many of which converge on the modulation of the mammalian target of rapamycin complex 1 (mTORC1, hereto referred to as mTOR). As the name suggests, mTOR was named due to its interaction with the pharmacological compound rapamycin, which is a commonly used autophagy activator that works via mTOR inhibition (Singh et al., 2016). As such, rapamycin is used throughout the experimental work of this thesis as an mTOR-dependent autophagy activator. The remainder of this section will discuss the mTOR-dependent autophagy signalling pathways of most relevance to this thesis. These are the nutrient sensing, insulin/growth factor sensing and the energy sensing pathways.

Nutrient sensing is a common mechanism for induction of autophagy. In mammals, there are two, well-defined pathways that respond to changes in nutrient levels. These are mTOR and the Ras-cAMP-PKA (Ras/cAMP-dependent protein kinase A) pathways. Firstly, mTOR acts as a negative regulator of autophagy, by inhibiting the formation of the complex between ULK1 and the Atg13-FIP200 complex, which initiates the formation of the phagophore at the phagophore assembly site (PAS) (C. H. Jung et al., 2009). Under basal conditions, influx of extra cellular amino acids into the cell cause Ras-related small GTPases, referred to as Rag proteins, to facilitate the translocation of mTOR to subcellular compartments enriched with the mTOR activator, Rheb (Ras homolog enriched in brain) (Eunjung Kim, Goraksha-Hicks, Li, Neufeld, & Guan, 2008; Sancak et al., 2008). Therefore, when intracellular amino-acid levels drop, there is less activation of mTOR by Rheb, thus enabling a rapid increase in the levels of phagophore formation (He & Klionsky, 2009). The second, well-described nutrient sensing signalling pathway in autophagy modulation is that of glucose sensing by the Ras-cAMP-PKA pathway, most of which knowledge comes from studies in yeast. In situations of ample nutrient availability, the Ras1 and Ras2 small GTPases (small guanosine triphosphatases) are activated and promote the production of cAMP (cyclic adenosine monophosphate). This in turn inhibits the function of protein kinase A (PKA), which then reduces the phosphorylation of yeast Atg1 (the mammalian homologs being ULK1 and ULK2). In brief, the activation of the Ras-cAMP-PKA pathway by ample glucose availability is suggested to reduce the availability of active ULK1 at the PAS thus negatively regulating autophagosome

synthesis (Budovskaya, Stephan, Deminoff, & Herman, 2005; Budovskaya, Stephan, Reggiori, Klionsky, & Herman, 2004; Fujita et al., 2007)

Insulin and insulin-like growth factor related pathways are also involved in modulation of autophagy. In the absence of insulin and insulin-like growth factors, the Akt/PKB (phosphatidylinositol (PI) 3-kinase/protein kinase B) signalling pathway, is activated via a complex process of protein interactions. Activation of the Akt/PKB pathway leads the phosphorylation of the tumour suppressor protein called TSC2 (tuberous sclerosis complex 2), which enables Rheb to directly bind and activate mTOR, thus inhibiting autophagy. Conversely, when such hormones are absent, mTOR is inactivated and autophagy is activated (Alessi et al., 1997; Arico et al., 2001; X. Long, Lin, Ortiz-Vega, Yonezawa, & Avruch, 2005; Manning, Tee, Logsdon, Blenis, & Cantley, 2002; Stokoe et al., 1997).

Autophagy activation during metabolic stress is a key response that ensures the survival of the cell. During bioenergetic stress, a decreased ATP/AMP ratio (adenosine triphosphate/adenosine monophosphate) activates AMPK (5'-AMP-activated protein kinase), which phosphorylates and thus activates the TSC1/TSC2 complex, ultimately inhibiting mTOR through Rheb, similarly to the insulin and insulin-like growth factor pathway (Inoki, Zhu, & Guan, 2003). The resulting increase in autophagic activity then increases the availability of nutrients for use in ATP production. The activation of autophagy via this method also crucially halts the cell cycle by activating $P27^{kip1}$ (cyclin-dependent kinase inhibitor 1B), allowing for the

activation of autophagy and the prevention of apoptosis in cases of both reduced availability of growth factors and nutrient deprivation (Liang et al., 2007).

1.2.3.3 mTOR-independent autophagy regulation

Autophagy can be regulated in an mTOR-independent manner by changes in intracellular Ca^{2+} via the Ca^{2+}/Calpain pathway. Increases in cytosolic levels of Ca^{2+} activate Ca^{2+}-dependent cysteine proteases called calpains(Goll, Thompson, Li, Wei, & Cong, 2003). Activated calpain proteins then inhibit autophagy by further activating heterotrimeric G-proteins (Gsα) (A. Williams et al., 2008),which increase the production of cAMP through increased adenylate cyclase activity, which ultimately reduces the phosphorylation of ULK1, without interacting with mTOR (Sato-Kusubata, Yajima, & Kawashima, 2000). In short, increases in intracellular Ca^{2+} inhibit autophagy by negatively regulating autophagosome synthesis through a reduction of the availability of active ULK1 at the PAS.

Intracellular concentrations of Ca^{2+} can be affected in several ways, for example, by ER (endoplasmic reticulum) stress which can cause the ER to expel much of its store of Ca^{2+} (He & Klionsky, 2009) or by the action of L-type Ca^{2+} agonists, which result in in the influx of extracellular Ca^{2+} ions into the cell (A. Williams et al., 2008). In contrast to the effects of L-type Ca^{2+} agonists, several pharmacological L-type Ca^{2+} antagonists have been identified which block the action of L-type Ca^{2+} channels, thus decreasing levels of intracellular Ca^{2+}, which in turn increase autophagosome synthesis. Of particular interest among these compounds is the autophagy activating, Ca^{2+} antagonist Pimozide, which was recently identified via a high-throughput screening of already FDA-approved compounds as an autophagy

regulator (L. Zhang et al., 2007). Due to the multitude of interactions that mTOR exhibits within the context of autophagy regulation, other non-autophagy related scenarios not-withstanding, it has been used as an mTOR-independent autophagy activator throughout the work exhibited in this thesis.

Our current knowledge of the complexity of autophagy signalling mechanisms presents a multitude of opportunities for investigations into targeted, pharmacological regulation in response to disease-linked autophagy dysregulation. Another method of pharmacological, mTOR-independent regulation of autophagy, that is of relevance to this work, is via disrupting autophagosome maturation. This is commonly done by the use of the autophagy inhibitor bafilomycin A1. Bafilomycin A1 is a v-ATPase (vacuolar H+ -ATPase) inhibitor that impairs acidification of lysosomes, which therefore impairs autophagosome maturation. This therefore prevents the degradation of sequestered cargoes, resulting in the intracellular accumulation of autophagosomes (Fass, Shvets, Degani, Hirschberg, & Elazar, 2006; Myeku & Figueiredo-Pereira, 2011; Yamamoto et al., 1998).

1.2.4 Neuronal autophagy and Disease

Neuronal cells are, on the whole, post-mitotic, long-lived cell types. As previously discussed, protein turnover is in constant flux during neuronal development and throughout the rest of the neuron's lifespan as it undergoes normal metabolic processes. As would be expected, an active and responsive autophagy system is essential to facilitate normal cell processes at basal levels, but in fact, much evidence

has emerged that links autophagy impairments to degenerative neurological diseases, emphasising the importance of autophagy in neuronal homeostasis.

It is important to understand basal autophagy in the neuron, and the axon in particular, before we consider its implications in potential disease states. Basal autophagy is known to be spatiotemporally specific within neurons. Autophagosome biogenesis is known to occur in both the cell body and axon, but within the axon, it is almost exclusively seen in the axon tip and rarely along the length of the axon (Maday & Holzbaur, 2014). As observed *in-vitro* in the axons of multiple rodent primary neuron types, including cortical, hippocampal and dorsal root ganglia neurons, autophagosomes formed in the axon tip will undergo rapid retrograde transport towards the soma upon maturation (S. Lee, Sato, & Nixon, 2011; Maday & Holzbaur, 2014; Manday, Wallace, & Holzbaur, 2012; Mizushima, Yamamoto, Matsui, Yoshimori, & Ohsumi, 2004b). These findings are also been backed up by *in-vivo* imaging studies in the neurons of C. elegans (Stavoe, Hill, Hall, & Colón-Ramos, 2016).

In broader neuronal terms, studies in transgenic mice using GFP-tagged autophagy marker proteins, such as LC3 and Atg5, have shown that basal autophagy in CNS neurons is relatively low in comparison to other cell types like heart and liver, and remain low even when exposed to starvation conditions (Jäger et al., 2004; Mizushima et al., 2004a). CNS neurons also generally exhibit a higher proportion of non-lipidated LC3 (LC3-I), compared to the autophagosome membrane-associated form, LC3-II, and healthy neurons also appear to exhibit low levels of double-

membrane vacuoles, in contrast to heart and liver cells with GFP-LC3, which show high levels of puncta present in healthy cells. This evidence suggests that healthy neurons maintain low levels of autophagy, likely due to their ability to exploit multiple sources of energy (Mizushima et al., 2004a; Yue, Friedman, Komatsu, & Tanaka, 2009). There is also the suggestion that while basal autophagy in neurons may not be as low as predicted, it is surely highly efficient with high rates of autophagosome clearance (Boland et al., 2008).

Considering basal autophagy levels with age is also an important factor, as the majority of human neurodegenerative conditions present themselves well into adulthood, and often in those of advanced ages. In the human brain in normal ageing, changes in the expression of various autophagy markers suggest a decline in overall autophagic flux with age. For example, the expression of Beclin 1, a regulator of autophagosome formation, was found to be decreased in normal ageing (M. Shibata et al., 2006), while genome-wide analysis of human brains older than 70 years of age found lower levels of Atg5 and Atg7 transcription compared to younger adult brains (Lipinski et al., 2010). The proportionally higher levels of autophagy in earlier age also point to the importance of autophagy in the crucial maintenance of homeostatic balance from a neurodevelopmental perspective. Studies have demonstrated that autophagosomes accumulate in developing neurons during brain development, and relatedly, that lysosomal activity is linked to *in-vivo* axon pruning (Nixon & Cataldo, 2006; J. W. Song et al., 2008). Changes to basal autophagy during development can lead to a variety of issues affecting the morphological plasticity of the neuron, including disruption of axon growth and interference in early axon

extension (Ban et al., 2013; Coupé et al., 2012). All of which suggests that during neurodevelopment, autophagy may play a role in fine tuning axon growth in order to facilitate normal development of connectivity. The disturbance of which is linked to abnormal brain connectivity in conditions such as autism, where mTOR-dependent autophagy defects have been linked to abnormal neuronal arborisation (K.-M. Lee, Hwang, & Lee, 2013), and schizophrenia, where the dysregulation of autophagy related genes has been found in brain loci associated with disease symptoms (Schneider, Miller, & Woesner, 2016).

Early links to some neuropathological states were shown to be due to excessive autophagy, and it was initially thought that autophagy was a mostly destructive process (Rubinsztein et al., 2005). However, in neurodegenerative diseases caused by protein mis-folding, the accumulation of protein aggregates in neuronal processes leaves axons particularly prone to damage and dysfunction (Zhao, Hong, Li, & Li, 2016) and implicates autophagy impairment in the cell's inability to clear such aggregates. In diseases where such a pathology is a hallmark, i.e., Alzheimer's disease, Parkinson's disease and Huntington's disease, autophagosome-like vesicles have been shown to be present in the dysfunctional and degenerating neurons of both human and relevant animal model tissues (Yue, Wang, & Komatsu, 2008).

Appropriate basal autophagy function has also been shown to be crucial to maintaining axonal stability and preventing axonal degeneration. Mounting evidence links defective autophagy to various neurodegenerative disease states and re-enforces the importance of intact autophagy mechanisms to axonal homeostasis in

particular (Yue et al., 2008). In the axons of healthy primary dorsal root ganglia cultures, newly formed autophagosomes in distal regions have been documented as being transported towards the soma (by recruitment of dynein motor proteins), where they undergo fusion and degradation (Cheng et al., 2015). It has been shown that a large proportion of autophagosomes are produced in the axon, but require retrograde transport to the cell body in order to undergo fusion and degradation with lysosomes (Q. J. Wang et al., 2006). This transportation process is highly susceptible to disruptions, and impairment has been shown to give rise to accumulation of P62 or ubiquitin inclusive aggregates – again, a pathological hallmark of neurodegenerative disease (Boland & Nixon, 2006; Eschbach & Dupuis, 2011). Further indication of autophagy's importance in axonal homeostasis comes from mouse models where the Purkinje neuron-specific deletion of either Atg5 or Atg7 resulted in axonal degeneration preceding both dendritic dystrophy and death of the cell body (Komatsu et al., 2007; Nishiyama et al., 2007).

1.3 Amyotrophic lateral sclerosis and associated frontotemporal lobar dementia (ALS-FTLD)

The increasing body of evidence emphasising the importance of autophagy in neuronal maintenance has been further strengthened by a myriad of published works that have begun linking autophagy defects, specifically implicating P62, to the ALS-FTLD (Amyotrophic lateral sclerosis and associated frontotemporal lobar dementia) pathology. In this section, we will introduce the cellular pathology of ALS-FTLD pathology and how our current knowledge of ALS-linked autophagy defects emphasises the need to explore potential new interventions.

1.3.1 ALS-FTLD neuroanatomy

Amyotrophic lateral sclerosis (ALS) and associated frontotemporal lobar dementia (ALS-FTLD) is a complex, fatal degenerative disease encompassing selective degeneration of motor neurons that leads to a severe loss of innervation of skeletal muscle and is accompanied by dementia-like cognitive defects (Ajroud-Driss & Siddique, 2015; Carrì, D'Ambrosi, & Cozzolino, 2016). A clinically severe disease, the pathology of ALS often leads to rapid death via respiratory failure within 3-5 years of symptom onset. This however can vary as most cases of familial ALS are early onset with a slower progression, while many sporadic ALS cases tend to have a much later onset (Fecto & Siddique, 2011a). In addition to the debilitating effects of motor neuron cell death, it is also known that at least 2-3% of ALS patients develop dementia-like symptoms often seen in cases of frontotemporal lobe dementia (FTLD) (Kiernan et al., 2011), affecting the frontal and temporal lobes of the brain (figure 6).

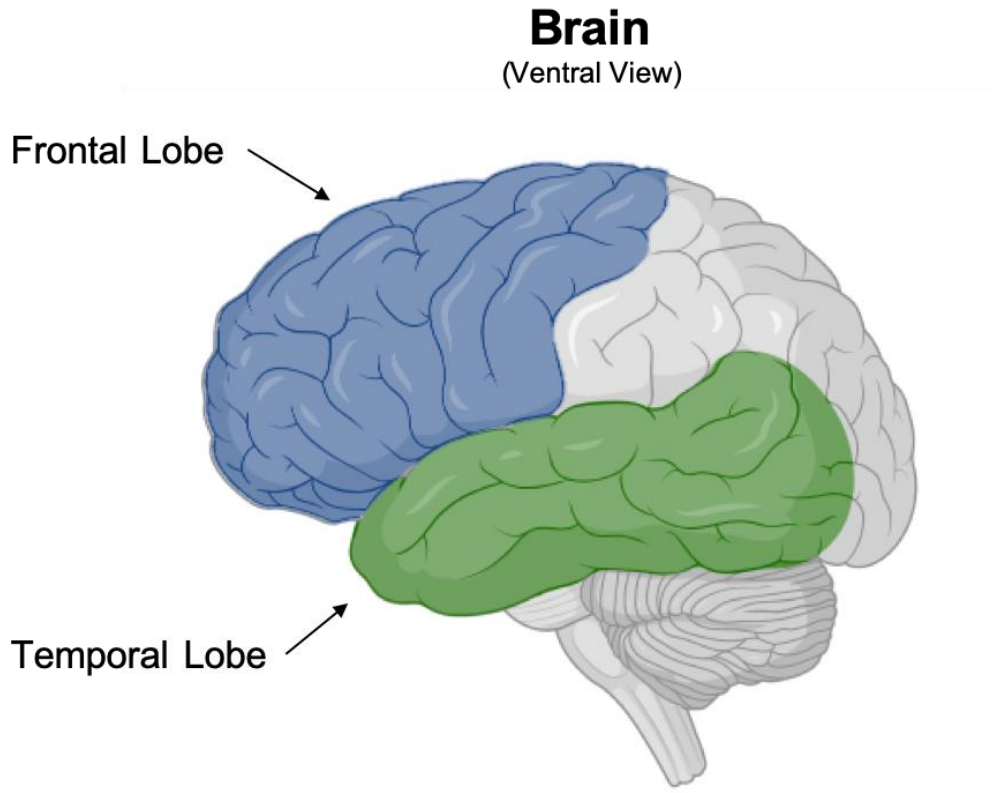


Figure 6. Brain regions affected in FTLD. Frontotemporal dementia is characterised by the atrophy of brain tissue of brain tissue in the both the Frontal (blue) and Temporal (green) lobes of the brain.

Typical features of ALS-FTLD at the intracellular level is the presence of misfolded protein aggregates, synaptic loss and ultimately neuronal cell death (Parakh & Atkin, 2016), with neuronal degeneration occurring in all components of the motor neuron circuitry - cortical, corticospinal tract and spinal (alpha) motor neurons (Jara, Villa, Khan, Bohn, & Özdinler, 2012). See figure 7 for a summary of motor neuron anatomy.

Lower motor neurons (LMNs) (also known as alpha motor neurons, anterior horn cells, or somatic motor neurons) originate in the spinal cord and are the effector component of motor neuron circuitry that controls voluntary movement. LMNs in the spinal cord are organised in a patterned column structure that reflects the

anatomical location of the relative muscle unit or units that each neuron innervates (de Carvalho & Swash, 2016; Jara et al., 2012). LMNs are typically unipolar, extending axons from nuclei located in the spine into peripheral tissues where a single neuron may interact with one or more muscle units. Control of alpha motor neurons is modulated by voluntary control where sensory input is received from corticospinal motor neurons located primarily in sensorimotor cortices of the brain. These cortical components of the motor neuron circuitry extend downwards into the brain stem and spinal cord, where interneurons relay input to alpha neurons (de Carvalho & Swash, 2016).

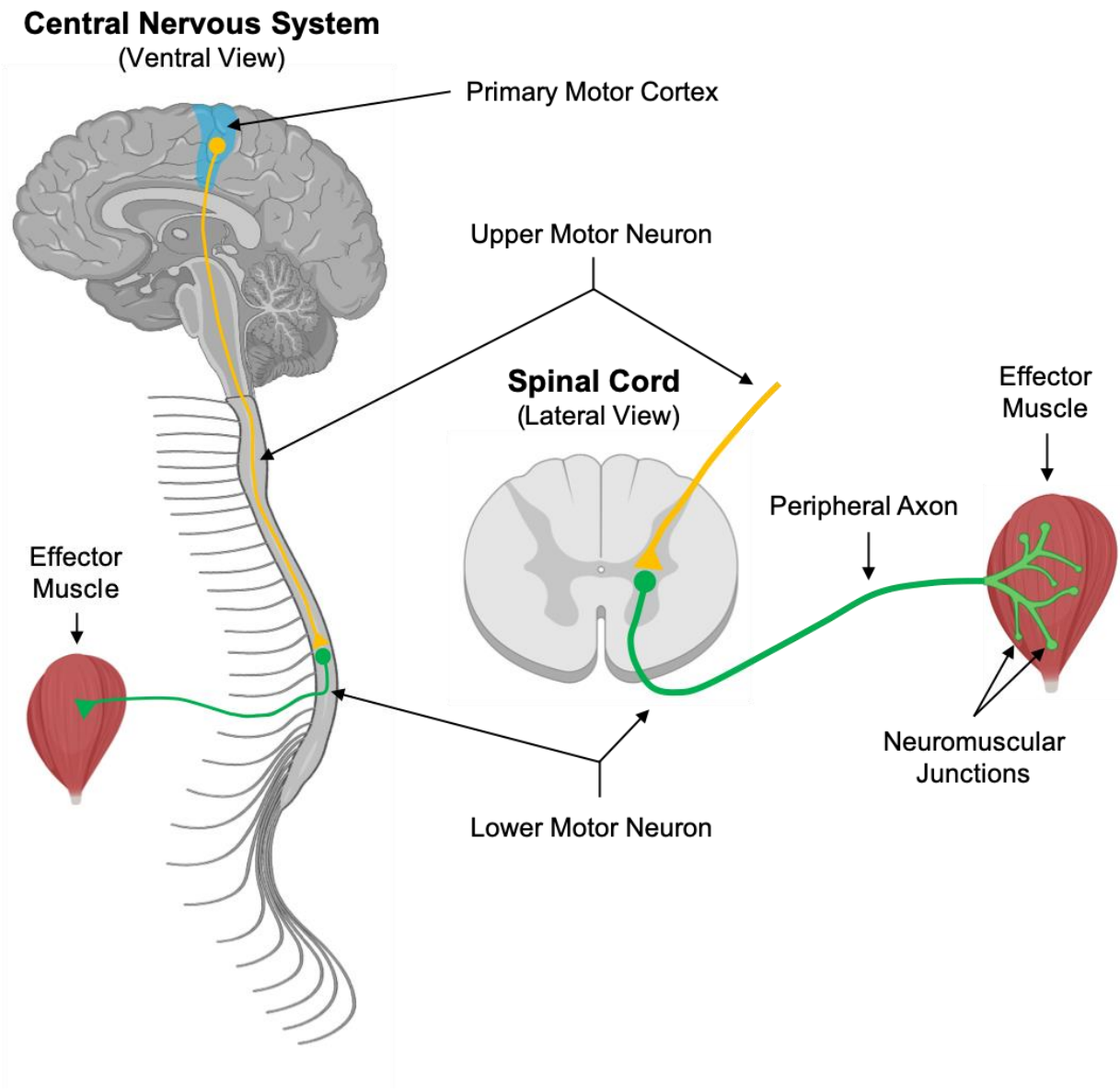


Figure 7. Motor neuron anatomy. Upper motor neurons (yellow) have their cell bodies located in the primary motor cortex (blue) of the brain and extend axons down through the spinal cord. Lower motor neurons (green) have cell bodies located within the spinal cord in loci that relate to the anatomical positions of the skeletal muscle groups they innervate. Lower motor neurons extend axons beyond the spinal cord, through the peripheral tissue to form connections with skeletal muscle tissue called neuromuscular junctions.

Both ALS and FTLD belong to the same clinical group of disorders and although both are known to be highly genetically heterogeneous, they have been linked by more than 30 gene and related defects, all of which display great variety in their causative mechanisms (Fecto & Siddique, 2011a). Approximately 90% of ALS cases are

sporadic, with familial ALS contributing to 5-10% of cases, via a Mendelian pattern of inheritance. Both sporadic and familial forms of the disease are genetically heterogeneous, with several genetic factors having been identified in recent years that are implicated in both forms of the disease (de Carvalho & Swash, 2016). In ALS more generally, mutations in a total of 147 genes loci have been identified, with 39 of these shown to have a causal relationship with the disease. These 39 comprise the loci ALS2, ALS3 and ALS7; and the genes ANG, ANXA11, ATXN2, CFAP410, C9orf72, CHCHD10, CHMP2B, DAO, DCTN1, ELP3, ERBB4, Erlin1, FIG4, FUS, HNRNPA1, LMNB1, MATR3, NEFH, NEK1, OPTN, PFN1, PRPH, SETX, SIGMAR1, SOD1, SPAST, SPG11, SQSTM1, TAF15, TARDBP, TIA1, TUBA4A, UBQLN2, UNC13A, VAPB and VCP (Dervishi et al., 2018). Here we discuss several notable and relevant ALS genes in further detail; SOD1, TDP-43 and FUS, UBQ2, OPTN, VCP and the more recently identified, SQSTM1/P62.

Mutations to the SOD1 (Cu/Zn superoxide dismutase) gene have been shown to be responsible for around 20% of familial and 1-3% sporadic ALS cases and were the first ALS genes to be identified (Ajroud-Driss & Siddique, 2015). Normally, SOD1 catalyses the dismutation of superoxide free radicals, thus performing a protective function in protecting the cell from molecular damage by the action of reactive oxygen species (Fecto & Siddique, 2011a). A commonly used mouse model of ALS is of transgenic mice which overexpresses SOD1 mutant proteins, most commonly the $SOD1^{G93A}$ mutant (Gurney et al., 1994). While the exact pathogenic mechanism of SOD1 mutations is still debated, it is known that mutant SOD1 becomes misfolded and leads to ER stress which can result in toxic effects including mitochondrial

dysfunction and disruptions to axonal transport (Dal Canto & Gurney, 1995; N. Shibata et al., 1996). Most SOD1 mutants result in the appearance of SOD1 in cytoplasmic aggregations in motor neurons (Bosco et al., 2010).

TDP-43 (TAR DNA binding protein 43 kDa) is a member of the heterogeneous ribonucleoproteins (hnRNPs) family, and is primarily found in the nucleus where, through its DNA/RNA binding ability, it is involved in processes such as RNA processing, RNA transport and the biogenesis of small, regulatory RNAs known as microRNA (Gao, Wang, Yan, Perry, & Wang, 2019). Mutations to TDP-43 can lead to its mis-localisation to the cytoplasm, where it has been identified as part of ubiquitin-positive protein aggregates in almost all cases of ALS, and more than half of patients with FTLD (Manuela Neumann et al., 2006; Van Deerlin et al., 2008; Winton et al., 2008) Similarly to TDP-43, FUS (fused in sarcoma) is also an hnRNP protein primarily localised to the nucleus. In normal physiology, FUS is involved in processes such as transcription, mRNA stabilisation, mRNA transport and has links to microRNA processing (Ishigaki et al., 2012; Kanai, Dohmae, & Hirokawa, 2004; Udagawa et al., 2015; L. Yang, Gal, Chen, & Zhu, 2014; Zhou, Liu, Liu, Öztürk, & Hicks, 2013). Multiple ALS-associated FUS mutations have been identified showing that in its aberrant form, mutant FUS becomes localised to the cytoplasm (just as with TDP-43 mutants), leading to a reduction of availability of FUS for regular processes, leading to the altered expression of key microRNAs, some of such as miR-9, miR-125b, miR-128, miR-132 and miR-143 have been implicated in neuronal functions (Morlando et al., 2004). Yet again, FUS is found to be present in ubiquitin-positive

inclusions in the brains and spinal cords of ALS and FTLD patients (Kwiatkowski et al., 2009; Vance et al., 2009).

Ubiquilin-2 (UBQLN2), through its UBA and ubiquitin-like domain (UBL) binds ubiquitinated substrates which it is then able to deliver to the proteasome (D. Y. Lee & Brown, 2012). UBQLN2 also demonstrates an indirect interaction with LC3 (Rothenberg et al., 2010). In HeLa cells, the deletion of its UBA domain results in a loss of its ability to associate with LC3, and a complete knock-down of UBQLN2 leads to reduced autophagosome formation, thus linking it to the clearance of autophagic cargoes (N'Diaye et al., 2009). UBQLN2 mutations have been identified in both familial and sporadic ALS patients, as well as in an X-linked, dominantly inherited sub- type of ALS with dementia (H.-X. Deng et al., 2011; K. Y. Zhang, Yang, Warraich, & Blair, 2014). UBQLN2 has also been identified as a component of cytoplasmic inclusions in spinal motor neurons, positive for ubiquitin, TDP-43 and FUS (K. L. Williams et al., 2012).

Similarly to UBQLN2, Optineurin (OPTN) is another ubiquitin binding protein that has been implicated in ALS. OPTN can simultaneously bind ubiquitin and LC3 to act as an autophagy receptor, and as such is involved in the autophagy clearance of mitochondria (mitophagy) (Wong & Holzbaur, 2014). Mutations to the OPTN gene have been identified in ALS patients, and have been found in TDP-43- and ubiquitin-positive cytoplasmic inclusions in both sporadic and familial ALS cases (Maruyama et al., 2010). The depletion of OPTN in zebrafish has also been shown to result in

increased cell death of motor neurons in a manner similar to other zebrafish models of ALS (Korac et al., 2013; Paulus & Link, 2014).

Valosin- containing protein (VCP) is also involved in autophagy, whereby it is able to directly interact with ubiquitinated substrates and as such, ubiquitin-linked processes like autophagy (Ju et al., 2009; Tresse et al., 2010) . In the context of ALS, VCP mutations can be found in 1-2% of familial cases, with some evidence linking VCP defects to TDP-43 and FUS as a result of their colocalization to hallmark intracellular inclusions in ALS-FTLD (Arai et al., 2006; Johnson et al., 2010; Miller et al., 2012; M. Neumann et al., 2009; Sasayama et al., 2012).

A common link between these highlighted ALS-FTLD mutations is that all of them can be found in ubiquitin-positive cytoplasmic inclusion in affected neurons. Furthermore, UBQLN2, OPTN and VCP all perform autophagy receptor-related functions, putting them in the same functional realm as P62. These links, combined with findings from several studies detecting and accumulation of autophagosomes in the motor neurons of patients with ALS-FTLD and associated neurodegenerative conditions (S. Chen, Zhang, Song, & Le, 2012; Fecto & Siddique, 2011a), solidly implicates defective autophagy in ALS-FTLD pathology, even without considering the impact of P62 and its related defects in the disease.

1.3.2 ALS-FTLD and P62

As described in 1.2.2, P62 is an autophagy adaptor protein capable of simultaneously binding ubiquitinated substrates and LC3 in order to facilitate the sequestration of

cargoes to autophagosomes. In several neurodegenerative diseases where potential autophagy impairment is implicated, P62 has been identified as a component of hallmark intracellular neuronal protein aggregates, such as Lewy bodies in Parkinson's disease and neurofibrillary tangles in Alzheimer's disease (Bjørkøy et al., 2005). Recent work by Bjørkøy et al. (2005) also showed that *in-vitro* depletion of P62 in a cellular model of Huntington's disease was linked to increased cell death due to higher accumulation of mutant huntingtin protein. The mounting evidence highlighting the accumulation of protein aggregates and ubiquitin-positive inclusion bodies in ALS-FTLD not only strongly links autophagy impairment to ALS-FTLD pathology, but the recent identification of numerous P62 polymorphisms in ALS and FTLD patients (Usategui-Martín et al., 2015) now directly link P62 dysfunction to the disease as well.

In particular, recent work showed that an L341V mutation in P62 that was identified in ALS-FTLD patients is confirmed to inhibit autophagy by reducing the binding affinity of P62 for LC3-II (Goode et al., 2016). This suggested that a failure of P62 to link poly-ubiquitinated protein aggregates with other autophagy machinery plays a role in the axonal degeneration and cell death seen in ALS-FTLD. The evidence thus-far discussed in relation to P62 and autophagy in ALS-FTLD, strongly suggests that regulation of autophagy machinery has a larger role to play in preventing axonal degeneration, and perhaps even modulating axon development.

1.3.2.1 Issues with pharmacological mTOR-dependent autophagy activation in ALS

As discussed in section 1.2.3.1, mTOR activity can be regulated by a number of different signalling pathways leading to the moderation of autophagy activity, with rapamycin being a pharmacological compound commonly used for this purpose. However, the promiscuous characteristic of mTOR means that autophagy activation by mTOR inhibition can often result in numerous undesired effects. The inhibition of mTOR by rapamycin has been shown to have conflicting effects on the regulation of key metabolic processes, such as insulin sensitivity and even adipocyte differentiation (Li, Kim, & Blenis, 2014), but of most concern, in the context of models of ALS, is the ability of mTOR inhibition to modulate immune function. Rapamycin is widely used as a potent immunosuppressant, capable of modulating both innate and adaptive immunity processes through mTOR inhibition, largely by inhibiting T-cell proliferation (Heitman, Movva, & Hall, 1991; Thomson, Turnquist, & Raimondi, 2009). In an ALS mouse model expressing the mutant SOD1 protein, rapamycin failed to have any effect on survival (Bhattacharya et al., 2012) and in a different SOD1 mutant mouse, rapamycin was only able to positively impact on survival in those mice lacking mature lymphocytes (Staats et al., 2013). This evidence strongly suggests that the immunosuppressive effects of mTOR-dependent activation of autophagy may counteract any potential benefits of in ALS disease models, making the case for a need for mTOR-independent autophagy activators.

1.4 Overview

Neuronal connectivity is critically important to the function of the nervous system, and in long-lived cells such as neurons, homeostatic processes like autophagy are

vital in modulating protein turnover during development through to maintaining their function throughout life. The axon is a vital sub-unit of the neuron which allows for the transmission of action potentials within the brain and throughout the body via its synaptic connections. Failures in the cellular maintenance process of autophagy have been strongly linked to the pathology of many neurodegenerative diseases, including the debilitating, fatal condition, ALS-FTLD. In particular, loss-of-function mutations to the protein P62, a key cog in the autophagy machinery, have been implicated in ALS-FTLD pathology. However, little is known as to how P62-linked autophagy dysregulation impacts the development and maintenance of the axon. Considering how the initial loss of connectivity from distal axonal degeneration is associated with being a direct cause of early degenerative disease symptoms, prior to any cell death, it is essential to understand the mechanisms at play which are affecting the axon.

For the purposes of clarity and flow, this work has been separated into three main areas of investigation. These are summarised in figure 8, but in brief, experiments will focus on ‘development’, ‘degeneration’ and ‘local’ investigations.

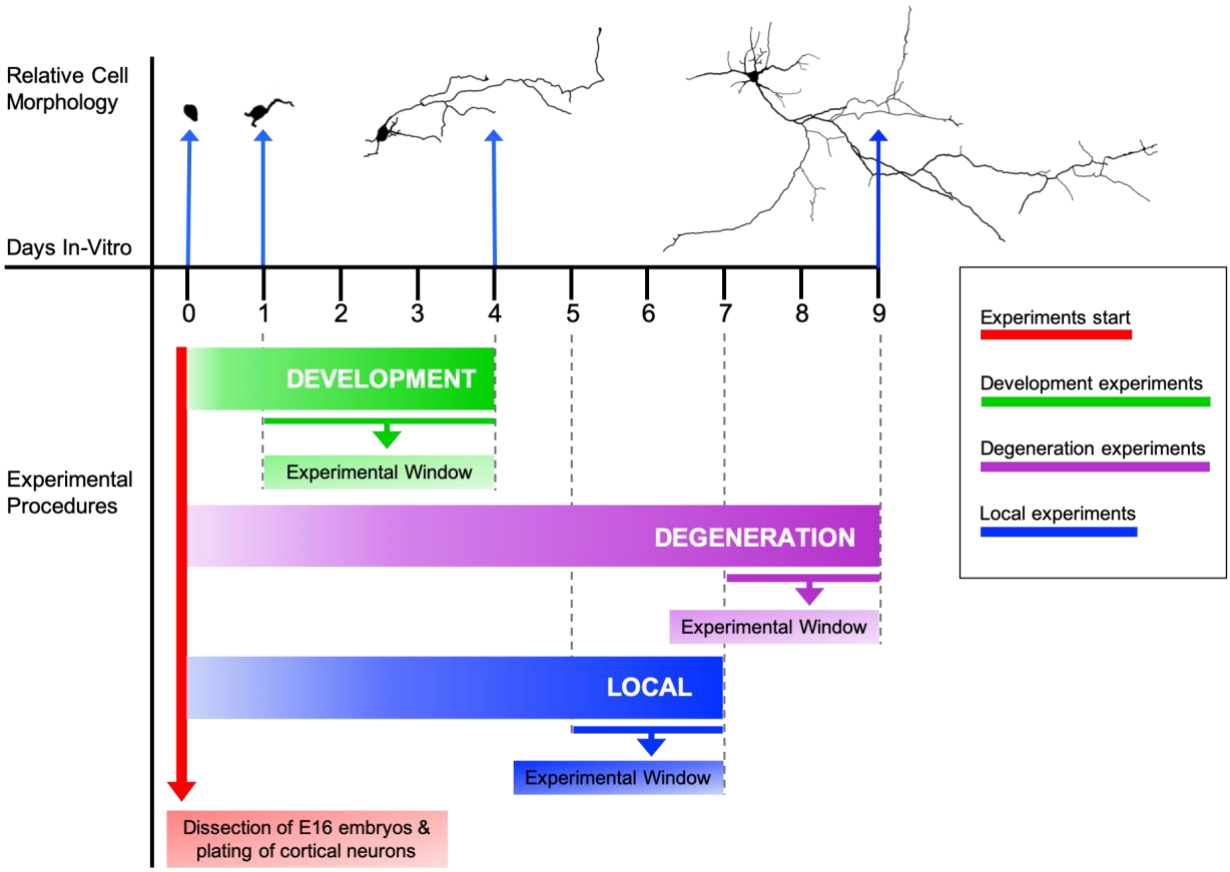


Figure 8. Relative timeline of experimental procedures. The work in this thesis uses primary cortical neuron culture models and is separated into three distinct areas of investigation. Days in-vitro and the relative cell morphology are shown as an indication of the stage of development of cells in the experimental model. 'Development' experiments used neurons up to DIV4, with interventions taking place between DIV1-4. 'Degeneration' experiments used neurons up to DIV9 in age with interventions taking place between DIV7-9. 'Local' experiments used neurons up to DIV7 with interventions taking place between DIV5-7.

1.4.1 Hypothesis

Throughout this thesis, the experimental evidence presented aims to explore the role of autophagy regulation and disease-linked P62 loss-of-function mutations in the development, maintenance and local control of the axon. In undertaking the experiments presented in this thesis, we hypothesise that;

1. Expression of the $P62^{L341V}$ mutation in cortical neuron axons during early neurodevelopment will lead to increased axonal outgrowth due to decreased autophagic activity, which is likely to be due to dysregulation of fine mechanistic control of this cellular growth process.
2. Expression of the $P62^{L341V}$ mutation in cortical neurons of more mature cultures will negatively impact the structural integrity of the larger axons, leading to the likely appearance of degenerative phenotypes, such as those seen in *in-vitro* pathology.
3. Downregulation of endogenous P62 expression locally within the axon will be sufficient to cause increased axon growth due to the impairment of axonal autophagy.

2. Methods

2.1 Primary cortical neuron cultures

2.1.1 Animals and isolation of primary cortical neurons

The use of animals and animal tissue in this study was carried out in accordance with the Animals (Scientific Procedures) Act 1986 and in full compliance with the animal welfare and husbandry standards of the University of Nottingham.

For all cultures, C57/BL6 mouse embryos at 15.5-17 days of embryonic development (E15.5-17) were used. At E16, pregnant females were humanely culled by cervical dislocation and embryos removed. Embryos were removed from the uterus and humanely culled by decapitation. Using micro-dissection spring scissors and fine forceps, brains were removed from skulls, both cortices were dissected out and meninges were removed. A maximum of 7 individual cortices were manually shredded with forceps and incubated in 2ml of Hanks Balanced Salt Solution (HBSS Ca2+ and Mg2+-free; Gibco) with 1mg/ml trypsin (Sigma) and 5mg/ml DNAse I (Sigma) for 30 minutes at 37°C and 5% CO_2, in a high humidity tissue incubator. Following this incubation period, 0.05% (v/v) trypsin inhibitor (Life Technologies) was added and the digested tissue solution was washed by transferring to a 50ml flacon tube containing 10ml of Neurobasal media (Gibco). Tissue was left to sink to the bottom of the tube before approximately 8ml of media was removed. 2.5mg/ml DNAse I was added to the remaining media and tissue, gently mixed, before repeated adding and removing more Neurobasal media to repeat the wash step. To the remaining 2ml of media with tissue, 5mg/ml DNAse I was added and using a 2ml

strippette, the tissue was mechanically dissociated by trituration. The resulting cell suspension was washed by adding 8ml of Neurobasal media and centrifuging at 250xg for 5min. Media was removed and the cell pellet resuspended in Neurobasal media supplemented with 1x GlutaMax (Gibco) and 2% (v/v) B-27 (Gibco) for plating. Where possible, all steps were carried out under sterile conditions in a class II laminar flow cabinet.

2.1.2. Dissociated primary cortical neuron cultures

Before cell seeding, 22x22mm glass coverslips (Menzel Glaser) were sterilised in 70% (v/v) ethanol and placed in the base of 6-well plates (Corning) and/or 8.8cm^2 Nunc dishes (Thermo Fisher). The dishes and coverslips were then coated by addition dishes in 50ug/ml poly-L-ornithine (PLO; Sigma-Aldrich) for a minimum of 4 hours at 37°C and 5% CO_2 in a high humidity tissue incubator. Following incubation, PLO solution was removed, and dishes/plates were washed twice with sterile, distilled water.

Cortical neuronal cells obtained from the dissection process were resuspended in supplemented Neurobasal media and seeded onto PLO coated glass coverslips at a density appropriate for experimental use and grown at 37°C and 5% CO_2 in a high humidity tissue incubator. For transfection experiments, cells were diluted to 10.0x10^6 cells/ml for a final density of 1.75x10^5 cells/cm^2 (referred to as high density). For experiments requiring measurement of axons from immunofluorescence stained cultures, cells were diluted to 2.5x10^5 cells/ml for a final density of 3.5x10^4 cells/cm^2 (referred to as 'low density' cultures). For cultures

used for 'degeneration' experiments, up to 9 days *in-vitro* (DIV9), 50% of the media was replaced at DIV5.

2.1.3 Compartmentalised microfluidic culture of primary cortical neurons

Experiments requiring microfluidic chamber cultures were setup using two-channel microfluidic devices with a 150μm microgroove barrier between the channels (SND150; Xona microfluidics) which allowed for the fluidic isolation of cortical neuron axons from their cell bodies – see figure 9. Before first use, devices were sterilised in 70% (v/v) ethanol and incubated overnight in HBSS to remove any potential toxic by-products from the manufacturing process. Devices were then washed in distilled water and sterilised again in ethanol. For repeat use, devices were washed in distilled water, sterilised in 70% (v/v) ethanol and placed in an ultrasonic water bath for 5mins, to aid the removal of any debris from the microgrooves. Devices were then left to dry completely before being mounted onto PLO-coated 8.8cm^2 Nunc dishes with or without PLO-coated glass coverslips. Immediately prior to seeding, supplemented Neurobasal media was added to the reservoirs of both channels and devices were left to equilibrate for 1hr at 37°C and 5% CO_2 in a high humidity tissue incubator. Following this, media was removed from both reservoirs of the channel to be seeded (to be referred to as the soma channel), and 50μl of a 4.0x10^6 cells/ml cell suspension was added to each side of the soma channel. Devices were allowed to sit in a tissue incubator for a minimum of 1hr to allow cells to settle and adhere to the PLO coated surface, after which additional supplemented Neurobasal media was added for a total volume of 100μl per each soma channel reservoir and 150μl per each axonal channel reservoir. Any media

replacements for the addition of pharmacological compounds or other reagents were made using media taken from wells with age-matched, high density cultures (referred to as conditioning media). Cultures were kept at 37°C and 5% CO_2 in a high humidity tissue incubator.

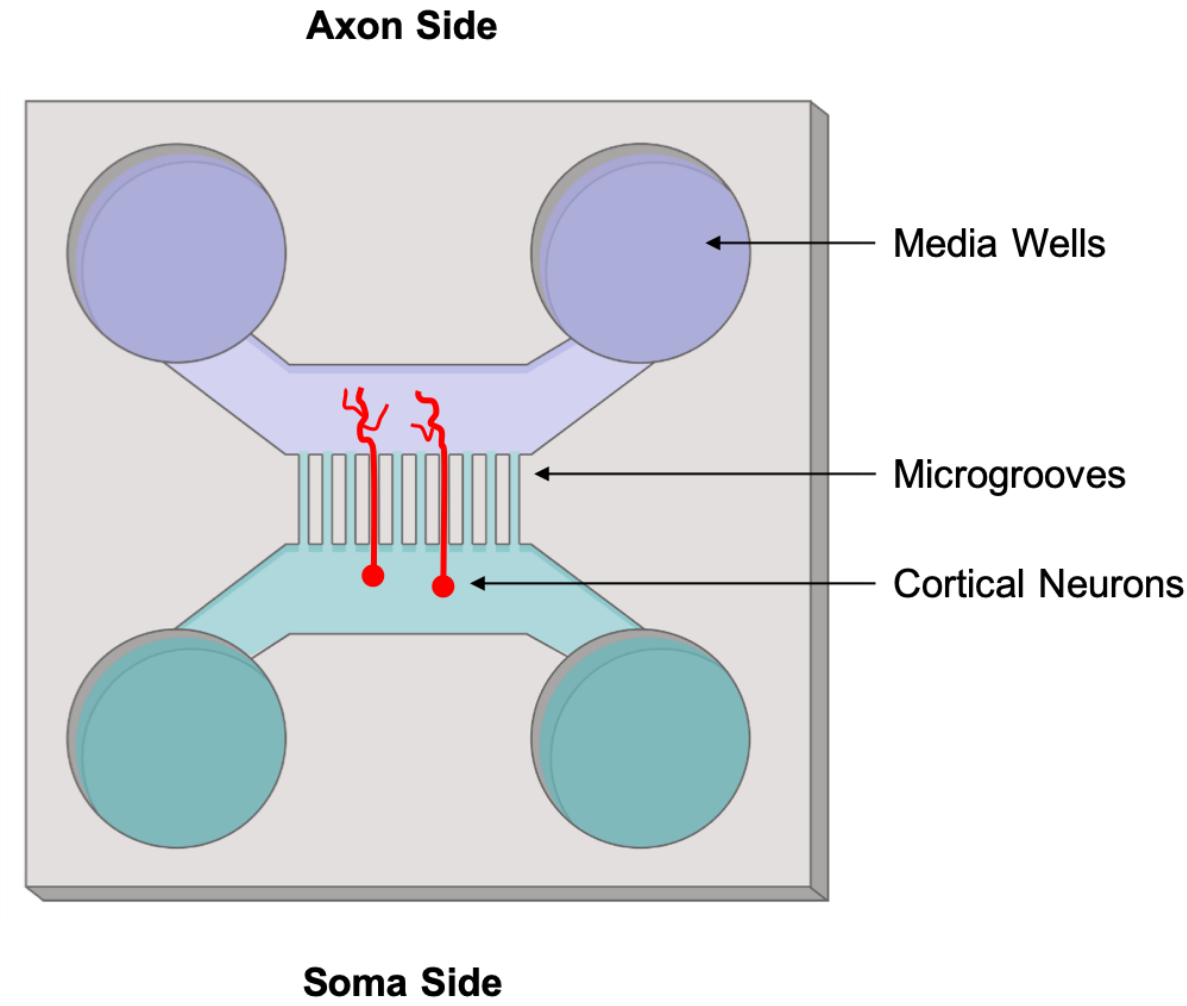


Figure 9. Schematic diagram of a microfluidic culture device. Dissociated primary cortical neurons (red) are plated into the soma side channel of the device. As the neurons develop, axons begin to grow through the microgrooves to become isolated in the axon side channel. Fluidic isolation allows for localised application of treatments one side or the other.

2.2 Pharmacological manipulation of autophagy

Three different autophagy modulating compounds were used in multiple experimental setups, in order to induce or inhibit levels of autophagy in primary cortical neuron cultures. The autophagy activating compounds used were rapamycin (Rapa; Sigma-Aldrich), always at a final concentration of 20nM, and pimozide (Pim; Sigma-Aldrich), always at a final concentration of 1μM. The autophagy inhibiting compound used was bafilomycin A1 (BafA1; Sigma-Aldrich), always at a final concentration of 20nM. When used in an experiment, these compounds were applied for 18hrs before the relevant cultures were fixed and analysed. All compounds were suspended in 100% dimethyl sulphoxide (DMSO; Sigma), with equivalent volumes of DMSO being used as a vehicle control in experimental settings.

2.3 Quantification of autophagy markers in pharmacologically treated cultures

2.3.1 Protein sample preparation

Following treatment of 'high density' cultures with pharmacological compounds (see 2.1.2), media was removed from wells, plates were put on ice and washed with ice-cold 1X PBS (ThermoFisher). For each condition, 2 wells of a 6-well plate were combined in order to obtain sufficient sample material. 100μl Triton Lysis Buffer (see table 1) with protease inhibitor cocktail (at 1:100; Sigma) was added to the first well and cells were scraped into buffer. Solution was collected and transferred to the second well where scraping was repeated. The cell-buffer mix was then transferred

to Eppendorf tubes and further homogenised by sonication for 10 seconds using an ultrasonic probe. Samples were then left on ice for 20mins before centrifugation at 13,000 xg at 4°C for 30mins, after which supernatant was removed to fresh Eppendorf tubes and kept on ice for immediate use or stored at -20°C

Protein samples were quantified using a BCA (bicinchoninic acid) assay BCA assay (Pierce) with bovine serum album (BSA; Sigma) standard, as per manufacturer's instructions. In brief, reactions were incubated in 96-well plates (Corning) in a shaking incubator at 37°C for 30 mins before being read by a MultiSkan FC plate reader (ThermoFisher). Between 50-100μg of protein was loaded for each sample.

2.3.2 SDS-PAGE

Protein samples obtained by the method described above (2.3.1) were separated by SDS-PAGE. This was carried out on 5-20% gradient gels, cast in house using a gradient mixer. 5% and 20% gel solutions were mixed to create a continuous gradient and topped with water-saturated butanol to ensure a level surface. Once the gel set and the butanol layer was removed, a stacking gel was poured, and a comb added to form wells.

Prior to loading, gel application buffer (GAB) was added to samples (10μl GAB per sample) and then denatured by heating at 100°C for 5mins on a heat block.

Denatured samples were loaded into wells alongside 5μl BLUeye Prestained Protein marker (Geneflow), and the gel run at 45mA for approximately 2hrs in electrode buffer (see table 1 for composition), or until the sample reached the bottom of the gel. The full composition of the gradient gels is detailed in table 1 (see 2.3.3).

2.3.3 Western blotting

After the separation of denatured protein samples by SDS-PAGE, gels were placed onto a 0.45μm nitrocellulose membrane (Amersham Protran, GE Healthcare) and sandwiched between layers of sponge and filter paper (Whatman), all pre-soaked in transfer buffer (see table 1 for composition). The transfer of denatured proteins to the nitrocellulose membrane was carried out blotting overnight at RT, at 40mA (Fisherbrand Electrophoresis Unit). The presence of proteins on the nitrocellulose membrane was confirmed by Ponceau-S staining, which was then washed off by x3, 5min washes in TBS-Tween 0.1% (Tween 20, Sigma). For blocking, membranes were sealed in a plastic sleeve with excess blocking buffer (see table1 for composition) for 1hr at RT. Membranes were then probed with primary antibodies diluted in blocking buffer, sealed in a plastic sleeve and incubated at 4°C overnight. Primary antibodies used were; α-β-Actin as loading control (at 1:25000; Sigma) and markers of autophagic flux, α-P62 (at 1:1500; Enzo) and α-LC3 (at 1:6000; MBL International). Following this, membranes underwent x3, 5min washes before incubation with a species-specific horseradish peroxidase (HRP)-conjugated secondary antibody (Rabbit α-mouse HRP, Swine α-rabbit HRP, both at 1:3000 in blocking buffer; Dako), sealed in a plastic sleeve at RT for 1hr. Prior to visualisation, membranes underwent a final set of x3, 5min washes, before being treated with Western Lightning Plus-ECL Enhanced Chemiluminescence (ECL) system (Perkin Elmer), according to manufacturer's guidelines. In a dark room, ECL-treated membranes were exposed to imaging film (Kodak) for durations of 30secs to 15mins, as needed. Film was then developed by immersion in developing solution (PQ universal, Ilford) and then fixer

solution (2000rt, Ilford), with washes in water after each immersion, then allowed to dry.

Table 1. Composition of buffers used in protein extraction, SDS-PAGE and Western blotting procedures as detailed in Section 2.4. All reagents were acquired from Sigma.

Component	Composition
5-20% Gradient Gels	
Acrylamide 30%	n/a
Ammonium persulphate (10%)	n/a
Buffer A	1.1M Tris, 0.1% (w/v) SDS, 30% (w/v) glycerol (pH 8.8); Sigma
Buffer B	1.1M Tris, 0.1% (w/v) SDS (pH8.8)
Sodium dodecyl sulphate (10%)	n/a
TEMED	n/a
Other Buffers	
Electrode buffer	0.025M Tris, 0.19M glycine, 0.1% w/v SDS
Gel application buffer (GAB)	0.15M Tris, 8M urea, 2.5% (w/v) SDS, 20% (v/v) glycerol, 10% (w/v) 2-mercaptoethanol, 3% (w/v) DTT, 0.1% w/v bromophenol blue (pH6.8)
Ponceau S solution	0.1% (w/v) Ponceau S, 10% (v/v) glacial acetic acid
TBS-Tween 0.1%	20 mM Tris, 150 mM NaCl (pH 7.5), Tween 01% (v/v)
Transfer buffer	25 mM Tris, 192 mM glycine, 20% (v/v) methanol
Triton Lysis Buffer	25mM HEPES, 100mM NaCl, 1mM EDTA, 10% (v/v) glycerol, 1% (v/v) Triton X-100; Sigma

2.3.4 Analysis of Western blot by densitometry

Developed Western blot films were quantified by way of densitometry using FIJI open-source image analysis software (Schindelin et al., 2012). Developed blot films

were digitally scanned to create .tiff image files. FIJI's gel analysis tools were then used to convert scanned images to 8-bit grayscale before creating density peak profiles of each antibody band. The area under each peak was then measured, producing a value for each peak as percentage of the total area of all peaks. These values were then used to calculate the relative intensity of each band, normalised to that of the loading control, β-actin.

2.4 Overexpression and Knock-down of P62

2.4.1 Production and purification of plasmids

All plasmid constructs were cultured from glycerol stocks, from which a sterile pipette tip was used to scrape the top of frozen stocks to inoculate 10ml of Luria-Bertani (LB) broth media (consisting of 10g/L tryptone, 5g/L yeast extract, 10g/L NaCl & 100μl/ml Ampicillin or Kanamycin). Inoculated media was cultured overnight at 37°C. Glycerol stocks were setup using 0.5 ml of overnight culture mixed with 30% (w/v) glycerol and stored at -80°C. Plasmid DNA was isolated from overnight cultures using a plasmid Miniprep kit (Qiagen), according to manufacturer's instructions.

2.4.2 Expression of SQSMT1/P62 constructs

For all 'Developmental Experiments', primary cortical neuron cultures were transfected 24hrs after plating, whereas 'Degeneration Experiments' were transfected at DIV7. All transfected cultures were plated with a cell density of approximately $1.75x10^5$ cells/cm^2, with the exception of experiments transfected for

the purposes of quantifying LC3 by immunofluorescence staining. These experiments where plated at an approximate cell density of $8.75x10^4$ cells/cm^2.

In brief, transfections were carried out by incubating 5μl Lipofectamine 2000 (Life Technologies) in 125μl Opt-MEM reduced serum media (Life Technologies), per well, for 15mins. In parallel, 1μg DNA per plasmid was added to 125μl Opti-MEM, per well. Both mixes were then combined and incubated for 30mins at RT before being added, drop-wise, to wells.

All plasmids were co-transfected with a pmax-FP-Green-C (Lonza) GFP-like reporter plasmid in order to identify cells successfully expressing transfected constructs. Previously successful work carried out by Dajas-Bailador et al has shown this manner of co-transfection to be effective and sufficient in primary cortical neurons used in this thesis (Dajas-Bailador et al., 2014). The following pDEST-EGFP-mCherry constructs were used to assess the effects of P62 mutant expression on axon length in the experiments detailed in chapter 3.3.1: $P62^{wildtype}$, $P62^{L341V}$, $P62^{K102E}$and $P62^{T430P}$. The pDEST-YFP-mCherry construct was used as a negative control. In all other experiments involving transfection of P62 constructs, the following were used: pDEST-HFN-empty vector control, $P62^{wildtype}$, $P62^{L341V}$, -$P62^{K102E}$and-$P62^{T430P}$.

In 'developmental' experiments, cultures were fixed and analysed 72hrs after transfection at DIV4, with the exception of cultures transfected for LC3 quantification, which were fixed and analysed 48hrs after transfection at DIV3. For rescue experiments, pharmacological compounds were added for 18hrs before the end of the 72hr post-transfection window.

For 'degeneration' experiments, cultures were fixed and analysed 48hrs after transfection, at DIV9. For rescue experiment in this time frame, pharmacological

compounds were added 2 hours after transfection. Cultures were then fixed and analysed 18hrs later. All 'degeneration' cultures had 500μl conditioning media added to each well at DIV5 to account for evaporation and nutrient depletion.

2.4.3 Knock-down of SQSTM1/P62 expression by siRNA in standard cultures

For the knockdown of endogenous P62 expression in standard cultures, siGENOME Human SQSTM1 siRNA (Dharmacon) was used alongside ON-TARGET plus Non-targeting Control Pool (Dharmacon), a pool of 4 non-targeting siRNAs, as control. The siGENOME siRNA and non-targeting control were introduced into cells using the same transfection protocol as described in the previous section, again 24hrs after plating. A final concentration of 50nM of siRNA and non-targeting control were used and were co-transfected with 1μg of pMAX-GFP reporter plasmid, used to identify successfully transfected cells. All siRNA experiments were fixed and analysed 72hrs after transfection, at DIV4

2.4.4 Knockdown of SQSTM1/P62 in microfluidic cultures

For P62 knockdown in microfluidic cultures, the cell permeable Accel Human SQSTM1 siRNA (dharmacon) was used alongside Accell Non-targeting Pool (Dharmacon) as control. Axonal compartments were imaged at approximately DIV5-6, when sufficient axons had crossed the microgrooves into the axonal compartment. Also referred to as Time 0 (T0). At this point, all media was removed from the soma channel reservoirs and replaced with a total of 100μl of conditioning media containing a final concentration of 1μM Accell siRNA or Accell non-targeting control. Axons growing in the axonal chamber were imaged again after 48hrs (T48).

For experiments involving the pharmacological rescue of siRNA treated microfluidic cultures, 30hrs after addition of the siRNA, media containing the siRNA was removed from the soma channel reservoirs and replaced with 150μl conditioning media per each reservoir. Axonal channel reservoir media was removed and replaced with 100μl conditioning media containing a final concentration of 1μM pimozide per each reservoir. Axonal compartments were imaged after 18hrs, which total 48hrs post-transfection.

2.5 Immunohistochemistry of primary neuronal cultures

Neurons cultured on glass coverslips were washed in 1X PBS before being fixed in 4% (w/v) paraformaldehyde (3.6% sucrose (w/v), 1XPBS, 5mM $MgCl_2$, pH 7.4; ThermoFisher) for 30 minutes at room temperature (RT). Coverslips were then washed 3 times with 10mM Glyceine in 1X PBS (Thermofisher). At this point, experiments transfected with a GFP reporter and not requiring further staining were mounted onto glass slides (Menzel Glaser) with Vectashield Hardset mounting media, with or without DAPI (Vectorlabs). Coverslips requiring immunohistochemical staining were permeabilised for 20mins at RT in PBS/Glycine-0.2% Triton (1x PBS, 10mM glycine, 0.2% (v/v) Triton X-100; Sigma), washed 3 times in 1X PBS, then blocked for 1hr at RT in 3% (v/v) Bovine Serum Albumin (BSA; Sigma) in 1X PBS. Coverslips were then placed onto 50μl droplets of primary antibody in 3% BSA in a make-shift humidity chamber (Foil covered petri dish with ddH_2O saturated paper taped to the inside of the lid and Parafilm (Sigma) lining the base onto which droplets of antibody solution were placed) and incubated overnight at 4°C. Following primary incubation, coverslips were washed 3 times in PBS-0.1% Trition (1x PBS,

0.1% (v/v) Triton X-100) and incubated with secondary antibodies in 3% BSA for 1hr at RT, again in make-shift humidity chambers. Finally, coverslips were washed 3 times with 1X PBS before mounting onto glass coverslips with Vectashield Hardset, with or without DAPI. Details of primary and secondary antibodies and dilutions used can be found in table 2.

Table 2. Antibodies and working concentrations used in immunohistochemical staining of fixed cell cultures.

Antibody	Dilution
Primary antibodies	
Mouse α-Acetylated tubulin (Sigma)	1:300
Rabbit α-β-III tubulin (Abcam)	1:300
Mouse α-Human P62/SQSTM1 (Enzo)	1:150
Rabbit α-LC3, rabbit (MBL International)	1:500
Secondary antibodies/stains	
Alexa Fluor 488, goat α-mouse (Sigma)	1:300
Alexa Fluor 488, goat α-rabbit (Sigma)	1:300
Alexa Fluor 568, goat α-mouse (Sigma)	1:300
Alexa Fluor 568, goat α-rabbit (Sigma)	1:300
Phalloidin 568 (Sigma)	1:100
DAPI (Sigma)	1:100

2.6 Microscopy image acquisition

All images of fixed and live cultures were captured using an Axiovert 200M inverted microscope (Zeiss) fitted with a CCD camera (Retiga 3000, QImaging) and μManager softare (Edelstein et al., 2014). Imaging was carried out using either a 10X, 10X phase

contrast, 20X or 64X objective, as required. All image analysis was carried out using FIJI open source software (Schindelin et al., 2012).

2.7 Autophagy assessment by quantification of fluorescence intensity

The quantification of the intensity of fluorescence staining was used to assess the relative levels of the marker of autophagic flux, LC3 in transfected cultures were quantification by SDS-PAGE and Western blotting was not possible.

The process of quantifying the fluorescence intensity of images is summarised in figure 10, below. Images of fixed coverslips were captured using a 64X objective, using the same exposure settings for all images in any given experimental set. Two-channel images were opened in FIJI where the free-hand drawing tool was used to delineate the outline of GFP-positive cells. By saving as a region of interest, this delineated border was then overlaid onto the second channel showing LC3 staining (Figure10, C1 and C2). With the parameters of area, integrated density and mean grey value selected, at least 20 cells were measured per condition in each individual experiment. Three background readings were taken for each cell (Figure 10, C3). Using the output measurements, corrected total cell fluorescence (CTCF) was calculated as; CTCF = Integrated Density - (Area of selected cell X Mean fluorescence of background readings). All CTCF values for each condition were averaged, normalised to their respective control group and presented as a percentage of the control.

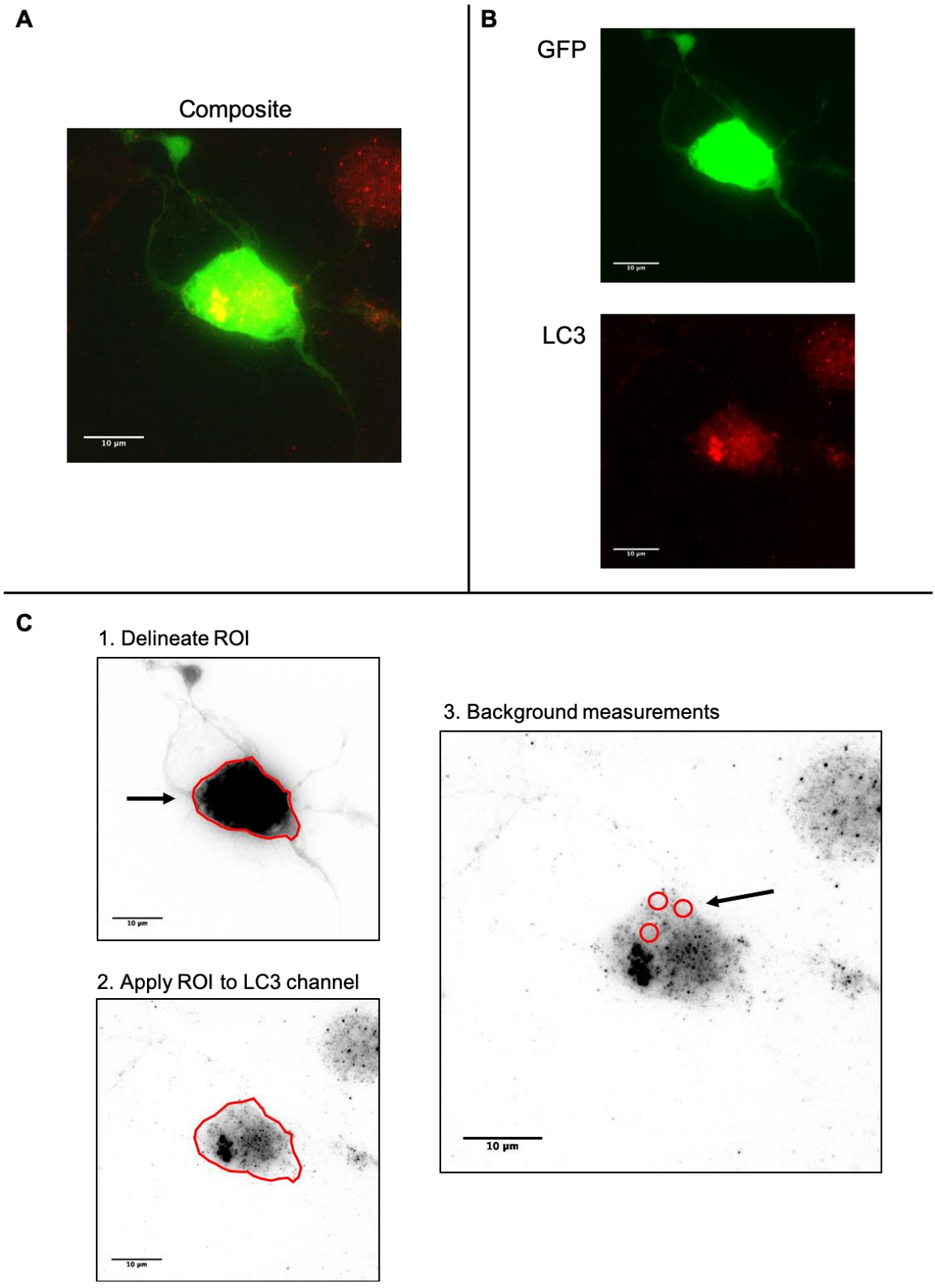


Figure 10. Workflow for the quantification of fluorescence intensity in FIJI. (A) The composite image and (B) breakdown of each component channel. Using the GFP channel image (C1), an ROI (Region of interest) is delineated (red) and applied to the same location in the LC3 channel image (C2) to measure fluorescence. (C3) Three background measurement were taken from inside the cell of interest, in the LC3 channel, in areas lacking LC3 puncta.

2.8 Axon length assays

2.8.1 Axon length in dissociated primary cortical neuron cultures

Images of fixed coverslips were captured using an Axiovert 200M inverted microscope (Zeiss) under a 10X objective on μManager (Edelstein et al., 2014) software. Images were analysed using FIJI software (Schindelin et al., 2012) where the freehand line tool was used to delineate the length of cortical axons from the cell body to the tip of the longest axonal projection, as shown in figure 11. For these measurements, an axon was defined as being at least 2x the total length of the cell body and at least 2x the length of any other projections. For each experiment of any given condition, 40-80 individual axons were measured. The average of measurements for each experimental set were then averaged overall to give a final value for that experimental condition. By using between n=4-6 experimental sets per condition, final mean values for each condition reflect the measurements of 160-240 unique axons. Each condition was normalised to its respective control and the data presented as a percentage of the control.

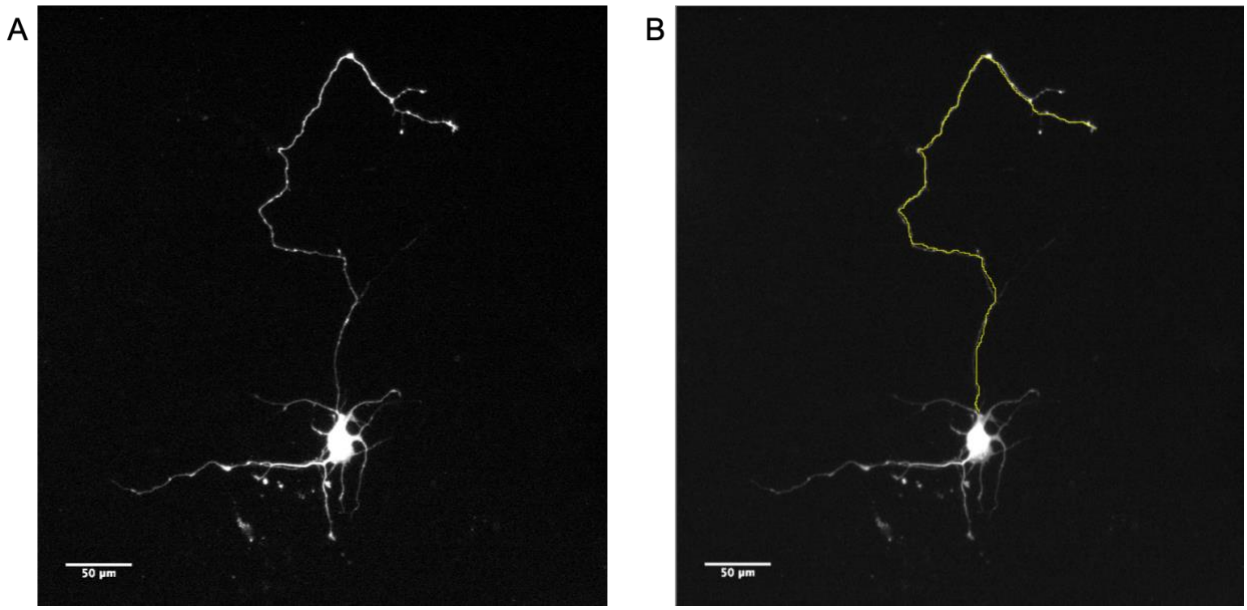


Figure 11. Delineation of cortical axons. Axons were traced from the base of the cell body, to their most distal point.

2.8.2 Axon length in microfluidic cultures

Cultures were grown to DIV5, or until axons began to cross the microgrooves from the cell body chamber into the axonal chamber. Due to the intrinsic variability in the pattern of axonal growth in each neuronal culture in a microfluidic device, axon length was assessed as the percentage change in length from before the application of pharmacological compounds (t=0hrs), and 18hrs after drug application (t=18hrs). As such, cultures were imaged while live, using 10X phase contrast objective. For each timepoint, images were taken across the entirety of the axonal chamber of the device. At t=0h the length of all visible axons was measured, from the base of the axonal chamber to the tip of their most distal projection. For t=18hrs images, intact axons which were previously measured at t=0h were measured again, discounting any new projections which had crossed the microgroove boundary since t=0hrs. This method is summarised in figure 12. By using between n=6-8 experimental sets per

condition, final mean values for each condition reflect the measurements of 8-104 unique axons. All measurements were averaged, normalised to an experimentally matched control and expressed as a percentage change in growth, relative to the control.

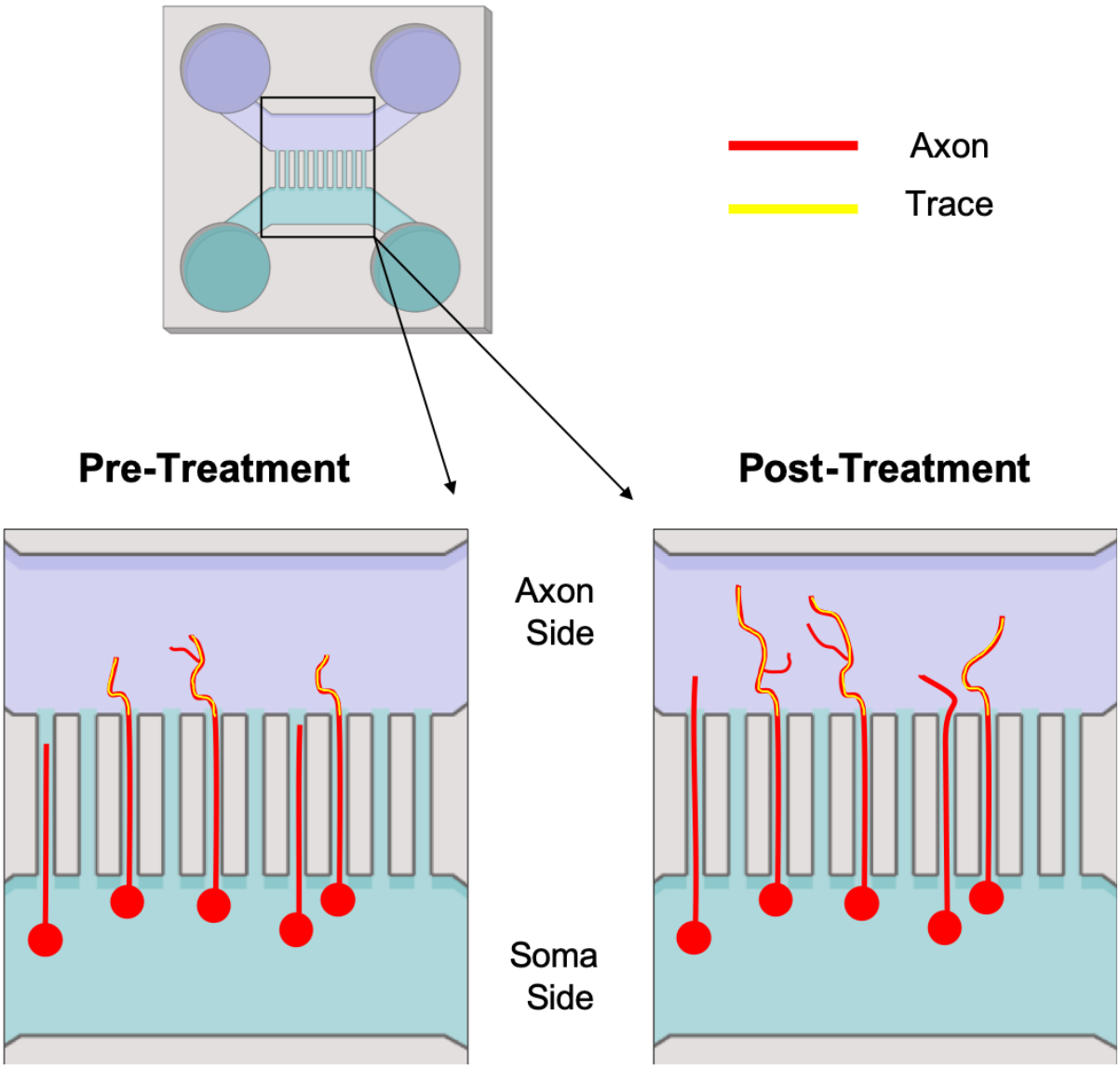


Figure 12. Measurement of axon length in microfluidic culture devices. Axons (red) are traced (yellow) from where they emerge from the microgroove to their most distal point. Post-treatment, the same axons are re-traced, ignoring new branches or axons newly emerging from the microgrooves.

2.9 Quantification of axon degeneration

2.9.1 Assessing axonal degeneration

The quantification of axon degeneration was used as a functional assay of axon health in 'Degeneration Experiments'. Fixed coverslips of DIV9 cultures were imaged using a 20X objective with the same exposure settings were used for all images in a given set. Between 15-50 fields showing networks of GFP-positive axons and excluding cell bodies or cell debris per condition in each individual experiment. Images were converted to 8-bit grayscale before manually applying a threshold at the same settings to all images of a given experimental set, in order to produce binary images. The ratio of degenerated to intact axons for each image was then automatically analysed using the Degeneration Index plugin (Sasaki, Vohra, Lund, & Milbrandt, 2009). In brief, the plugin calculated the ratio of fragmented axonal area to the total axonal area after subtraction of background noise, thus defining the Degeneration Index value as the ratio of area of intact axons to area of detectable axon fragmentation. Example visual outputs of this process are given in figure 13. By using between n=4-5 experimental sets per condition, final mean values for each condition reflect the measurements of 7-43 unique images. Each condition was normalised to its respective control and the data presented as a percentage of the control.

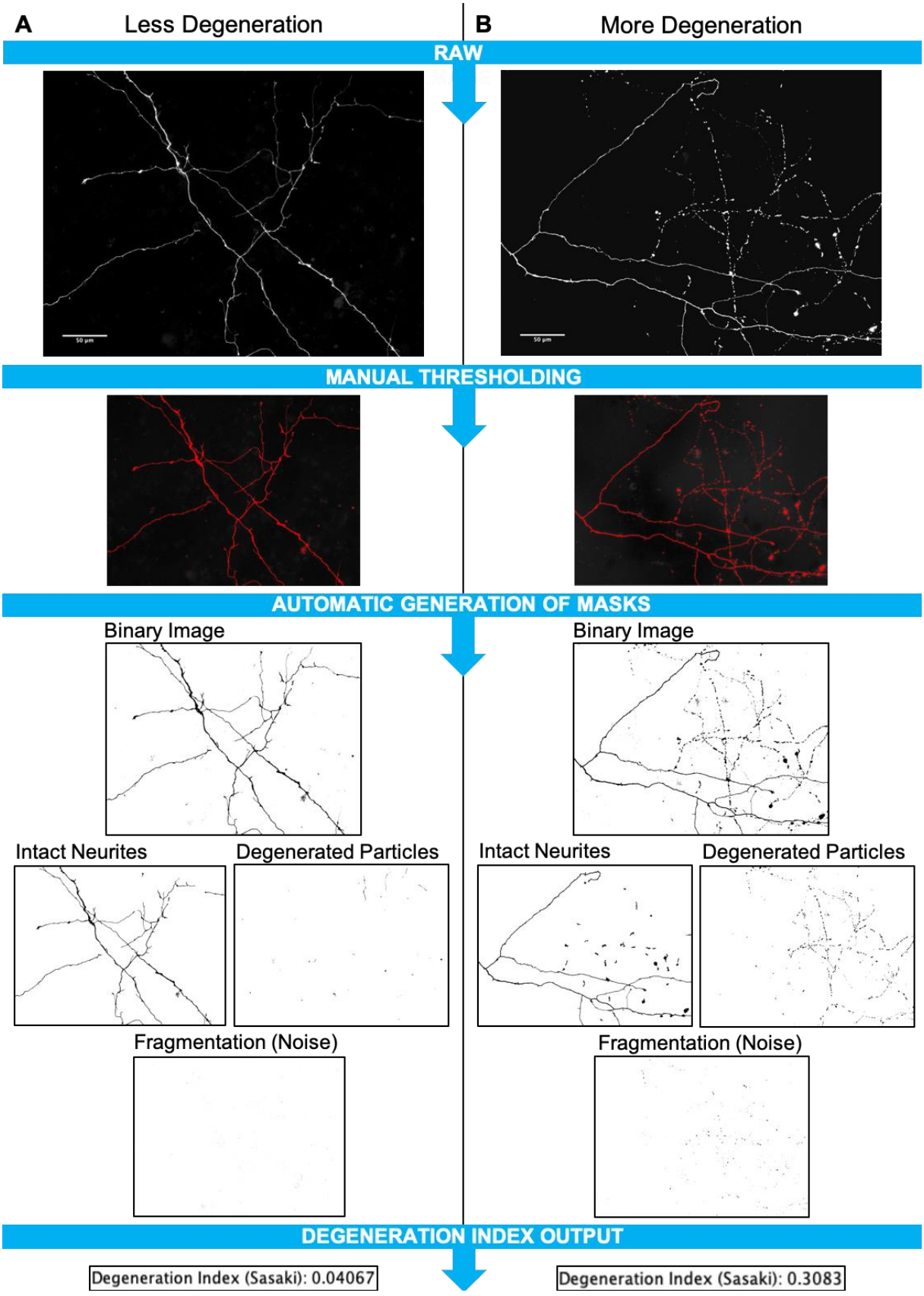


Figure 13. Workflow for quantifying axonal degeneration in GFP-positive neurons of DIV9 cultures. A threshold is applied to raw images, allowing for automatic analysis of the degeneration index.

2.10 Statistical analysis

All statistical analysis of data was carried out using GraphPad Prism (Version 8.1.0 for macOS, GraphPad Software, La Jolla California USA, www.graphpad.com). All data sets were assessed for normal distribution by way of Shapiro-Wilk normality test. In axon outgrowth experiments, the inter-experimental variability of the absolute values of length distribution is a confounding factor for analysis. To account for this, length data was normalised to the mean length of the control neurons within each experimental set, therefore the mean of the control length becomes 100% for all biological replicates. Statistical evaluation between two groups was performed using One-Sample Student's t-test, considering a hypothetical value of 100 which defines the control group. Analysis of more than two groups were carried out using One-way ANOVA with Tukey's or Dunnett's *post hoc* analysis. All data are presented as ± standard error of mean (mean±S.E.M.). Significance was determined where $p \leq 0.05$.

3. Exploring P62 dysfunction and autophagy regulation in early axonal development

3.1 Introduction

3.1.1 Primary cortical neuron culture as a model of axonal development

The initial investigations of this thesis sought to probe the role of P62 and autophagy in the context of early neuronal development. Traditional, 2-dimensional (2D) primary cortical neuron culture is an ideal model by which to investigate the impacts of autophagy impairment on the developing axon, as the growth and development of primary cortical neurons *in-vitro* has been well characterised (Barnes & Polleux, 2009). Not only is the course of growth of this neuronal cell type well described, but 2D cultures also allow for convenient application of experimental interventions and subsequent imaging of individual cells and their entire axons, especially within the early stages of growth. These features make the model ideal for assessing axonal development by quantifying axon outgrowth over time.

3.1.2 Pharmacological inhibitors and activators of autophagy

There is considerable variety in the types of pharmacological compounds available that modulate autophagic activity, all with varying mechanisms of action. However, the most commonly used compounds for activation and inhibition of autophagy in in-vitro studies are rapamycin and bafilomycin A1, respectively. The v-ATPase inhibitor, bafilomycin A1 inhibitions autophagy by inhibiting lysosomal functioning which prevents the degradation of autophagosomes. In PC12 cell induced into sympathetic neuron-like cells, bafilomycin has been shown to induced neurite

outgrowth (Tamura & Ohkuma, 1991). Rapamycin, which modulates autophagy via the interaction with the mTOR pathway (Singh et al., 2016), has previously been used in primary cortical neuron cultures where it resulted in reduced axonal outgrowth in early neurons (Ban et al., 2013). Despite its widespread use, rapamycin poses a problem due to the potential for undesired, off-target effects that can be elicited through mTOR activation via its wide array of molecular functions (Takei, Nawa, Luikart, Tarassishin, & Einstein, 2014). In light of this, the following experiments include pimozide as an additional autophagy activating compound. Unlike rapamycin, pimozide activates autophagy in an mTOR-independent manner via calcium ion channels (L. Zhang et al., 2007), and thus reduces the potential for off-target effects. Its use is also relevant in that it has previously been used as a therapeutic agent in a zebrafish model of ALS (Patten et al., 2017).

3.1.3 Neuronal development and autophagy

Basal autophagy is considered a vital homeostatic function and is pivotal in neuronal development processes (K.-M. Lee et al., 2013). Neurons are cells which undergo complex morphological changes as they develop, from polarisation, to axon specification and axonal outgrowth, guided by a highly responsive growth cone (Polleux & Snider, 2010). As such, autophagy is involved in modulating the highly dynamic protein turnover involved in this process (Marsh & Dragich, 2019). Dysfunctional autophagy during neuronal development has even been linked to aberrant connectivity and functional issues later in life, where, for example, mutations to PTEN (phosphatase and tensin homolog deleted

on chromosome ten) leading to mTOR dysregulation have been strongly linked to autism spectrum disorders (Tommy L Lewis, Courchet, Polleux, & Polleux, 2013). While ALS-FTLD is not a disease of the young, in order to understand the mechanisms behind the disease and it's familial P62-linked mutations, it is important to understand how such changes could impact the developing axon.

3.1.4 Autophagy and P62 mutations in ALS-FTLD

Considering the recent identification of P62 mutations in some instances of ALS-FTLD (Majcher et al., 2015), there is a strong suggestion that regulation of autophagy machinery via P62 may have a considerable role to play in modulating axon development. In particular, recent work showed that an L341V mutation in P62 that was identified in ALS-FTLD patients is confirmed to inhibit autophagy by reducing the binding affinity of P62 for LC3-II (Goode et al., 2016). While the L341V mutation affects the LC3-binding region (LIR) of P62, the binding of LC3 to this region is not exclusively responsible for the correct functioning of P62. The binding of ubiquitinylated cargoes to its ubiquitin-associated domain (UBA) and its Phox and Bemp1 (PB1) domain also play vital parts in the protein's function (Pankiv et al., 2007). As such, the ALS-associated mutants $P62^{K102E}$ (Rea, Majcher, Searle, & Layfield, 2014; Teyssou et al., 2013) and $P62^{T430P}$ (Z. Deng et al., 2017; A Goode et al., 2016) affecting the PB1 and UBA regions, respectively, are also included in the experiments presented in this chapter.

3.2 Aims and hypothesis

P62 mutations are known to be present in ALS-FTLD, and it is suspected that the inhibitory effect on autophagy produced by such loss of function mutations may be the contributing factor that results in a pathological phenotype. However, to more fully understand how these mutations affect the axon, it is necessary to explore the role of autophagy and P62 more specifically at earlier stages of growth and development. In the following experiments, the autophagy activating compound pimozide will be tested alongside well-established autophagy modulators to assess its effects on axon outgrowth during early development. The effects of P62 loss of function mutants will also be explored, with the aim of characterising the effects of such mutants on primary cortical neuron axons and to what extent the effects can be counteracted pharmacologically.

The specific hypothesis being tested via the experiments in this chapters predicts that;

- Both expression of the $P62^{L341V}$ mutation and downregulation of endogenous P62 by way of siRNA in cortical neuron axons during early neurodevelopment will lead to increased axonal outgrowth due to decreased autophagic activity.
- The application of autophagy activators Pimozide and Rapamycin will be sufficient to cause a decrease in axon growth, with the application of Bafilomycin A1 will be cause an increase in the length of developing axons.
- Pharmacological activation by of autophagy will increase axonal branching, while inhibition of autophagy will reduce axonal branching.

- Autophagy activation through the use of Rapamycin and Pimozide will be sufficient to rescue the effect of increased axon growth caused by autophagy inhibition through $P62^{L341V}$ expression.

3.3. Results & Discussion

As an overview, the methodological approach to experiments detailed in this section are summarised in figure 14. In brief, all 'Development' experimental interventions were carried out in primary cortical neuron cultures between DIV1 and DIV 4, with experiments coming to completion for analysis at either DIV3 or DIV4.

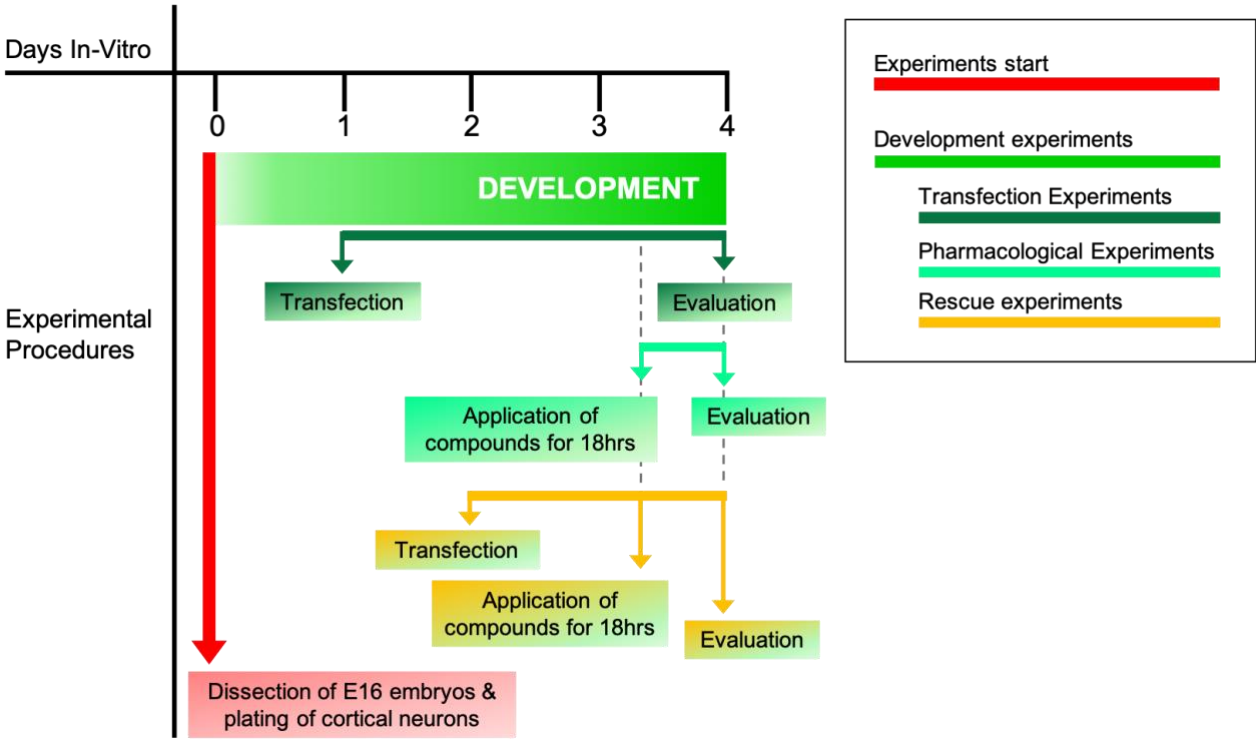


Figure 14 Timeline of Development Experiments. All experimental interventions were conducted between DIV1 and DIV4. Transfection of P62 constructs was carried out at DIV1 with experiments terminating 48hrs later at DIV3 for rescue experiments, and 72hrs later at DIV4 for all other experiments.

3.3.1 Autophagy activation reduces axonal outgrowth

The purpose of this initial experiment was two-fold. Firstly, it was necessary to establish what the resulting effects of autophagy activation and inhibition in our model of primary cortical neuron culture would be. Secondly, this experiment sought to identify whether mTOR-independent activation of autophagy using pimozide would prove effective in this model, compared to the widely used mTOR activator, rapamycin. Preliminary evidence from imaging studies in the motor neuron-like NSC34 cell line, kindly provided by Dr. Daniel Scott at the University of Nottingham, presented in figure 15, B, shows that in a cell culture model, pimozide is able to rescue the autophagy inhibition phenotype produced by the expression of the $P62^{L341V}$ mutant. These data help support the decision to use pimozide as an alternative autophagy activator in the experiments presented in this section.

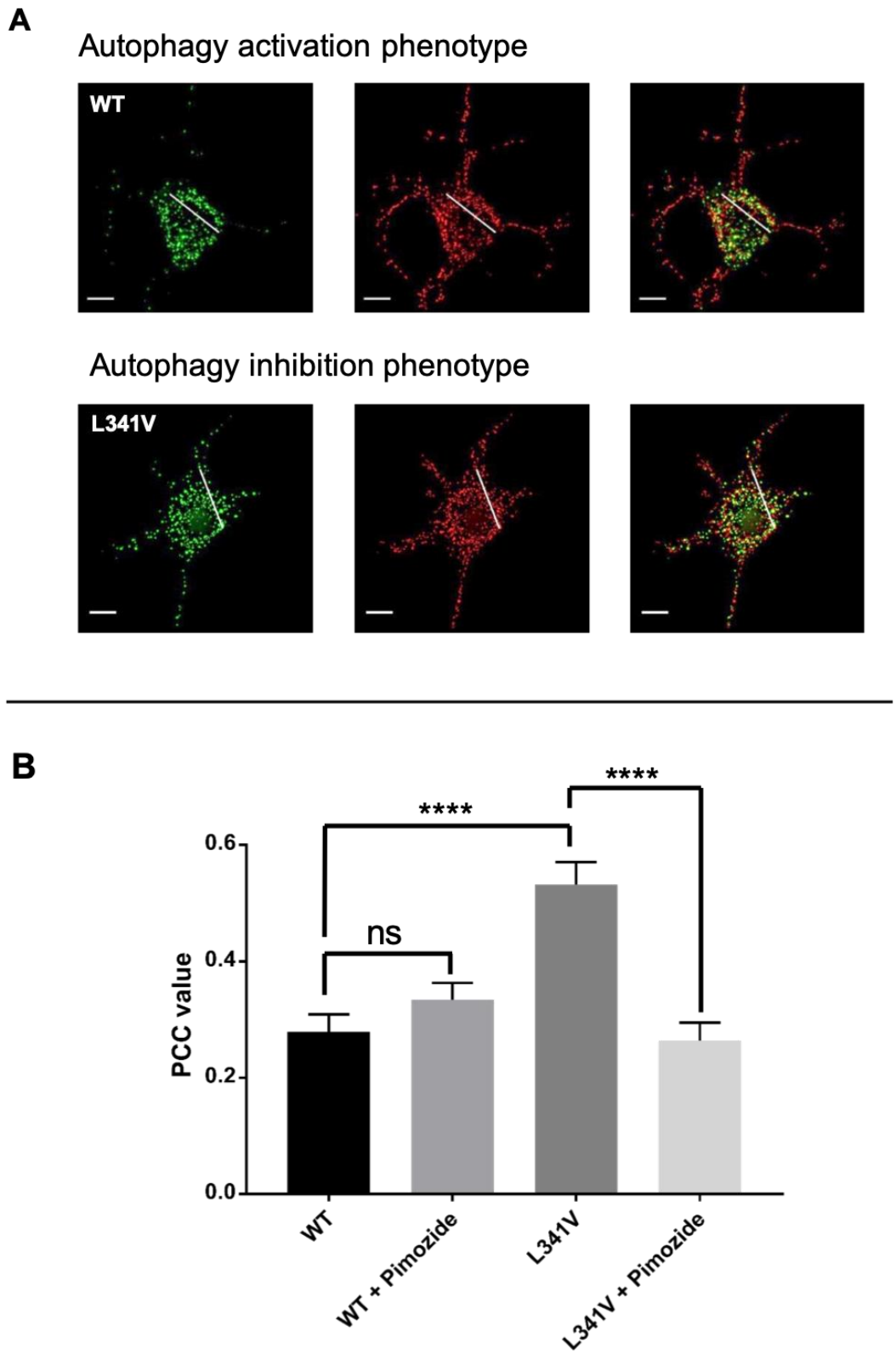


Figure 15. Pimozide rescues the P62^{L341V} autophagy inhibition phenotype in NSC34s. NSC34 cells were transfected with GFP-mCherry tagged wild-type (WT) P62 or P62^{L341V}. (A) Representative images of GFP-mCherry-wild-type and GFP-mCherry-P62^{L341V}. Upon incorporation into mature, acidic autophagosomes, the green GFP signal is quenched, leaving only the red, mCherry signal. Yellow puncta indicate the

overlap of GFP and mCherry signals and thus incomplete autophagy. (B) The portion of overlap between GFP and mCherry was assessed as a measure of autophagic flux. Data represents pooling of >25 cells per condition, where n=3 independent replicates for each condition. Data is presented as ±SEM. Images and data kindly been provided by Dr Daniel Scott.

For the experiments presented below in figure 16, primary cortical neuron cultures were treated with rapamycin, pimozide and bafilomycin A1, with DMSO as control vehicle, for 18 hours on DIV4. After fixing, immunohistochemical staining was carried out for the structural protein acetylated tubulin which allowed for the identification and measurement of cortical neuron axons.

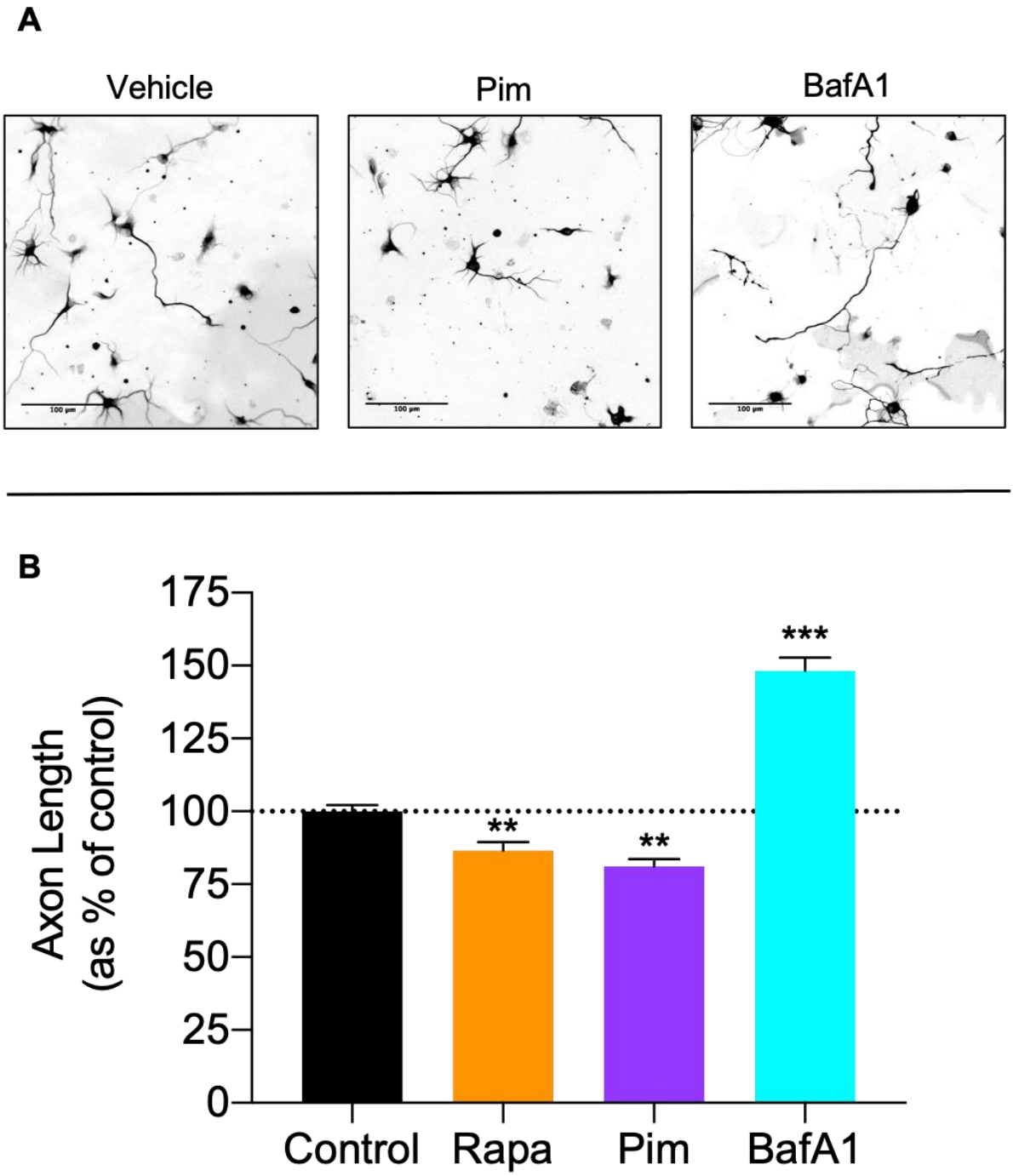


Figure 16. Pimozide decreases axonal outgrowth via activation of autophagy. (A) Pharmacological activation of autophagy by use of rapamycin and pimozide led to a reduction on axonal outgrowth, compared to vehicle treated controls. Conversely, inhibition of autophagy by bafilomycin increased outgrowth, compared to the control. Cultures were treated for 18hrs at DIV4. Data is expressed as mean ± SEM. Each group represents n≥3 individual experiments, where each experiment is comprised of the measurements of n≥40 axons. Student's t-test, ** p≤0.01, *** p≤0.001 and **** p≤0.0001. (B) Images are representative of the majority of neurons measured, stained here for acetylated tubulin. Scale bars are 100µm.

The results in figure 16 show that pharmacological inhibition of autophagy in cortical neurons at DIV4 by use of bafilomycin A1 induced a significant increase in axonal outgrowth, compared to controls. Bafilomycin A1 is a commonly used autophagy inhibitor (Myeku & Figueiredo-Pereira, 2011) and has been previously shown to increase autophagy in mouse hippocampal neurons (Maday & Holzbaur, 2016) and increase neurite length in the PC12 neuronal cell line. (Tamura & Ohkuma, 1991). The results in figure 16 corroborate these previous findings from the literature and show that in this culture model of cortical neurons, the inhibition of autophagy, at least by the inhibition of lysosomal formation by Bafilomycin A1, has a positive correlation to axon outgrowth producing axons almost 1.5X the length of controls.

Conversely to the action of bafilomycin A1 on axon length, pharmacological activation of autophagy in cortical neurons at DIV4, by both rapamycin and pimozide, resulted in an overall reduced axon length, compared to controls. This is in-keeping with previous findings such as those by Ban et al., 2013, wherein autophagy activation by rapamycin in primary cortical neurons produced a phenotype of reduced axon length at an even earlier stage of neuronal development (DIV2). Additionally, the ability to produce the autophagy activation phenotype seen by mTOR-dependent activation with rapamycin, but by mTOR-independent activation with pimozide provides further justification of the use of pimozide as an autophagy activating compound in this model.

During axon development, a highly dynamic balance of protein synthesis and degradation is at play, particular within the growth cone, as the axon extends

outwards towards its pre-synaptic target (Lowery & Van Vactor, 2009). As the polymerisation of microtubules in the distal axon drives the growth the axon (Kunda et al., 2001), the dynamic extension and retraction of actin filament components of the growth cone allow for the fine control or 'steering' of axonal growth, in response to intra- and extra-cellular cues (Dickson et al., 2002). Considering the dynamic interplay between protein synthesis and degradation required during this process, it is therefore reasonable to expect that affecting basal autophagy levels at this stage of development is likely to alter these molecular controls and could be a cause of the differential effects in axon length seen in these results. For example, an increase in autophagy could result in less/slower microtubule polymerisation, leading to a reduced axon length, while a decrease in autophagy may be affecting axon guidance processes by limiting the plasticity of the growth cone. Overall, these results demonstrate the suitability of this primary cortical neuron culture model for further investigations into the role of autophagy in the axon

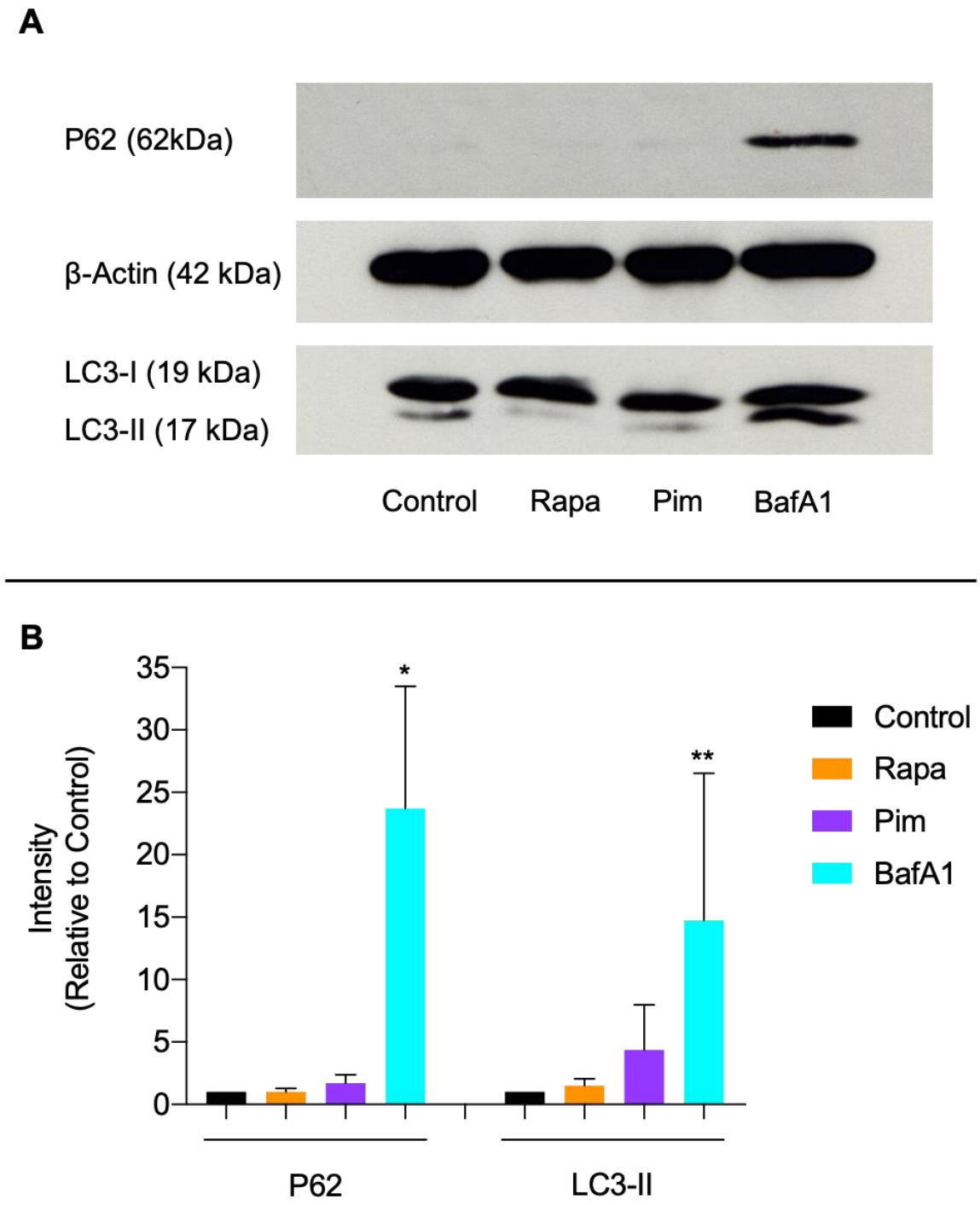


Figure 17. Western blot densitometry confirmation of pharmacological action on autophagy flux. (A) The image shown is the cleanest example of the blots resulting from this experiment but is not necessarily representative. They show a visible increase in P62 and LC3 in BafA1 treated cultures, as shown by the graph in part B. (B) Densitometry analysis of western blots confirm that bafilomycin treated cultures showed a 23.72 fold and 14.75 fold increase in the expression of P62 and LC3-II, respectively. Data is expressed as mean ± SEM. Each group represents n≥4 individual experiments. Student's t-test, * p≤0.05 and ** p≤0.01.

Following the assessment of their effects on axon length, it was necessary to confirm whether the action of these drugs did indeed affect any expected changes in relevant autophagy markers, reported here in figure 17. After treatment with rapamycin, pimozide and bafilomycin A1 for 18hours at DIV4, antibodies were used to probe western blots of protein extracts for the presence of two commonly used markers of autophagic flux, LC3 and P62. In brief, higher levels of P62 and LC3-II indicate the accumulation of autophagosomes and thus a reduction in autophagic flux, while a decrease in levels is indicative of higher flux.

In keeping with its strong effects on axon length, decreases in autophagic activity in cortical neurons through the treatment of bafilomycin A1 resulted in a marked increase in both P62 and LC3-II. These results are congruent with the reported effects of bafilomycin A1 on autophagy markers in cortical neurons in the literature (Redmann et al., 2017). Final densitometry analysis did not yield any statistically significant reductions in the levels of LC3-II and P62, compared to controls, that would be expected from the action of rapamycin and pimozide.

While a high LC3-I to LC3-II ratio is common in neuronal cell types, especially in the brain where LC3-I is known to be more abundant, the conversion of LC3-I to LC3-II is also known to be cell specific, and specific to the method of cellular stress. This means that it can be difficult to demonstrate a moderate induction of autophagy through LC3 quantification (Padman, Bach, Lucarelli, Prescott, & Ramm, 2013; D.-S. Yang et al., 2011). The expression of autophagy related proteins like P62 and LC3 has been shown to increase over time throughout the development of cortical neurons (Ban et al., 2013). This explains the low total levels of P62 in control lysates from

DIV4 cultures, meaning that the western blot technique was likely not sensitive enough to adequately detect small decreases in P62 expression that would result from autophagy activation. Despite difficulties in directly demonstrating protein level changes as a result of autophagy induction, rapamycin is still a widely accepted autophagy activator (Berger et al., 2006) and LC3-II quantification in rat primary cortical neurons treated with pimozide have shown that the compound does indeed induce autophagy activation (Tsvetkov et al., 2010). The very nature of autophagic flux also adds to the difficulty of identifying moderate decreases in LC3-II, as all cells within a single culture are unlikely to all be functioning at the same level of autophagic activity, despite the overall effect across the cell population (Klionsky et al., 2016).

3.3.2 siRNA knock-down of endogenous P62 results in axon elongation

Given the results described in the previous section, we next sought to begin our investigation into the nature by which the function of P62 and autophagy may impact axonal development. For the following experiment, 50nM P62 siRNA was co-transfected into cells with GFP, 24hrs after plating. Cultures were then left for 72hrs to allow for a substantial change in protein expression levels before being fixed and analysed for axon length.

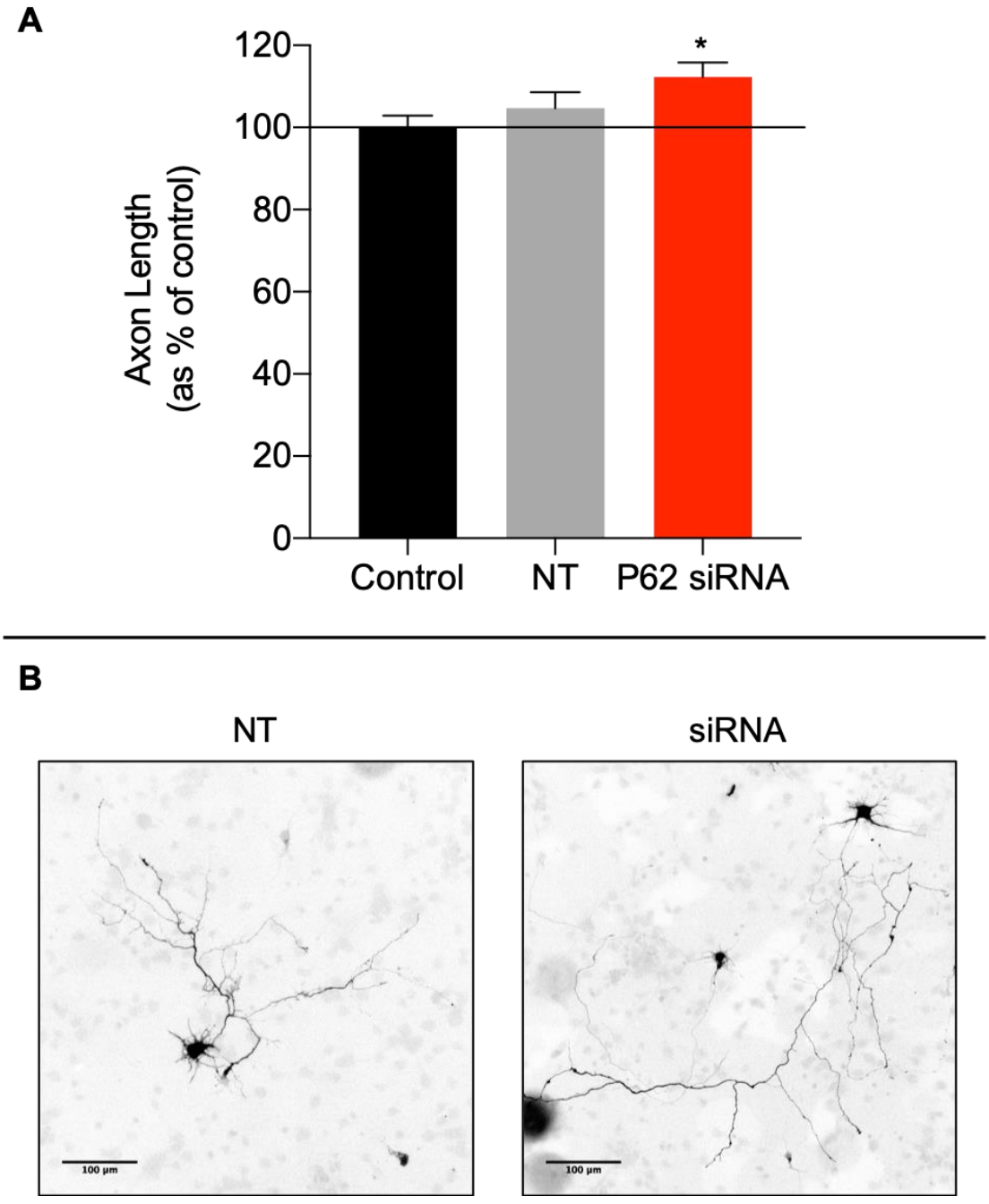


Figure 18. Knock-down of endogenous P62 by P62 siRNA increases axonal outgrowth. (A) Quantification of GFP-positive axons show that cultures transfected with P62 siRNA demonstrate increased axon lengths of 12.3%, compared to the control. Cultures were transfected 24hrs after plating and fixed 72hrs after transfection (DIV4). Data is expressed as mean ± SEM. Each group represents n≥8 individual experiments, where each experiment is comprised of the measurements of n≥40 axons. Student's t-test, * p≤0.05. (B) Representative images of GFP-positive neurons. Scale bars are 100µm.

Downregulating the endogenous expression of wild-type P62 in cortical neurons by P62 siRNA resulted in axons 12.3% longer than controls. As P62 is a marker of autophagy itself (Klionsky et al., 2016), it's therefore reasonable to expect that this result could be due to a decrease in levels of autophagy as a direct result of the decreased availability of endogenous P62. This phenotype of increased axonal outgrowth is mimicked by results from experiments in the previous section which show that pharmacological inhibition of autophagy results in greater axon growth. This comparison corroborates the idea that decreased availability of P62 in this experiment might be contributing to a decrease in basal autophagy.

Given the identification of loss of function P62 mutants in ALS and ALS-FTLD (Goode et al., 2016; Majcher et al., 2015), the results of this experiment support the notion that disturbances to P62 function (and thus autophagy) are linked to clinical pathologies seen in ALS-FTLD. This then raises the question as to whether this elongation phenotype would also be seen in the presence of the expression of loss of function mutants, such as $P62^{L341V}$.

3.3.3 The loss-of-function $P62^{L341V}$ mutant increases axonal outgrowth during development

Armed with evidence of the phenotypic effects that autophagy inhibition and P62 knock-down has on developmental axon morphology, the following experiment sought to examine the role of the $P62^{L341V}$ mutation on the axon in this context. To briefly recap, the $P62^{L341V}$ mutant is a recently identified, familial mutation in ALS

patients (Y. Chen et al., 2014) which has been shown to have a reduced binding affinity for LC3 in P62-mediated autophagy (Goode et al., 2016).

In the experiments detailed below, cortical neurons were co-transfected with GFP and the $P62^{L341V}$ construct 24hours after plating. The length of GFP-positive axons was then assessed at 72hours later, at DIV4. Transfection with $P62^{WT}$ was included as a negative control, while transfection with GFP only was used as the control. Transfection with $P62^{K102E}$ and $P62^{T430P}$ ALS-linked mutants was also included in this experiment for comparative purposes, to determine whether or not any effects seen would be attributed solely to $P62^{L341V}$.

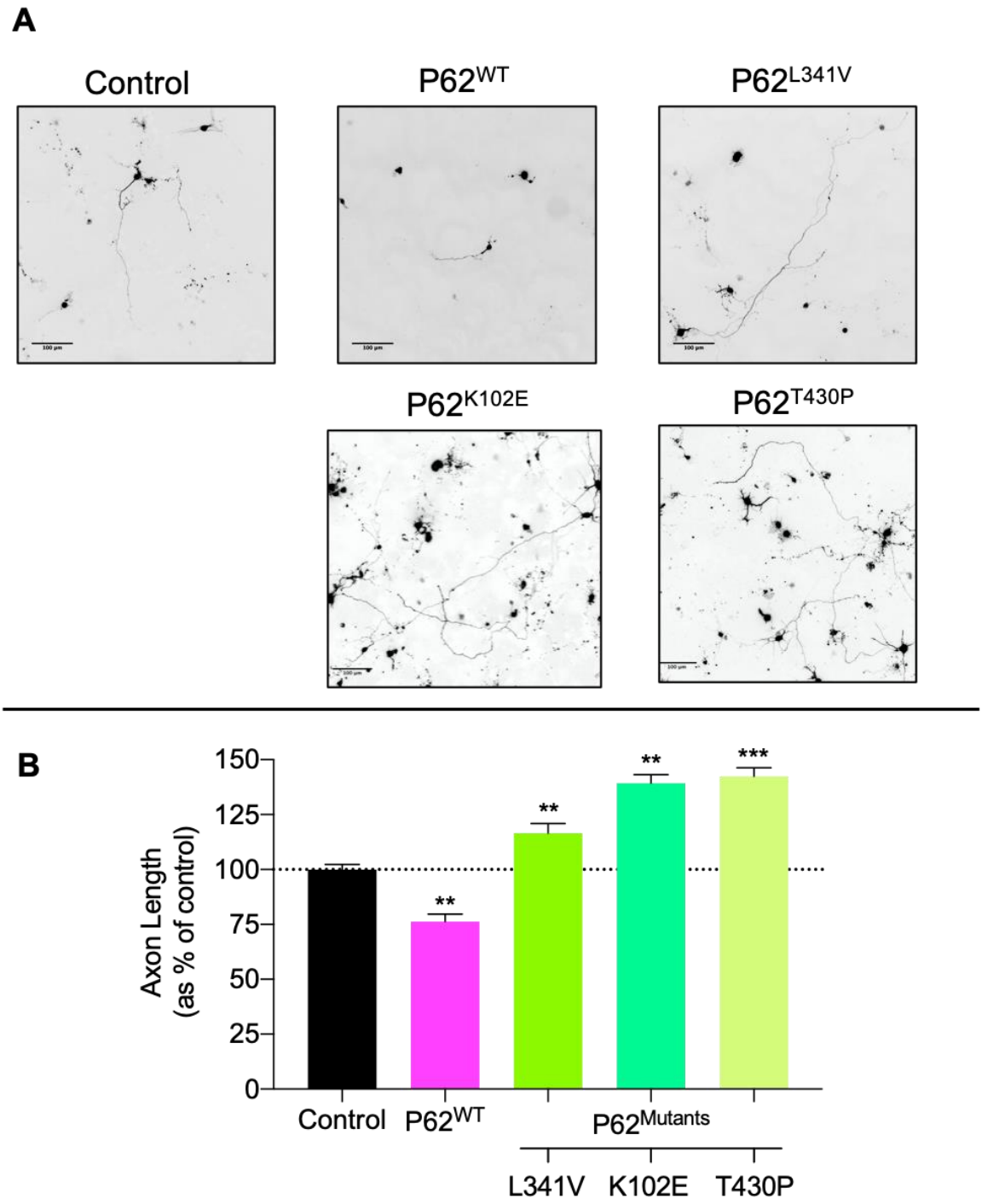


Figure 19. Expression of the loss-of-function mutant P62^{L341V} increases axon outgrowth. (A) Quantification of axon lengths of GFP-positive, transfected neurons show that expression of the P62^{L341V}, P62^{K102E} and P62^{T430P} ALS mutants result in increases in axon length of 16.6%, 39.3% and 42.5%, respectively. Conversely, overexpression of P62^{WT} led to a 23.7% decrease in axon length. Cultures were transfected 24hrs after plating and fixed 72hrs after transfection. Data is expressed as mean ± SEM. Each group represents n≥6 individual experiments, where each experiment is comprised of the measurements of n≥40 axons. Student's t-test, **

p≤0.01 and *** p≤0.001. (B) Images of GFP-positive neurons are representative of the majority of cells measured. Scale bars are 100μm.

The data displayed in figure 19 show that expression of the L341V mutant (as well as the K102E and T430P mutants) results in a phenotype of significantly increased axon outgrowth, compared to the control. This elongated axon phenotype was also seen in experiments in section 3.3.1 where autophagy was inhibited via the application of BafA1. These particular outcomes begin to suggest that the phenotype produced by ALS P62 mutants in these cortical neuron axons may be as a direct result of decreased autophagic activity by attenuated P62 functionality. These results corroborate previous findings by our group that show that the ALS-associated L341V mutation reduces the binding affinity of P62 for LC3 (Goode et al., 2016). It therefore adds to the argument that decreased efficiency of P62 function is likely altering the autophagy process to the extent that it has an effect on the fine control processes governing axonal outgrowth during development. In a wider context, these results also corroborate the idea that autophagy dysregulation plays a role in ALS pathology.

Conversely to the effect of the mutants, over expression of *wild-type* P62 resulted in axons with significantly less outgrowth than controls. This finding mimics the effect on axons seen when pharmacologically inducing autophagy using rapamycin and pimozide (section 3.3.1). Seeing this effect as a result of over expression of the wild-type protein is not unexpected, considering that increasing levels of P62 has been shown to increase levels of autophagy (Klionsky et al., 2016).

Considering the effects that the expression of loss of function P62 mutants raise in relation to the regulation of axonal development processes, we also investigated whether there was an impact on axonal branching (see figure 20). Our findings however did not find any significant differences in axonal branching of cortical neurons overexpressing either wild-type P62 or the L341V mutant, compared to the control.

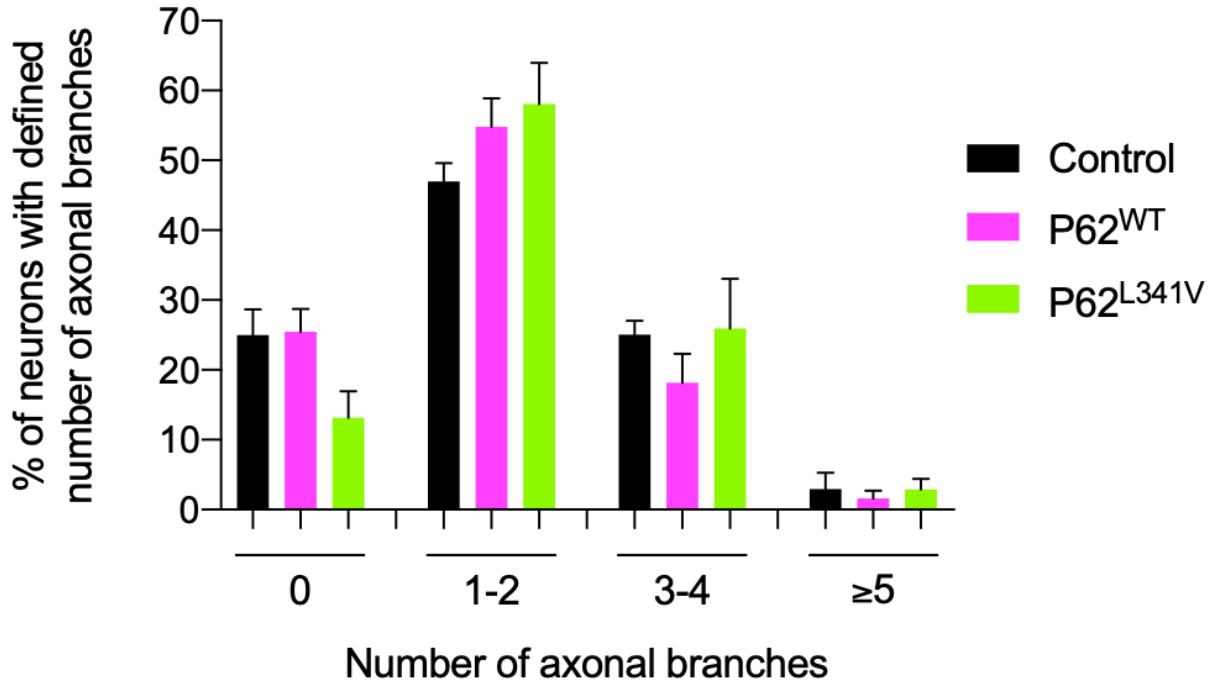


Figure 20. Assessment of axonal branching in cells expressing P62 constructs. Analysis of GFP-positive neurons expressing either $P62^{WT}$ or $P62^{L341V}$ showed no differences in axonal branching, compared to controls. Cultures were transfected 24hrs after plating and fixed 72hrs after transfection. Each neuron assessed was assigned a score based on the number of branches coming from the longest axonal projection. Analysis of groups was carried out using One-way ANOVA with Tukey's post hoc analysis. All data are presented as ± standard error of mean (mean±S.E.M.). Significance was determined where $p \leq 0.05$. Each group represents n=4 individual experiments, where each experiment is comprised of the assessment of n=30-82 neurons.

Following these experiments examining the effect of expression of the various P62 constructs, we sought to quantify the levels of the autophagy marker protein LC3 in

neuron cell bodies to confirm the impact that that said constructs were having autophagic flux. Given the overall low transfection efficiency of primary cortical neurons and the lack of sensitivity of available Western blotting techniques, we took the approach of quantifying the intensity of LC3 staining in fixed cultures. Cells were co-transfected with a GFP reporter and the relevant P62 construct 24hrs after plating. Cultures had been plated at half the standard density for transfection experiments in order to maximise the clarity of staining of individual cells for image analysis.

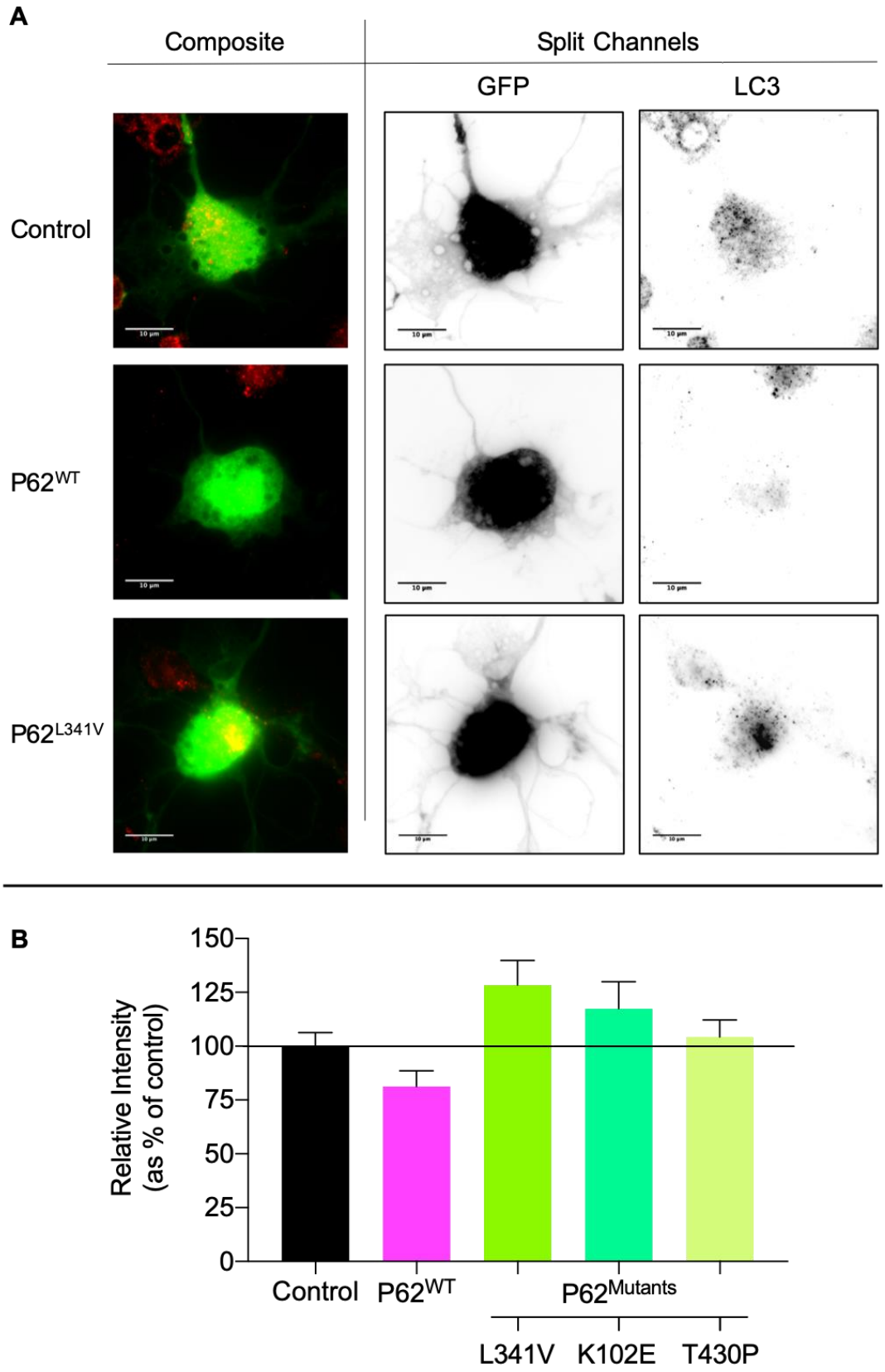


Figure 21. LC3 quantification in cells expressing P62 constructs. (A) images of GFP-positive neurons (green) stained for LC3 (red). Shown here as composite images in colour and individual channels in monochrome. Scale bars are 10μm. (B) Quantification of fluorescence intensity of LC3 staining in GFP-positive cell bodies

could not directly confirm whether expression of $P62^{WT}$, $P62^{L341V}$, $P62^{K102E}$ or $P62^{T430P}$ affected endogenous levels of LC3 in the cell body. Cultures were transfected 24hrs after plating, fixed 48hrs after transfection (DIV4) and subsequently stained for LC3. Data is expressed as mean $\pm$ SEM. Each group represents n=3 individual experiments, where each experiment is comprised of the measurements of $n \geq 20$ soma

Analysis of fluorescence intensity of LC3 staining (figure 21) did not yield any statistically significant differences between conditions and the control. Despite this, there are strong trends present in the data. Based on the observed trends, levels of LC3 appear to decrease in cells overexpressing $P62^{WT}$, compared to the control. This trend is in-keeping with existing knowledge of autophagy wherein as levels of P62 increase, so does autophagic flux (Klionsky et al., 2016; Sahani, Itakura, & Mizushima, 2014). In contrast, neurons expressing the $P62^{L341V}$ mutant appear to have increased levels of LC3 compared to the control. As increased levels of LC3 indicate decreased levels of autophagic activity, this supports previous work which shows that the $P62^{L341V}$ mutant has a much reduced binding affinity for LC3 (Goode et al., 2016). Additionally, preliminary imaging data from experiments expressing a dual tagged GFP-mCherry-$P62^{L341V}$ construct in NSC34 cells produced an autophagy inhibition-like phenotype (see figure 15, A). There is no definite trend purporting any particular change in LC3 levels in the cases of the $P62^{K102E}$ or $P62^{T430P}$ mutants, compared to the control.

There are multiple possible reasons why the observed trends were not found to be statistically significant. Firstly, an inadequate number of repeat experiments is likely to have led to the experiment being statistically under-powered. A 2-sample, 2-sided power calculation for an experiment of n=3, assuming 5% error, reported a power

value of 1−β=0.5903, below the desired threshold of 1−β=0.8. The same calculation suggest n=8 would have been required for 80% power. In reference to cultures overexpressing $P62^{WT}$ there can be difficulties in detecting small decreases in LC3 associated with autophagy activation, which could lead to increased variation in the data. This is discussed in more detail in section 3.3.1. Overall, a major factor contributing towards the margin of error in this experiment could be that the α-LC3 antibody available was also not able to distinguish between LC3 isoforms, and as such variations in ratios of LC3-I to LC3-II could have easily obscured any smaller changes in LC3-II. Observing the differences in LC3-I to LC3-II conversion in bafilomycin A1 treated cultures compared to controls, seen in section 3.3.1, it's likely that the high LC3-I to LC3-II ratio present in this primary cortical neuron model would have obscured any changes to LC3-II levels that would have been detectable through this method. One crucial reason for this lack of significant results is likely to lie in the large variability resulting from difficult to remove background in our attempt to quantify levels of LC3. Additionally, a certain level of non-specific, diffuse background is also expected from the immunohistochemistry techniques used to stain for LC3. The presence of high basal levels of LC3 expression, coupled with a diffuse background is likely to have contributed to an increased margin of error.

3.3.4 Autophagy activation rescues the effect of $P62^{L341V}$ expression on axon outgrowth

The idea being developed throughout this chapter is that the ALS-associated $P62^{L341V}$ mutation elicits a significant axon elongation phenotype in developing cortical neurons, potentially by dysregulating the balance of protein turnover in the growing

axon through inhibiting autophagy by ineffective functioning of P62. To further scrutinise this idea, a logical progression was to test whether activation of autophagy could rescue the effects of $P62^{L341V}$.

For these experiments, cortical neuron cultures were co-transfected with GFP and either the $P62^{L341V}$ or relevant empty vector control, 24hrs after plating. Approximately 54hrs after transfection, either 1μM pimozide, 20nM rapamycin, or control vehicle were added. After 18hrs incubation with the compounds, a total of 72hrs post-transfection, cultures were fixed, and the length of GFP-positive neurons measured.

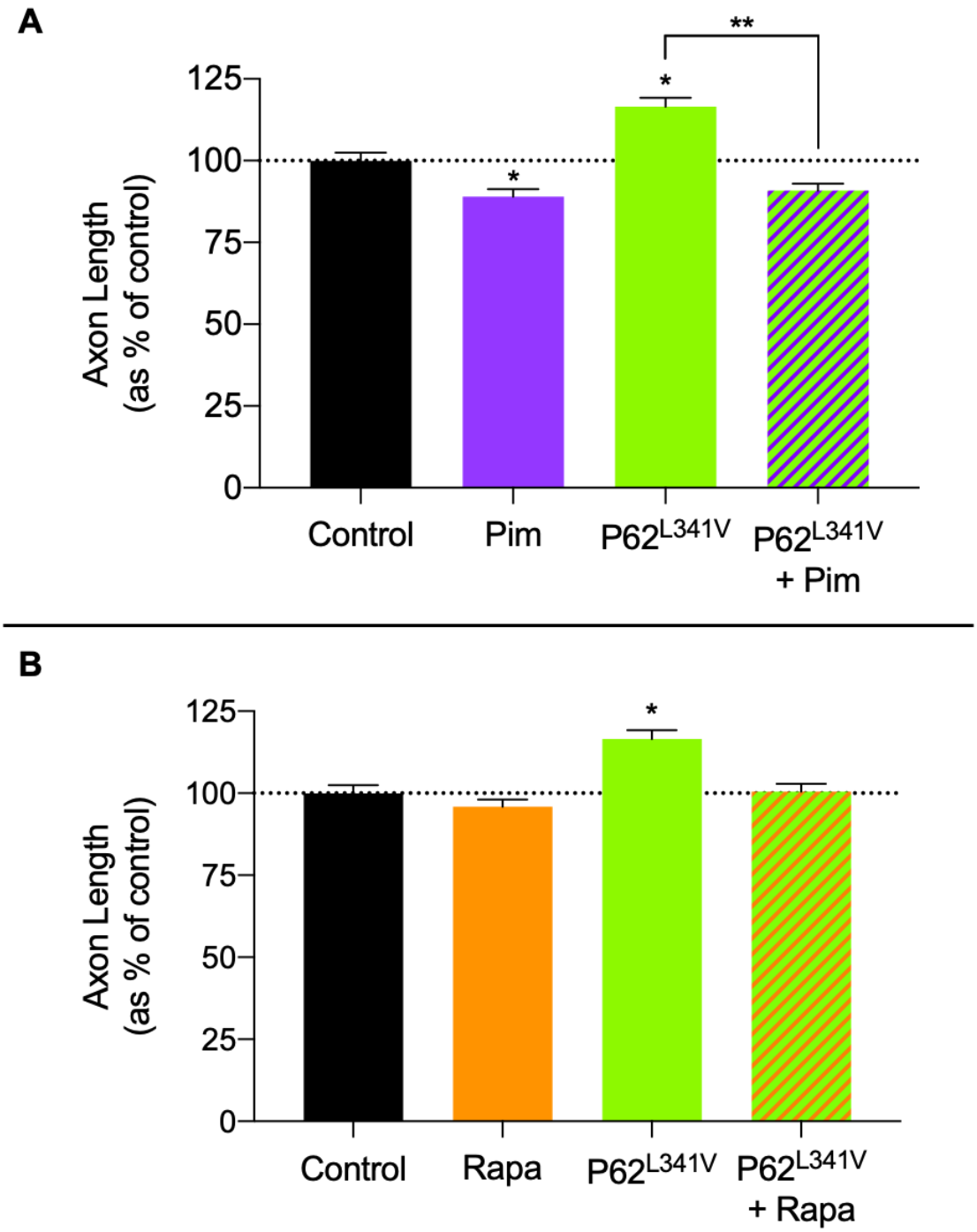


Figure 22. Pimozide rescues the effect of increased axon length in P62^{L341V} transfected cultures. (A) Axon length data shows that the addition of pimozide to neurons over expressing P62^{L341V} counteracts the increase in length of P62^{L341V} alone. The addition of pimozide to control experiments transfected with only an empty vector plasmid control results in a decrease in axon outgrowth, compared to controls. (B) When added to transfection controls and neurons expressing P62^{L341V}, rapamycin did produce any significant change in axon length, compared to controls. Data is expressed as mean ± SEM. Each group represents n≥7 individual experiments, where each experiment is comprised of the measurements of n≥40 axons. Student's t-test, * p≤0.05 and ** p≤0.001.

The outcome of the rescue experiments detailed in figure 22 showed that the addition of pimozide to P62^{L341V} transfected cultures was able to successfully rescue the elongated axonal phenotype typical of the mutant protein's expression in this model. In contrast to the effectiveness of pimozide in this experiment, rapamycin did not exhibit the same effects. Despite its overall negative effect on axon outgrowth when applied to cultures in isolation, our results show that rapamycin only produced a partial rescue the P62^{L341V} mutant phenotype, which was not statistically significant. A likely culprit for this could be rapamycin's mechanism of inducing autophagy via mammalian target of rapamycin (mTOR) related pathways. The mTOR signalling pathway is involved in a multitude of cellular processes, including protein synthesis, gene expression and control of cellular growth process (Lipton et al., 2014). For example, studies on the role of mTOR in spinal and optic neurons have widely implicated its function in in the process of neuronal regeneration (Wei, Luo, & Chen, 2019). Therefore, it's reasonable to suspect that rapamycin may have induced one or more undesired effects in a system where external genetic constructs have already been introduced by lipid membrane transfection. The activation of mTOR is known to activate both protein synthesis and protein degradation (by autophagy) (Takei et al., 2014), and as such these two systems may have acted in a contradiction to each other in this instance.

3.3.5 Concluding remarks

The experiments discussed in this chapter point towards a conclusion that inhibition of autophagy through a loss of P62 functionality has a significant effect in deregulating the growth process of the axon in an early, developmental context.

While intuitively it is easy to interpret an increase in axon length as a positive phenotypical observation, this is mainly a consequence of the extensive research carried out in degenerative models. In fact, it's important to remember that during neuronal development, the development and growth of all neuronal axons towards their appropriate pre-synaptic targets is essential for the proper formation of functional neuronal networks (Kon et al., 2017) and excessive growth might have serious consequences on neuronal wiring and network connectivity. In fact, autophagic activity has been documented as declining with age (Barbosa, Grosso, & Fader, 2019), suggesting that increased autophagy levels are necessary during neuronal development when highly dynamic growth processes are at play.

However interesting the results are of the experiments in this chapter, an important point to note here is that ALS-FTLD is a disease that does not present any known symptoms during stages of neuronal development, with the average age-range of disease onset being 30-70 years of age (Cruts, Engelborghs, Van Der Zee, & Van Broeckhoven, 1993). While familial ALS is more closely associated with an early onset of the disease, a hallmark cellular pathology of ALS diseases is the degeneration of axons in the brain and spinal cord (Ajroud-Driss & Siddique, 2015; Fecto & Siddique, 2011b). While the experiments discussed here are useful in providing an insight into the effects of autophagy dysregulation in axonal development, the next question that arises is how does the presence of dysfunctional P62 impact axonal integrity in a more mature system with different autophagic requirements.

4. P62 dysfunction and autophagy regulation in the stability of more mature axons

4.1 Introduction

4.1.1 Axonal degeneration in ALS-FTLD

As discussed in Chapter 1, the axon is highly reliant on homeostatic mechanisms to tightly maintain its structural integrity and function, with autophagy being a crucial, contributory mechanism. Mounting evidence in recent years has begun linking autophagy impairments to neurodegenerative diseases, with the identification of autophagy relevant disease mutants (such as $P62^{L341V}$) and the presence of tell-tale signs in the pathology of affected neurons, such as an accumulation of autophagosomes and the presence of ubiquitin-positive intracellular inclusions and protein aggregates (Goode et al., 2016; Majcher et al., 2015).

Given the experimental results presented in chapter 3, where autophagy impairment had a significant impact on axon development, it is important to understand how the axon is affected by the presence of an ALS-FTLD P62 mutant in a context more directly linked to that of disease-affected neurons *in-vivo*. In ALS-FTLD, a disease occurring only in adults, the neurons begin to show signs of dysfunction and early degeneration long after undergoing any active growth processes, and thus their autophagic requirements will differ from younger, actively developing neurons. As discussed previously, evidence from ALS mouse models suggests that differing levels of basal autophagy are necessary at different life stages (Rudnick et al., 2017), as such, in an experimental setting where development and growth is less active,

disruptions to autophagy could have different effects to those seen in a developmental setting.

4.1.2 Mature primary cortical neuron cultures

In order to model a neuronal context similar to that of disease affected neurons *in-vivo*, experimental interventions in this section have been applied to primary cortical neuron cultures at a later stage than in the experiments detailed in chapter 3. By using this more mature model of cortical neuron growth, it is possible to avoid the earlier stages of neuronal development like polarisation and early neurite outgrowth. This method also allows us to work with primary cortical neurons within a window where they will still respond to transfection, as it has been observed (Dajas-Bailador, F. et al, Personal Communication, October 2018) that the efficiency of lipid transfection in primary neuronal cultures dramatically decreases from the first 5-7 days of culture, onwards. Initial preliminary experiments confirmed this where attempts to transfect DIV14, DIV 12 and DIV10 cultures proved unsuccessful, resulting in too low a number of successfully transfected cells to make the experimental outputs reliable. However, further preliminary experiments demonstrated that the time point of DIV7, with evaluation on either DIV8 or DIV9 could be successfully used in the following experiments.

4.2 Aims and hypothesis

The experiments presented in chapter 3 demonstrated that the expression of mutant P62 is impacting autophagy and the axon in a developmental context resulting in a phenotype of increased axonal outgrowth. Following this, the experiments in this chapter are intended to investigate the role of the ALS-FTLD $P62^{L341V}$ mutant in a more mature and more disease-relevant neuronal context.

The experiments in this chapter aim to address the hypothesis that;

- Expression of the $P62^{L341V}$ mutation in a late model of primary cortical neuron culture will negatively affect axonal integrity and cause increased axonal degeneration.
- Pharmacological activation of autophagy by the compound pimozide will be sufficient to counteract the negative effects of $P62^{L341V}$ expression in these late cultures and thus rescue the degenerative phenotype.

4.3 Results and discussion

As an overview, the methodological approach to experiments detailed in this section are summarised in figure 23. In brief, all 'Degeneration' experimental interventions were carried out in primary cortical neuron cultures between DIV7 and DIV 9, with experiments coming to completion for analysis between DIV8 and DIV9.

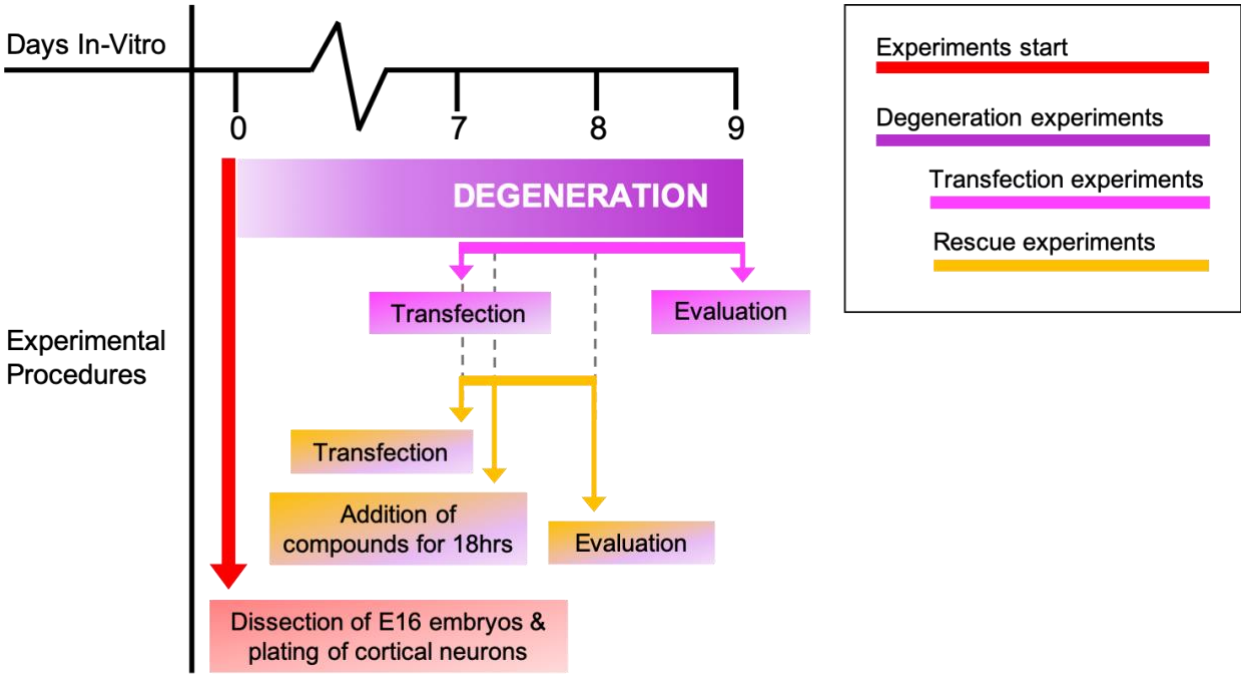


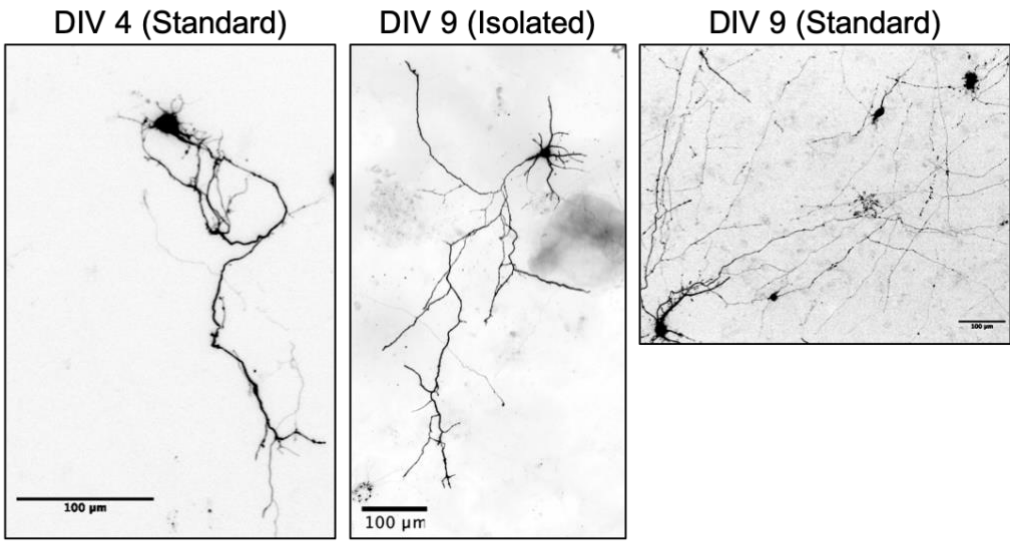
Figure 23. Timeline of Degeneration Experiments. All experimental interventions were conducted between DIV7 and DIV9. Transfection of P62 constructs was conducted at DIV7, with experiments terminating 48hrs later at DIV9, except for rescue experiments which were terminated 24hrs after transfection.

4.3.1 $P62^{L341V}$ expression increases axonal degeneration in mature primary cortical neuron cultures

Experiments carried out in Chapter 3 assessed axonal growth, as determined by their increase in length, and was evaluated after experimental interventions taking place between DIV1-4, whereas the experiments used in this section sought to assess axonal integrity after interventions between DIV7-9. For this reason, the purpose of this first experiment, detailed in figure 24, was to demonstrate the difference in axonal length between the DIV4 culture model used in experiments outlined in Chapter 3, in comparison to those in the DIV9 culture model used in the experiments detailed here in Chapter 4. For neurons assessed at DIV9, cultures were transfected at DIV7 and fixed 48hrs later at DIV9 where axons are reaching maximum growth, having become significantly longer (see figure 24, A). When analysing all conditions,

neurons displaying any features of degeneration or damage to the axon and/or cell body, such as “fragmentation” of processes, “beading” of the axon or “swelling” of the soma, were excluded. This ensured only live cells were analysed. Compilation images were generated for axonal measurement by imaging only GFP-positive neurons isolated enough from surrounding cells in order to clearly trace the most distal axon from terminus to its cell body.

A



B

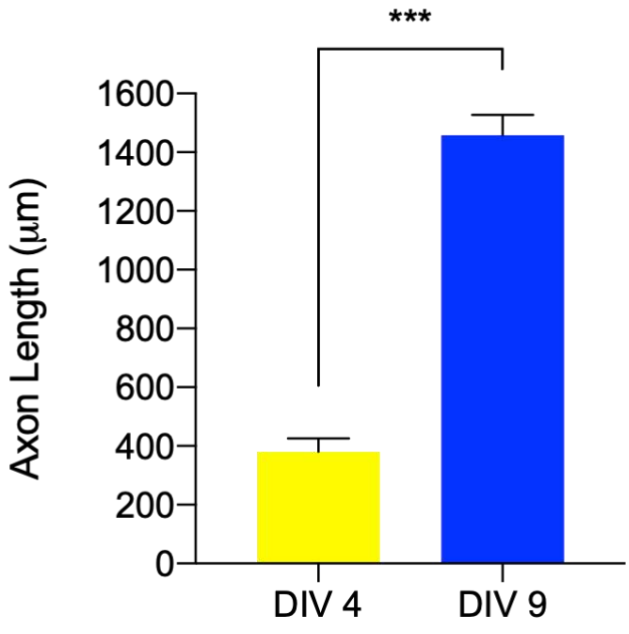


Figure 24. Differences in axon length between DIV 4 and DIV 9 cultures. The axons of GFP-positive control neurons were measured for length. (A) Neurons at DIV 4 were measured by standard means whereas only DIV 9 that were isolated from other axonal structures were able to be measured as the standard presentation of DIV 9 cultures is that of a web-like structure. (B) Axons at DIV 9 are significantly longer than those of DIV 4 neurons. Data is expressed as mean ± SEM. Each group represents n=3 individual experiments. Each DIV4 experiment comprises the measurements of n=59-74 axons while each DIV9 experiment comprises n=12-14 axons. Scale bars are 100μm. Student's t-test, *** $p \leq 0.001$.

The results of the experiment detailed in figure 24 demonstrate the large increase in axonal length in control cultures between DIV4 and DIV9, where on average, the axons of DIV9 primary cortical neurons are 383% longer than those of DIV4 neurons. The average length being 381.1μm for DIV4 neurons and 1458μm for DIV9 neurons. The remarkable growth that takes place between these two time points therefore makes it difficult to assess axon length at such time points, as illustrated by Fig 24, A: DIV9 (standard). In most DIV9 cultures, extensive axonal outgrowth results in a web-like network of cortical neuron axons, even when non-GFP-positive neurons are excluded. This resulting axon web makes the use of axon length as an assessment of the effects of protein expression in this late model, unreliable due to much decreased number of measurable neurons (DIV4 n=59-74 and DIV9 n=12-14, per each experiment). Moreover, beyond DIV9, cortical neurons are beginning to reach synaptic maturation in-vitro (Ichikawa, Muramoto, Kobayashi, Kawahara, & Kuroda, 1993) and are therefore focusing on the parameter of axonal outgrowth becomes decreasingly reliable with increasing number of days *in-vitro*.

The next experiment discussed in this section, shown in figure 25, investigated the effect of mutant P62 expression in a mature cortical neuron culture model. Earlier

experiments (see Chapter 3) revealed that the expression of $P62^{L341V}$ in the early stages of neuronal development had the effect of dysregulating axon growth through autophagy disruption, resulting in an elongated axon phenotype. In the experiments presented in this Chapter, we sought to understand what effect mutant P62 expression would have on axonal integrity in a more mature model system, where axonal growth is expected to be much reduced and demands on maintenance of a now much larger axonal compartment would be greatly increased. For this experiment, primary cortical neurons were plated and allowed to grow until DIV7, when they were transfected with the P62 mutant constructs. After 48hrs, cultures were fixed on DIV9 and axon degeneration assessed by use of the Degeneration index tool (Sasaki et al., 2009). Degeneration Index value is defined as the ratio of area of intact axons to area of detectable axon fragmentation.

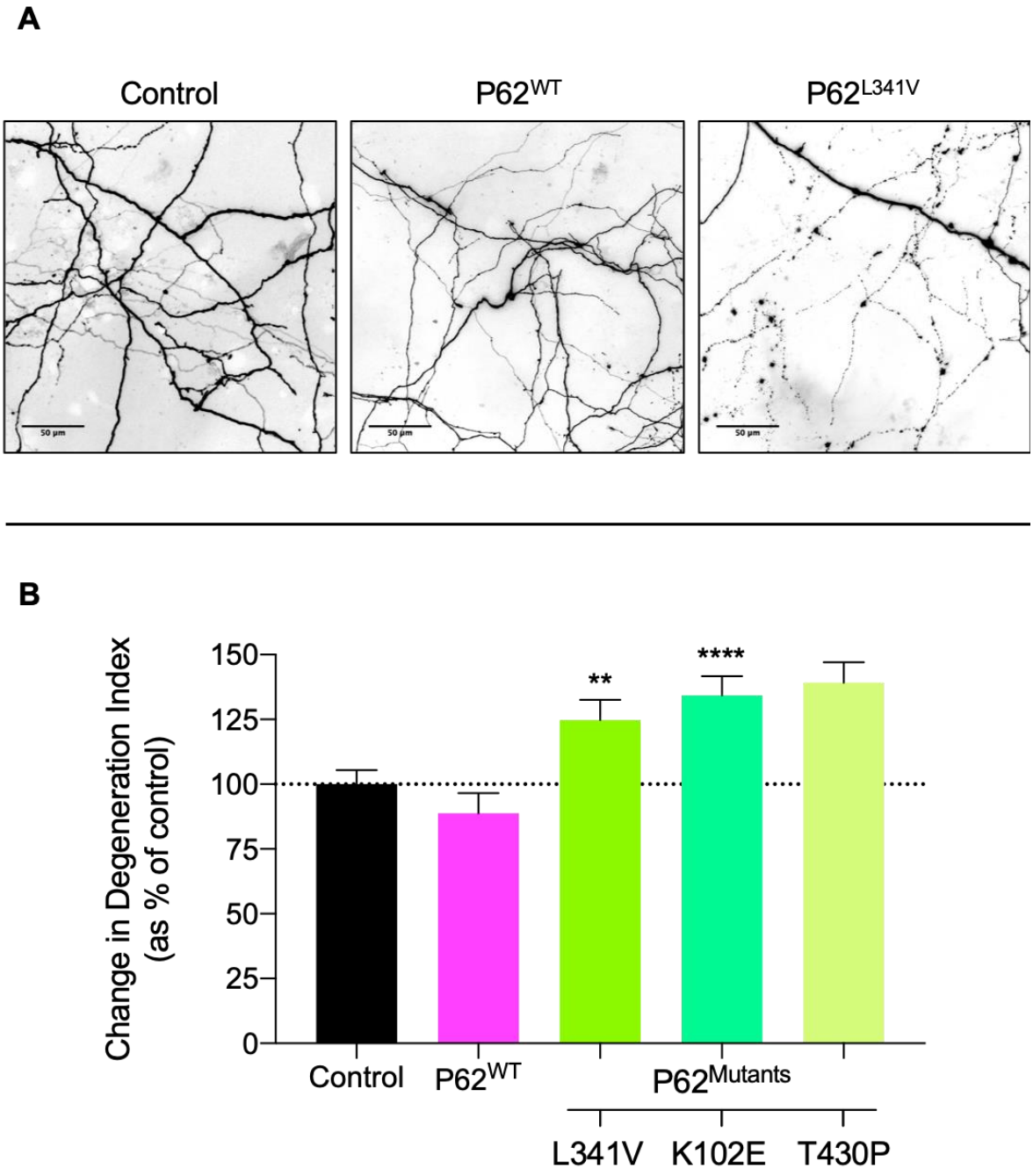


Figure 25. P62^{L341V} expression in late-stage cultures results in increased axonal degeneration. (A) Images of axon networks imaged at DIV 9, 2 days after transfection. Images are clearest examples of each condition & not necessarily representative of the results displayed below. (B) Compared to control, the expression of P62^{L341V} and P62^{K102E} in late-stage cultures resulted in significantly higher levels of axonal degeneration. There was no significant effect on axon degeneration by P62^{WT} or P62^{T430P} expression. Data is expressed as mean ± SEM. Each group represents n=5 individual experiments, where each experiment is comprised of the measurements of n=7-38 images. Scale bars are 50µm. Student's t-test, ** p≤0.001 and **** p≤ 0.0001.

The results of this experiment assessing axon degeneration showed that cells expressing the $P62^{L341V}$ mutant exhibited an increased proportion of degenerated axons, compared to controls. Additionally, cultures expressing the $P62^{K102E}$ and $P62^{T430P}$ mutants also showed an increase in degenerative features, albeit the latter without statistical significance.

It is important to note that while in neurodegenerative disease, such as ALS-FTLD, axonal degeneration is known to precede cell death, axon degeneration is a specific process in and of itself. It occurs during development as part of axonal pruning, as well as in response to multiple stressors in later life, but most importantly in this context, axonal degeneration is a hallmark pathology of ALS-FTLD which leads to the characteristic symptoms of dementia and loss of motor coordination associated with the disease (Lingor, Koch, Tönges, & Bähr, 2012; Salvadores, Sanhueza, Manque, & Court, 2017).

The effect of increased axonal degeneration resulting from $P62^{L341V}$ expression is in-keeping with pathological process in ALS-affected neurons, where axonal degeneration is considered to be a feature of the early stages of neurodegeneration. In fact, axonal degeneration is a common pathology seen in *in-vivo* models of ALS such as mutant SOD1 mice (Salvadores et al., 2017). This degenerative effect in response to autophagy impairment is also seen as a major phenotype in *in-vivo* models of basal autophagy impairment. For example, Atg5 knock-out mice were shown to develop motor function deficits as a result of generalised neuronal degeneration, axonal swelling of Purkinje neurons, and ubiquitin-positive intracellular inclusions (Hara et al., 2006). Similarly, an Atg7 knock-out mouse model also exhibited severe neuronal degeneration accompanied by motor coordination

deficits with a distinct presence of ubiquitin-positive intracellular aggregates (Hara et al., 2006). Although in cases such as these, it is important to note that due to the nature of the genetic interventions, autophagy impairments were present from early, pre-natal development as well as during stages of maturity.

There is also some evidence to suggest that these degenerative effects may not be applicable to all neuronal cell types (Munemasa & Kitaoka, 2015). In one example, a mouse model expressing a mutant rhodopsin protein susceptible to misfolding in its retinal ganglion cells, was either treated with hydroxychloroquine or underwent cell-specific deletion of Atg5 in order to inhibit autophagy (Yao et al., 2018) Their findings suggested that both methods of autophagy inhibition reduced degeneration. Despite this finding, it is worth noting that there are known degenerative conditions of retinal ganglion cell linked-to autophagy impairment, but there were no pre-existing autophagy impairments in this model, and the outcomes do still emphasise the importance of autophagy as a maintenance process in neuronal cells.

4.3.2 P62 mutant expression does not cause axonal degeneration in early cultures

After confirming that expression of the $P62^{L341V}$ (and $P62^{K102E}$) construct lead to increased axonal degeneration in mature cultures, it was necessary to investigate whether this degeneration phenotype was present in earlier cultures, as used in 'Development' experiments in Chapter 3. It would be expected that in Development cultures, where $P62^{L341V}$ expression resulted in increased axon outgrowth, axonal degeneration would not be a significant phenotypic factor. In this experiment, primary cortical neurons were transfected 24hrs after plating and fixed 72hrs later at

DIV4. GFP-positive neurons were scored as either exhibiting features of axon degeneration or not. Cells were considered to have degenerating axons if they had one or more of the following features; a clear axon displaying irregular “beading” morphology or GFP-positive cytoplasmic fragments the occupy the distal portion or all of the axon.

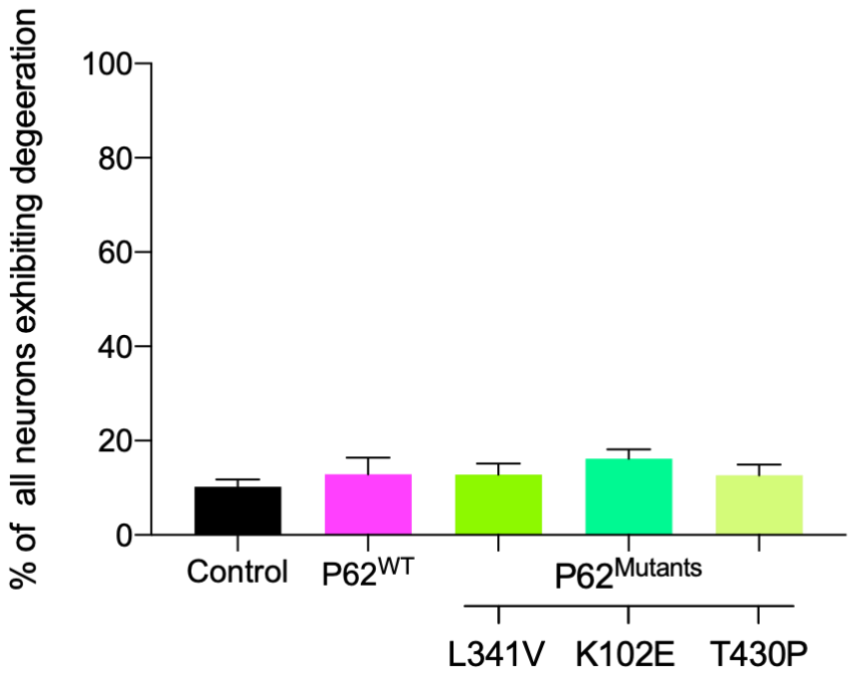


Figure 26. Expression of P62 mutants does not increase the proportion of degenerated axons in DIV 4 cultures. The axons of GFP-positive transfected neurons were scored as either exhibiting degeneration or not. The number of axons presenting with a degeneration phenotype are shown here as a percentage of all neurons measured. Data is expressed as mean ± SEM. Each group represents n=3 individual experiments, where each experiment is comprised of scoring n=111-570 neurons.

While there was some base level of axon degeneration seen in all conditions (~10%), regardless of the construct being expressed, this is commonly observed in neuronal transfections and is likely due to the well-known sensitivity of primary cortical neurons to the process of lipid transfection and its associated reagents.

It is important to note that manual scoring was used to assess axonal degeneration in this experiment because at DIV4, the total surface area covered by the cortical neuron axons in culture is too low for the automated Degeneration Index tool to reliably score.

4.3.3 Activation of autophagy rescues the degeneration phenotype

With a clear degeneration phenotype present in axons expressing $P62^{L341V}$, we next wanted to examine whether the mOTR-independent autophagy activator, pimozide could be used to ameliorate the emergence of the degeneration phenotype. A shorter window of construct expression was used in this experiment to mitigate any effects that the dual acts of transfection and drug application may have on reducing the number of viable axon-covered areas to measure. Initially, assessment of degeneration was repeated where cultures were transfected at DIV7 and fixed 24hrs later at DIV8. For the pharmacological rescue experiments, cultures were transfected at DIV7 and treated 4hrs later with pimozide, for 18hrs, after which they were fixed and assessed for axonal degeneration.

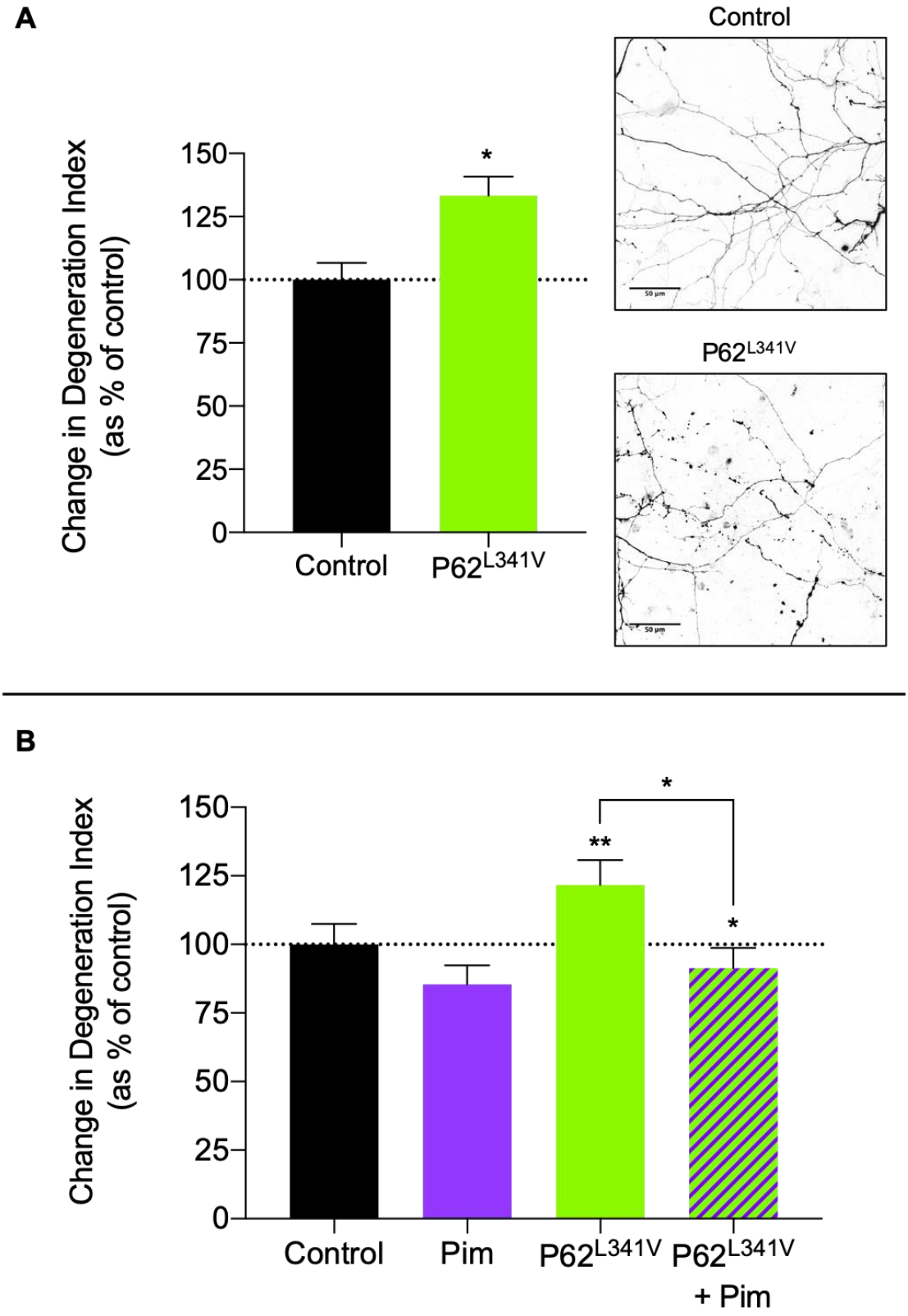


Figure 27. Pimozide rescues axonal degeneration in late cultures expressing P62^{L341V}. (A) Late cultures were transfected at DIV 7 and degeneration of GFP-positive axons assessed 24 hours later. Expressing P62^{L341V} significantly increased the proportion of axonal degeneration, compared to control. (B) pimozide was added to late cultures, 4 hours after transfection on DIV 7. After 18 hours, degeneration of

GFP-positive axons was assessed. $P62^{L341V}$ expression significantly increased axonal degeneration, compared to the control, while addition of pimozide rescued this effect and resulted in lower levels of degeneration, compared to the control. The addition of pimozide to the transfection control did not significantly alter the proportion of axonal degeneration, compared to control. Data is expressed as mean $\pm$ SEM. Each group represents $n \geq 4$ individual experiments, where each experiment is comprised of the measurement of n=14-43 images. Scale bars are 50μm. Student's t-test, * $p \leq 0.05$ and ** $p \leq 0.001$.

Firstly, the assessment of degeneration 24hrs after transfection (figure 27, A) demonstrated that $P62^{L341V}$ expression results in a significant increase in axonal degeneration, compared to the control, even 24hrs after initial transfection.
The results of the rescue experiment (figure 27, B), reveal that autophagy activation by the application of pimozide can mitigate and prevent the appearance of the axon degeneration phenotype seen in mature cultures, and even significantly reduced the overall level of axon degeneration, compared to the control. While the application of pimozide alone also reduced the level of degeneration in line with the $P62^{L341V}$ + pimozide condition, it was however, not found to be statistically significant.

These findings fit well with a mounting body of evidence in the literature reporting the protective effects of autophagy activation in models of other neurodegenerative contexts. In a *Drosophila* model of Alzheimer's Disease, autophagy activation by use of rapamycin has shown to decrease the toxicity of the expression of mutant forms of tau (Berger et al., 2006), while the use of autophagy activators (rapamycin again) in a mouse model of FTLD presenting with TDP-43 proteinopathies demonstrated the ability of autophagy activation to rescue learning and memory impairments in the mice (I. F. Wang et al., 2012). Of particular relevance is an in-vivo study performed in zebrafish embryos, where a motor deficit phenotype produced by the knock-down of

endogenous P62 expression, was rescued by autophagy activation via pharmacological means (using rapamycin) and genetic means (over expression of wild-type P62) (Lattante et al., 2015). Specifically with regards to the rescue of the P62 mutant phenotype by autophagy activation with pimozide, in both the developmental context presented in Chapter 3 and the degenerative context here, such results are supported by previous studies in which pimozide was used to successfully restore varying degrees of functionality to C. elegans, zebrafish and mouse models of ALS (Patten et al., 2017).

It is worth noting that although rapamycin has been used in the vast majority of such studies investigating the benefits of autophagy activation in a neurodegenerative disease context, there are still multiple instances where the use of this mTOR-dependant autophagy activator has actually increased cytotoxic effects in disease models. Not only has it been shown to aggravate neuronal cytotoxicity in a *Drosophila* Alzheimer's Disease model (Ling, Song, Garza, Neufeld, & Salvaterra, 2009), but mTOR-dependent autophagy activation has even been shown to exacerbate motor deficits in the $SOD1^{G93A}$ mouse model of ALS (X. Zhang et al., 2011).

These previous studies illustrate is that the results of the experiments presented in this section are in-keeping with the prominent idea in the literature that pharmacological autophagy activation can be used as a therapeutic approach to rescue the effects of genetic mutations linked to degenerative conditions. And

what's more, while they have all shown these effects using mTOR-dependent autophagy activation, our results present a novel, mTOR-independent approach.

4.4 Concluding remarks

There are two main conclusions from the experiments discussed in this chapter. First is the occurrence of the degeneration phenotype in cells expressing $P62^{L341V}$. In contrast to the experiments presented in chapter 3, which showed an overall developmental phenotype of axon elongation in early neurons, the impairment of autophagy via P62 at this later point in our *in-vitro* model resulted in the 'destructive' phenotype of degeneration. Not only do the findings from this 'mature' model begin to demonstrate a phenotype more directly applicable to the pathology of ALS-FTLD patients, the contrast between the elongation and degeneration phenotypes occurring within the same system reinforce the notion that autophagy requirements differ with age and stage of development, and as a result, its impairment will incur different effects (Rudnick et al., 2017).

The second main point to note from these experiments is the extent to which pimozide is able to attenuate the effects of mutant P62 expression in leading to axonal degeneration. The results presented here show that as an mTOR-independent autophagy activator, pimozide is able to induce a neuroprotective effect, suggesting that it may be a contender for pharmacological interventions in clinical cases, at least at an early stage of disease pathology.

The results from the experiments presented over these last 2 chapters have established clear phenotypes resulting from P62-linked autophagy inhibition on axonal outgrowth and integrity. Across these experiments, entire cells were subjected to the experimental interventions carried, and as such it has not yet been possible to determine to what extent a change in the function of P62 would have locally within the axon. Using the outcomes of the experiments presented thus far, the next chapter seeks to explore the issue of axon-local autophagy control.

5. Results 3 – Local Experiments

5.1 Introduction

5.1.1 The importance of neuronal connectivity in ALS-FTLD

The maintenance of neuronal connectivity is crucial for communication between neurons and their effector cells, and thus brain function. In cases of neurodegenerative disease, the breaking of this connectivity through loss of synaptic connections, prior to any cell death, is well established as an pathological step, in many cases preceding disease symptoms (Bae & Kim, 2017). The axon of neuronal cells is a large sub-cellular compartment that must perform vital, highly dynamic functions a great distance away from the central machinery of the cell body and nucleus. In order to maintain neuronal connectivity and function, it is vital that certain essential processes can occur locally within the axon without relying solely on the transport of necessary protein components to and from the cell body (Spaulding & Burgess, 2017). As such, several important processes have been show to occur locally, to varying extents, within the axon, such as protein synthesis and autophagy (H. Jung et al., 2012; Stavoe & Holzbaur, 2019). Within the context of this thesis, it is therefore important to understand to what extent the local control of autophagy in the axon may impact its function and stability.

5.1.2 Local control of autophagy in the axon

Basal autophagy is known to be spatiotemporally specific within neurons, and even within the axon where the majority of autophagosome biogenesis has been noted to take place in the axon tip and rarely along the length of the axon (Maday & Holzbaur,

2014). Autophagosome formation, maturation and subsequent, rapid retrograde transport towards the soma from the axon tip has been observed *in-vitro* in the axons of multiple rodent primary neuron types, including cortical, hippocampal and dorsal root ganglia neurons (S. Lee et al., 2011; Maday & Holzbaur, 2014; Manday et al., 2012; Mizushima et al., 2004b). These findings are also been backed up by *in-vivo* imaging studies in the neurons of C. elegans (Stavoe et al., 2016).

In addition to local autophagy control under basal conditions, there is also evidence that control of autophagy at the axon tip is vital as the axon responds to damage. In cases of axonal damage by axotomy, the process of axon regeneration that follows requires significant local protein degradation, as well as synthesis, in order to generate a new axon growth cone to facilitate new growth (Gumy, Tan, & Fawcett, 2010; Verma et al., 2005). Evidence such as these points toward the importance of axon-local autophagy, not only in basal conditions, but particularly in the response of the axon damage. It further emphasises the importance of understanding axon-local autophagy in disease states where damage, such as axonal degeneration can occur.

5.1.3 Neuronal cytoarchitecture and compartmentalisation of axons in microfluidic culture chambers

In the brain, neurons do not exist in a disordered tangle of processes, but instead develop in a highly ordered structure, forming discrete layers of neuronal cell bodies, as in the cortex, together with distinct deep nuclei (grey matter), such as the basal ganglia, and a complex set of axonal connections (white matter). This is also true in the peripheral nervous system where specialised neurons such as lower motor

neurons have the cell bodies within the spinal cord and extend axons towards effector muscles through peripheral tissue (Mancall & Brock, 2010). This highly organised structuring of the nervous system gives rise to specific microenvironments, meaning that *in-vivo*, the local environment of axon terminals of say cortical neurons will be different to that of their own cell bodies.

While many 2D and 3D culture models have been successfully used to test potential therapies in *in-vitro* models of neurodegenerative diseases, many of the findings from such experiments have not translated to effective therapies in-vivo or in clinical settings (Osaki, Shin, Sivathanu, Campisi, & Kamm, 2018). Compartmentalised microfluidic culture devices have a unique advantage over 2D and 3D culture techniques in that they allow the experimenter to mimic components of highly organised in-vivo structures and their resulting microenvironments, such as those present in neuronal networks (Osaki et al., 2018).

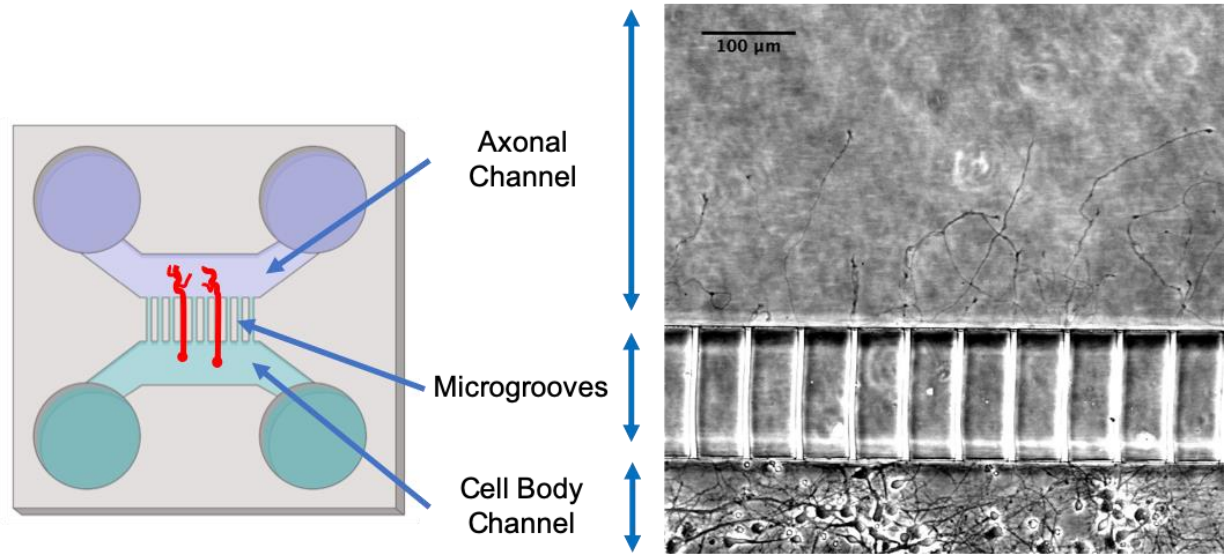


Figure 28. Microfluidic cell culture. Primary cortical neurons in a microfluidic culture device. Image was taken of live cultures on DIV5, at 10X magnification using phase contrast imaging. Microgrooves can be seen separating the two chambers, with cell bodies in the lower chamber and growing axons emerging into the axonal chamber above.

An example of a microfluidic culture of primary cortical neurons, as used in the experiments discussed in this chapter, is shown in figure 28. Here, it's possible to see how this more technically sophisticated approach clearly separates much of the axon compartments from their cell bodies, as the neurons grow. Not only are microfluidic culture devices able to physically separate axons from their cell bodies, but depending on the type of device and its setup, are able to use fluid pressure gradients between the two sides to replicate dynamic mechanical stresses and even biochemical gradients (Bhatia & Ingber, 2014). The diagram in figure 29 below details how, in the microfluidic devices used in this thesis, varying the hydrostatic pressure between the two channels can be used to isolate the application of a reagent to one compartment or the other.

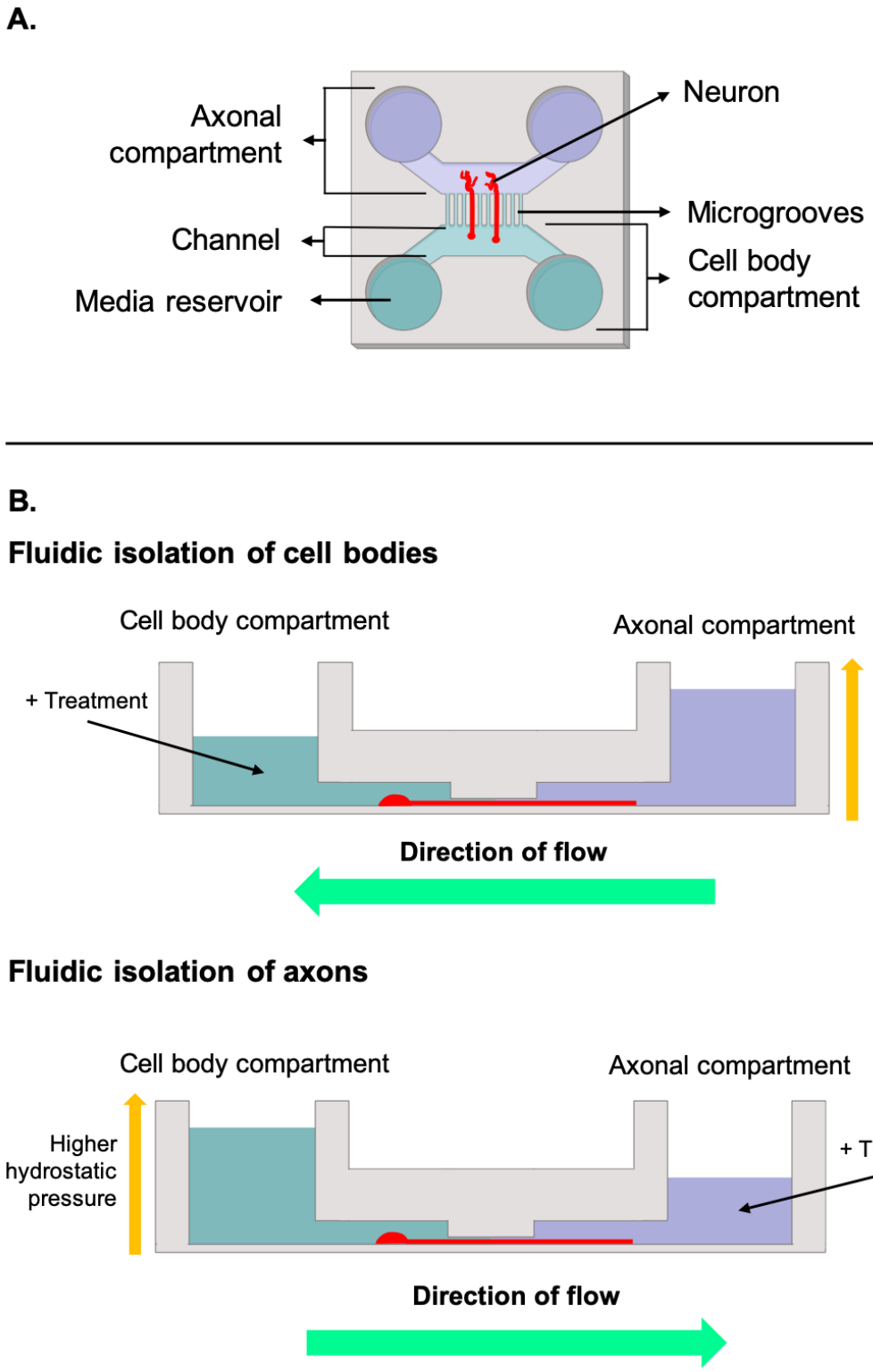


Figure 29. Establishing fluidic isolation in microfluidic cultures devices. (A) Aerial view of the microfluidic culture setup. (B) Lateral view of a microfluidic culture setup. The desired compartment can be fluidically isolated by maintaining a hydrostatic gradient where the opposing compartment has a higher fluid content than the compartment to be treated. The difference in hydrostatic pressure maintains a unidirectional flow, thus preventing diffusion of the treatment out of the target chamber.

5.2 Aims and hypothesis

The experiments detailed in both previous chapters have highlighted the role of autophagy linked to p62 loss of function in axon development and degeneration, demonstrating the capacity of the autophagy activating compound pimozide to rescue the observed effects of increased axon outgrowth and increased axonal degeneration, respectively. The following experiments detailed in this chapter sought to understand to the impact of local autophagy intervention in the axon, in the context of P62-related autophagy inhibition.

The experiments in this chapter will address the hypothesis which states that;

- Activation of autophagy by local application of Rapamycin and Pimozide to the axon will cause a decrease in axon outgrowth, while autophagy inhibition by local application of Bafilomycin A1 to the axon will cause an increase in axon outgrowth.
- Knock-down of endogenous P62 expression, by way of P62siRNA, in cortical neuron cell bodies will be sufficient to cause an increase in axon outgrowth, through decreased levels of autophagy.
- Axon-local activation of autophagy by addition of Pimozide to axonal compartments will increase autophagy sufficiently to prevent increased axon outgrowth caused by application of a P62siRNA.

5.3 Results and Discussion

Figure 30, below, gives a graphical overview of the timeline and methodology of the 'Local' experiments discussed in this section. In short, all experimental interventions

took place between DIV5 and DIV7. Axons from primary cortical neurons which were analysed in these-experiment were only measured from the points at which each projection emerged into the axonal chamber from a microgroove. From previous experience in the group using this model of primary cortical neuron culture, DIV5 is on average the day when axons have grown enough in length to have completely crossed the microgrooves. As such, DIV5 was the chosen start point for 'Local' experimental protocols.

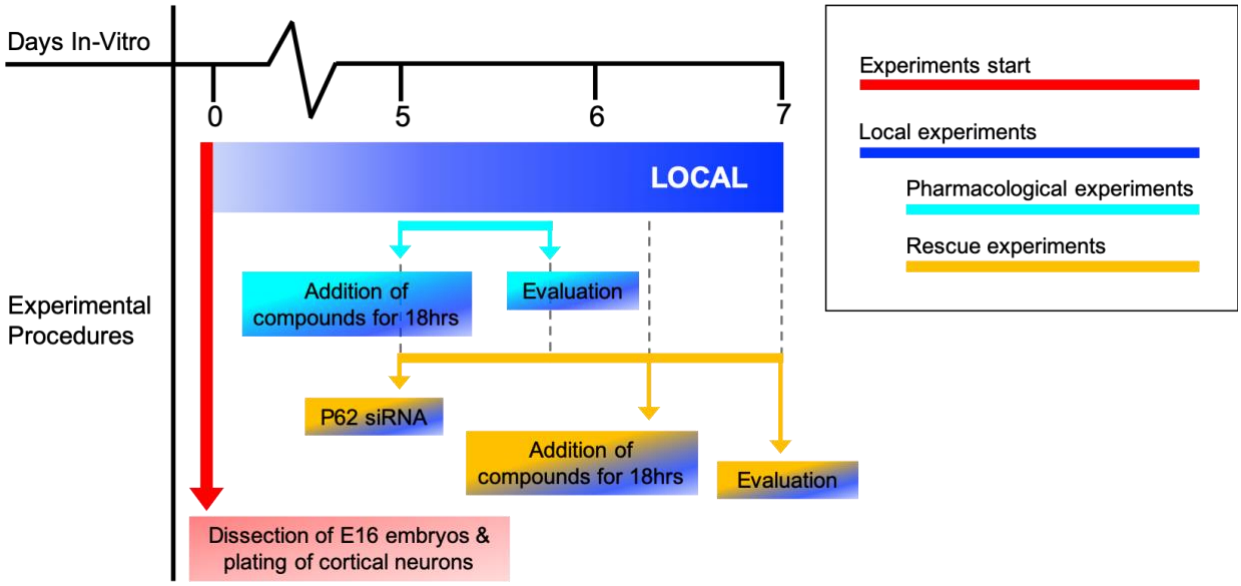


Figure 30. Timeline of Local Experiments. All experimental interventions were conducted between DIV5 and DIV7. For pharmacological experiments, rapamycin, pimozide or bafilomycin A1 were added to either cell body or axon compartments at DIV5, for 18 hours. For rescue experiments, P62 siRNA was added to cell bodies at DIV5 and pimozide added axons 18hrs prior to termination at DIV7.

5.3.1 Local regulation of autophagy in the cell body and axon.

The experiments detailed here in figure 31 were intended to identify to what extent the axon outgrowth phenotypes demonstrated by experiments in normal 2D cultures would be reproduced as the result of localised application of the compounds. Before

attempting to recreate conditions of autophagy inhibition through manipulation of P62 expression, it was necessary to understand whether the autophagy manipulating compounds, pimozide in particular, would elicit any expected effects when applied locally to either the cell body or the axon. To do this, one channel (the cell body channel) of microfluidic culture devices was seeded with primary cortical neurons. The cells were incubated until DIV5, at which point growing axons began to emerge into the axon chamber from across the microgrooves separating the two fluidically isolated compartments. On DIV5, the length of all axons visibly emerging from the base of the microgrooves were measured (t=0hrs), after which either rapamycin, pimozide, bafilomycin or the DMSO vehicle control was added to either the cell body chamber or the axon chamber. On DIV6, after 18hrs incubation with the compounds (t=18hrs), the same axons measured at t=0hrs were measured again, ignoring all newly emerging axons or any pre-existing axons which had degenerated or grown in a manner which made tracing the entire axon uncertain.

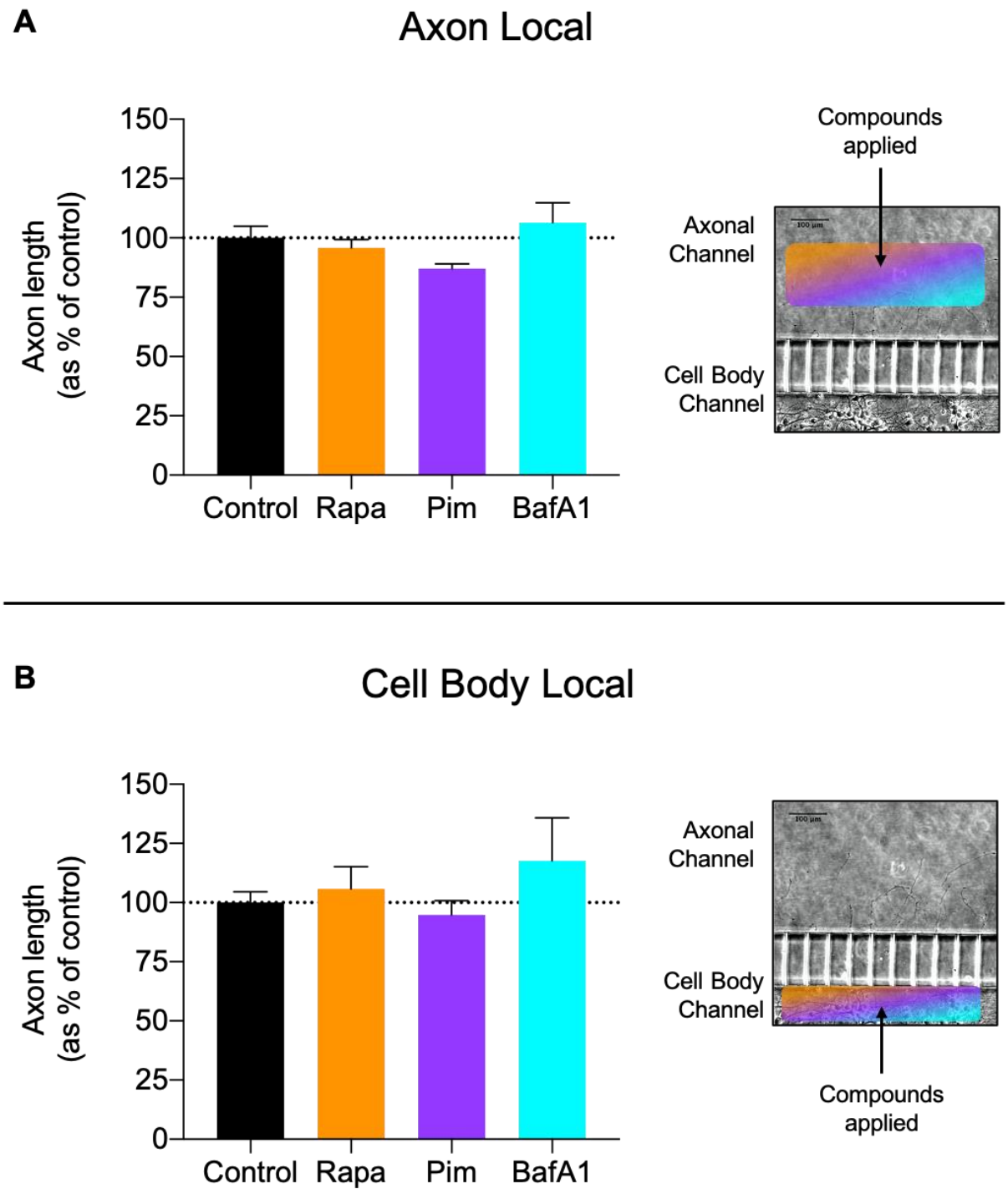


Figure 31. Local regulation of autophagy in the cell body and axon. The change in length of axons was measured after the application compounds to either the axonal chamber (A) or the cell body chamber (B). Axons crossing the microgrooves into the axonal chamber were measured at DIV5 before the application of compounds (t=0hrs) and the same axons measured again 18 hours later (t=18hrs). The change in axon length between t=0hrs and t=18hrs is presented as a percentage of the control and is expressed as mean ± SEM. Each group represents n=6 individual experiments for axon-local application (A) and n=8 for cell body-local application (B). Each individual experiment is comprised of the measurements of n=8-91 axons.

The results of these experiments did not yield any statistically significant results, however, there was a noticeable trend of decreased axon outgrowth as a result of pimozide, as seen in the axon-local results (figure 31, A) compared to the cell body local-results (figure 31, B). Interestingly, in the axon-local experiments, pimozide was the only compound to show a trend in reduced axonal outgrowth that resembled the results from standard cultures where pimozide led to a reduced axon length (see figure 16, section 3.3.1). While not statistically significant, appearance of this trend is in-keeping with previous findings in this thesis. In contrast, there are no obvious trends for any of the 3 compounds when applied locally to the cell body.

The reduction in axon outgrowth as a result of pimozide is more apparent when applied locally to the axon, compared to the cell body. This difference could be a result of one of two scenarios. Firstly, it may simply be the case that pimozide is indeed activating levels of autophagy in the cell body, but this stimulation is also needed locally at the axon to elicit any effects on axonal outgrowth. Secondly, there could be differences in the levels of calcium channel proteins in the axon, compared to the cell body, which may limit the effects of pimozide. As an mTOR independent autophagy activator, pimozide is an L-type Ca^{2+} channel antagonist which theoretically increases basal autophagy by occupying Ca^{2+} channels, preventing influx of extracellular Ca^{2+}, which maintains a lower intracellular concentration of Ca^{2+} that in turn reduces activation of calpains that would otherwise inhibit autophagic activity (Goll et al., 2003; L. Zhang et al., 2007). Calcium channels are enriched in the nervous system as they are crucial for processes such as neurogenesis, neurotransmission, synaptic transmission and even gene transcription (Wegierski &

Kuznicki, 2018). Given their abundance in neurons, it is plausible that because neurites comprise the majority of the neuronal cell surface area (GJ, BW, & RW, 1999) that there may be a larger proportion of calcium channels present in isolated axons, compared to cell bodies, upon which pimozide can act.

The lack of statistical significance resulting from these experiments is likely due to it not being possible to carry out a sufficient number of repeat experiments. A 2-sample, 2-sided power calculation was performed, assuming 5% error, which confirmed that these experiments at n=6 and n=8 were underpowered, where $1-\beta = 0.5881$ and $1-\beta = 0.7122$, respectively. Considering the limitation on time which prevented the addition of further experimental replicates, the decision was therefore made to pursue further investigations into axon-local control of P62-linked autophagy inhibition because of the presence of the reduced axon outgrowth trend from pimozide application that closely reflected previous experiments in 2D cultures.

5.3.2 P62 knock-down and attempted axon-local rescue

Following on from the results presented in section 5.3.1, the experiments detailed in this section sought to understand whether autophagy inhibition by downregulation of endogenous P62 expression in this culture model would produce similar results to those seen in experiments in sections 3.3.2 and 3.3.3, and whether the local application of pimozide to the axon could affect such a phenotype. To do this, the effects of local knock-down of P62 using a cell permeable P62 siRNA were tested in tandem with a potential 'rescue' experiment where pimozide was applied locally to the axon (as depicted in figure 32, A). Primary cortical neurons were cultured until

DIV5, at which point axons began to cross the microgrooves separating the cell body and axonal compartments. At t=0hrs on DIV5, all viable axons visible in the axonal channel were measured for length, after which point the P62 siRNA was added to the cell body channel. Pimozide was then added to the axonal channel 18hrs prior to the end of the of the 48hrs siRNA incubation period. The final measurements were then carried out at t=48hrs on DIV7.

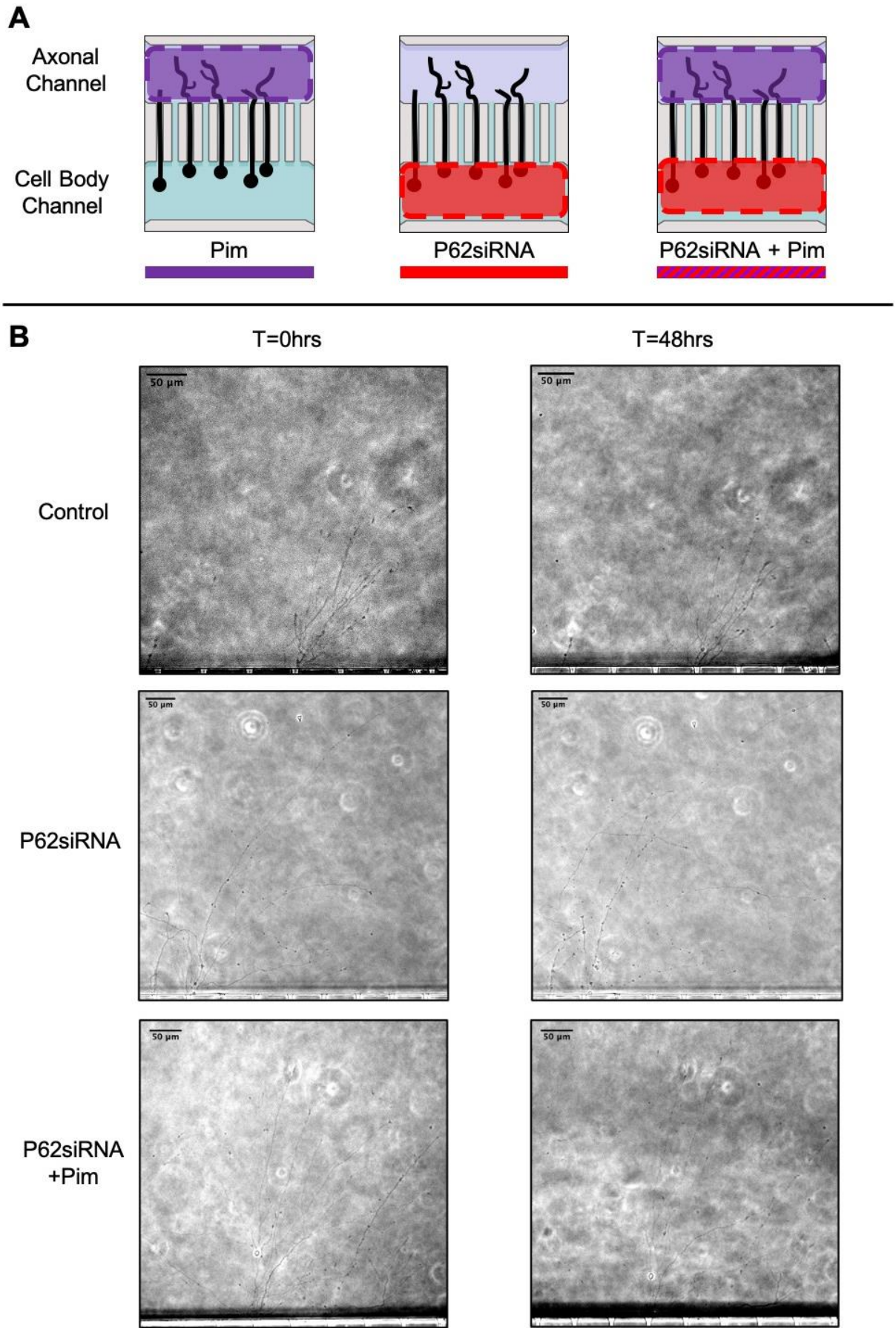

A
Axonal Channel
Cell Body Channel
Pim
P62siRNA
P62siRNA + Pim
B
T=0hrs
T=48hrs
Control
P62siRNA
P62siRNA +Pim
50 µm

Figure 32a. Application of P62 siRNA and attempted local rescue. (A) Graphical summary of the experimental setup. Pimozide was applied to the axonal chamber, cell permeable P62siRNA to the cell body chamber, and both were applied to each respective chamber in the rescue set. (B) Representative images of the change in axon outgrowth in the control, P62siRNA and P62siRNA + Pim conditions.

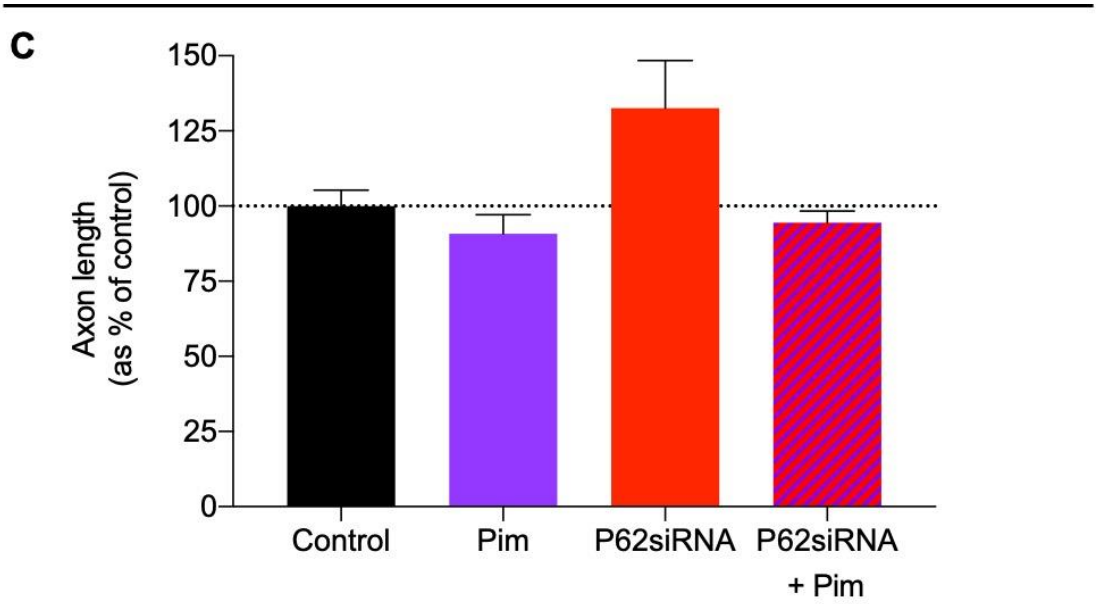


Figure 32b. Application of P62 siRNA and attempted local rescue. (C) The change in length of axons was assessed after the application of a P62 siRNA to the cell body chamber at DIV5 (t=0hrs), and the subsequent addition of compounds for 18hrs on DIV6, until DIV7 (t=48hrs). The change in axon length between t=0hrs and t=18hrs is presented as a percentage of the control and is expressed as mean ± SEM. Each group represents n=6 individual experiments. Each individual experiment is comprised of the measurements of n=9-104 axons.

In the results shown in figure 32, C, while there were no overall statistically significant results, there were obvious trends that mimic the expected results. Control cultures where pimozide was applied locally to the axonal chamber showed a trend of reduced axonal outgrowth, compared to the controls, whereas those treated with P62 siRNA demonstrated a trend towards increased in axonal

outgrowth. When pimozide was added locally to the axonal channels of P62 siRNA treated cultures, this trend reversed to resemble the result of the Pimozide control.

Similar to the previous microfluidic chamber experiments, insufficient experimental replicates were the likely cause of the observed lack of statistical significance. A 2-sample, 2-sided power calculation assuming 5% error reported that this experiment was indeed underpowered at n=6, where $1-\beta=0.5903$, below the desired threshold of $1-\beta=0.8$. Despite this, the results so far would suggest that p62 knock-down can be rescued locally by promoting autophagy after pimozide addition, reinforcing the view that autophagy does indeed act as a fine control mechanism in axonal growth (as previously suggest by Ban et al., 2013) and that P62 is a key regulatory component. The results from this experiment also suggest that local activation of autophagy by pimozide, may be sufficient to counteract deficits in local degradation at the axon tip where basal autophagic activity appears to be of significant importance axon (Stavoe & Holzbaur, 2019).

5.4 Concluding Remarks

Despite the limitations of time, which lead to a lack of statistically significant outcomes from 'Local' experiments, most likely due to underpowered experimental sets, the strong trends across the data remain consistent with previous findings. The likely local activation of autophagy by application of pimozide to axons (figure 31, A) led to decreased axon outgrowth, consistent with findings in 2D cultures treated with pimozide when show significant reductions in axon length. Local P62 knock-down using a cell-permeable P62 siRNA (figure 32, B) showed increased axon

outgrowth compared to controls; an effect seen to be significant in 2D cultures transfected with a P62 siRNA. Additionally, there is also a potential role for local regulation of autophagy in the axon in response to rescuing the effects of P62 knock-down (figure 32, B) through the local activation of autophagy by pimozide which reversed an autophagy inhibition phenotype. This adds to the proven rescue potential of the autophagy activator pimozide by similar, significant outcomes from previous experiments in 2D cultures where its application was able to rescue the $P62^{L341V}$ phenotypes of both increased axon outgrowth (in a developmental context) and increased axonal degeneration (in a more mature context). All such experiments continue to support previous evidence in the literature of the compound's efficacy in reversing ALS-FTLD-associated symptoms (Patten et al., 2017)

The observation of these non-significant, yet consistent trends in 'local' experiments suggests two main conclusions. Firstly, it reinforces the findings of our group that primary cortical neurons cultured in microfluidic devices appear to exhibit a level of heterogeneity between individual experiments, compared to that of standard 2D culture experiments, thus requiring larger experimental numbers to meet sufficient statistical power. Secondly, these trends suggest that a regulatory mechanism local to the axon, at least with regards to the P62-evoked mechanisms, may be a key mechanism by which neurons can alter basal levels of autophagy in response to relevant stressors and local environmental cues. That said, further experiments would need to be added to existing experimental data sets in order to confirm these effects.

6. Conclusion

6.1 Summary

The experiments detailed and discussed throughout this thesis have sought to better understand how defective autophagy resulting from expression of the hereditary ALS-FTLD $P62^{L341V}$ mutation would affect axon development and degeneration in cortical neurons, and to what extent autophagy regulation could be used to mitigate the resulting effects.

The outcomes of this work have led to several insights into the impact of the human $P62^{L341V}$ mutation on axon development and degeneration, as well as the role of autophagy in these processes. The work presented has shown that autophagy dysfunction, resulting from genetic impairment of P62, leads to differential effects on the axon, depending on the neuron's stage of development and maturity. Rescue experiments also demonstrated the ability of pharmacological autophagy activation to rescue the effects of P62 impairment, even when regulated locally in the distal axon.

6.2 Main findings

6.2.1 P62 dysfunction in developing neurons increased axon outgrowth.

The loss-of-function $P62^{L341V}$ mutant increases axonal outgrowth during development, with mTOR-independent activation of autophagy rescuing the phenotype. This result adds to the existing body of work emphasising the importance of autophagy to neuronal development (Ban et al., 2013; K.-M. Lee et al.,

2013) and suggests that autophagy dysregulation in early neurons is likely to have serious consequences *in-vivo* in later life. If such a phenotype is repeated in the 3D environment of the human body, the loss of this fine control over axonal outgrowth is likely to affect axonal pathfinding, and ultimately, the resulting connectivity of the brain and peripheral nervous system, which could produce aberrant cognitive and physical traits.

6.2.2 P62 dysfunction in more mature neurons leads to increased axonal degeneration

Disturbing basal autophagy by expressing $P62^{L341V}$ in more mature neurons leads to increased levels of axonal degeneration. The mTOR-independent activation of autophagy is also shown to prevent the development of this phenotype. These results are highly relevant to our current understanding of ALS-FTLD pathology, supporting previous works demonstrating the therapeutic benefits of autophagy activation in neurodegenerative models (Patten et al., 2017; Periyakaruppiah et al., 2016), and thus suggests that pharmacological activation of autophagy could be a potential option for an early intervention therapy. Additionally, the difference in outcomes between $P62^{L341V}$ expression in early compared to more mature neurons reinforces the notion that different levels of basal autophagy are necessary at different stages of life, and as such, the effects of autophagy dysregulation will also differ accordingly (Amin et al., 2015).

These findings also relate to the wider context of P62 and disease, for example, in relation to Padget's Disease of Bone (PD). PD is effectively a disorder of homeostatic imbalance in the metabolic activity of bone cells, resulting in expanded, poor quality

bone (Tuck, Layfield, Walker, Mekkayil, & Francis, 2017). P62 has been well characterised as a genetic link to PD (Albagha, 2015) and as such, the work in this thesis may be able to provide insights into how the fine control of proteostatic processes in bone, similar to those at play in the axon growth cone, may be regulated by P62.

6.2.3 mTOR-independent activation of autophagy with pimozide effectively rescues the effects of P62 dysfunction in the axon

The results of this work have shown that autophagy activation in an mTOR-independent manner with pimozide can effectively rescue the effects of likely autophagy disruption resulting from P62 dysfunction. This method was not only able to mitigate the effects of P62 dysfunction in the developing axon, but also in the more disease-relevant context of more mature neurons. These outcomes support recent evidence that has shown pimozide (Patten et al., 2017) to be an effective therapeutic agent in models of ALS. Furthermore, given the implications of P62 and autophagy dysfunction in cancers (Sánchez-Martín & Komatsu, 2018), Pimozide may provide a useful tool for further studying the relationship of autophagy homeostasis to cancer pathogenesis.

6.2.4 Local regulation of autophagy is important to the distal axonal

The aim of the final part of this work was to investigate the sub-cellular localisation of the autophagy mechanisms relevant to axon development. For this, experiments involved the microfluidic isolation of axons from their cell bodies. In this experimental setup, knock-down of P62 expression in the cell body resulted in a

trend towards an increased axon outgrowth phenotype, which could be rescued by local mTOR-independent activation of autophagy in the axon. If confirmed by additional experiments, these results would corroborate previous studies which indicate the importance of axon-local autophagy to axonal processes (Stavoe & Holzbaur, 2019; Y. Yang, Coleman, Zhang, Zheng, & Yue, 2013), and also suggest a role of localised interventions in disease.

6.3 Future directions

There are several different lines of investigation that would be useful to build upon, following the experimental results presented in this thesis. Firstly, it would be of significant benefit to the work to-date to acquire addition experimental repeats to the 'local' experiments detailed in Chapter 5. Following this, a logical follow-on would be to investigate the importance of P62 locally in the axon by axon-local knock-down of P62. This line of investigation would be able to shed light onto whether local translation of P62 within the axon is crucial to local autophagy processes, or whether this is maintained more via transport of proteins from the cell body.

To better understand the aetiology of ALS-FTLD, and the relevance of the findings from this thesis, it would be useful to investigate the extent to which other hereditary ALS-FTLD genes might replicate the effects seen in the primary cortical neuron culture models shown here. For example, FUS, TDP-43 and C9orf72 proteinopathies have all been identified as components of typical ALS-FTLD cytoplasmic inclusions, as well as having functions relevant to autophagy. Expressing

known pathogenic mutants of these other key ALS-FTLD genes in our primary cortical neuron model could expand the field's understanding of autophagy dysfunction in neurodegenerative disease. In particular, if the loss of function of these proteins could replicate the axonal degeneration phenotype shown in this thesis in later cortical neuron cultures, it would suggest that there is perhaps a singular mechanism of ALS-FTLD pathogenesis which can may require the presence of one or more of these dysfunction to occur. If expression of these other gene products showed different phenotypes from the ones produced by the $P62^{L341V}$ mutant in these works, then it might suggest a more complex interaction is at play with multiple diseases mechanisms being responsible for the pathogenesis of ALS-FTLD, other than solely some form of autophagy dysfunction.

In a more clinical context, it would also be of great interest to understand to what extent pathological changes in ALS-FTLD are localised to the axon. Considering that the mature axon, *in-vivo*, constitutes ~90% of the total neuronal cell volume and surface area, and that distal degeneration of the axon is a hallmark pathology that leads to the ALS-FTLD phenotype, better understanding the axon-local control of autophagy may create a pathway to developing better therapeutic agents which could potentially stabilise neuromuscular junctions, for example, and thus delay or even prevent the onset of ALS-FTLD symptoms. A possible approach for investigating this would be to use iPSCs created from ALS-FTLD patients with known pathogenic mutations. The cells could be cultured in microfluidic chambers, possibly even co-cultured with myocytes in order to model the neuromuscular junction and allow for through investigation of how autophagy regulation is affected. This type of approach

could also convey many benefits over traditional in-vivo models in organism such as the mouse and zebrafish, which are yet to accurately replicate human ALS-FTLD pathology.

Overall, the work in this thesis has brought to light new molecular mechanisms that are potentially involved in the disease relevant P62 mutations and autophagy regulation, with particular emphasis in axon biology. It emphasises not only the need to further understand the role of autophagy in the pathogenesis and treatment of neurodegenerative conditions like ALS-FTLD, but to better understand the relevance of the axon as a key cellular component in the context of neurodegenerative disease.

7. References

A.T. Nijholt, D., De Kimpe, L., L. Elfrink, H., J.M. Hoozemans, J., & Scheper, W. (2011). Removing Protein Aggregates: The Role of Proteolysis in Neurodegeneration. *Current Medicinal Chemistry*, *18*(16), 2459–2476. https://doi.org/10.2174/092986711795843236

Ajroud-Driss, S., & Siddique, T. (2015). Sporadic and hereditary amyotrophic lateral sclerosis (ALS). *Biochimica et Biophysica Acta (BBA) - Molecular Basis of Disease*, *1852*(4), 679–684. https://doi.org/10.1016/j.bbadis.2014.08.010

Albagha, O. M. (2015). Genetics of Paget's disease of bone. *BoneKEy Reports*, *4*. https://doi.org/10.1038/bonekey.2015.125

Alegre, F., Moragrega, Á. B., Polo, M., Marti-Rodrigo, A., Esplugues, J. V, Blas-Garcia, A., & Apostolova, N. (2018). Role of p62/SQSTM1 beyond autophagy: a lesson learned from drug-induced toxicity in vitro. *British Journal of Pharmacology*, *175*, 440. https://doi.org/10.1111/bph.14093

Alessi, D. R., James, S. R., Downes, C. P., Holmes, A. B., Gaffney, P. R. J., Reese, C. B., & Cohen, P. (1997). Characterization of a 3-phosphoinositide-dependent protein kinase which phosphorylates and activates protein kinase Bα. *Current Biology*, *7*(4), 261–269. https://doi.org/10.1016/S0960-9822(06)00122-9

Amin, N. D., Bai, G., Klug, J. R., Bonanomi, D., Pankratz, M. T., Gifford, W. D., … Goulding, M. (2015). Loss of motoneuron-specific microRNA-218 causes systemic neuromuscular failure. *Science (New York, N.Y.)*, *350*(6267), 1525–1529. https://doi.org/10.1126/science.aad2509

Anderson, S. A. (2002). Distinct Origins of Neocortical Projection Neurons and

Interneurons In Vivo. *Cerebral Cortex*, *12*(7), 702–709. https://doi.org/10.1093/cercor/12.7.702

Arai, T., Hasegawa, M., Akiyama, H., Ikeda, K., Nonaka, T., Mori, H., … Oda, T. (2006). TDP-43 is a component of ubiquitin-positive tau-negative inclusions in frontotemporal lobar degeneration and amyotrophic lateral sclerosis. *Biochemical and Biophysical Research Communications*, *351*(3), 602–611. https://doi.org/10.1016/J.BBRC.2006.10.093

Arico, S., Petiot, A., Bauvy, C., Dubbelhuis, P. F., Meijer, A. J., Codogno, P., & Ogier-Denis, E. (2001). The tumor suppressor PTEN positively regulates macroautophagy by inhibiting the phosphatidylinositol 3-kinase/protein kinase B pathway. *The Journal of Biological Chemistry*, *276*(38), 35243–35246. https://doi.org/10.1074/jbc.C100319200

Arimura, N., & Kaibuchi, K. (2007). Neuronal polarity: From extracellular signals to intracellular mechanisms. *Nature Reviews Neuroscience*, *8*(3), 194–205. https://doi.org/10.1038/nrn2056

Bae, J. R., & Kim, S. H. (2017). Synapses in neurodegenerative diseases. *BMB Reports*, *50*(5), 237–246. https://doi.org/10.5483/BMBRep.2017.50.5.038

Ban, B.-K., Jun, M.-H., Ryu, H.-H., Jang, D.-J., Ahmad, S. T., & Lee, J.-A. (2013). Autophagy negatively regulates early axon growth in cortical neurons. *Molecular and Cellular Biology*, *33*(19), 3907–3919. https://doi.org/10.1128/MCB.00627-13

Barbosa, M. C., Grosso, R. A., & Fader, C. M. (2019). Hallmarks of Aging: An Autophagic Perspective. *Frontiers in Endocrinology*, *9*, 790. https://doi.org/10.3389/fendo.2018.00790

Barnes, A. P., & Polleux, F. (2009). Establishment of Axon-Dendrite Polarity in Developing Neurons. *Annual Review of Neuroscience*, *32*(1), 347–381. https://doi.org/10.1146/annurev.neuro.31.060407.125536

Berger, Z., Ravikumar, B., Menzies, F. M., Oroz, L. G., Underwood, B. R., Pangalos, M. N., … Rubinsztein, D. C. (2006). Rapamycin alleviates toxicity of different aggregate-prone proteins. *Human Molecular Genetics*, *15*(3), 433–442. https://doi.org/10.1093/hmg/ddi458

Bhatia, S. N., & Ingber, D. E. (2014). Microfluidic organs-on-chips. *Nature Biotechnology*, *32*(8). https://doi.org/10.1038/nbt.2989

Bhattacharya, A., Bokov, A., Muller, F. L., Jernigan, A. L., Maslin, K., Diaz, V., … Van Remmen, H. (2012). Dietary restriction but not rapamycin extends disease onset and survival of the H46R/H48Q mouse model of ALS. *Neurobiology of Aging*, *33*(8), 1829–1832. https://doi.org/10.1016/J.NEUROBIOLAGING.2011.06.002

Bjørkøy, G., Lamark, T., Brech, A., Outzen, H., Perander, M., Overvatn, A., … Johansen, T. (2005). p62/SQSTM1 forms protein aggregates degraded by autophagy and has a protective effect on huntingtin-induced cell death. *The Journal of Cell Biology*, *171*(4), 603–614. https://doi.org/10.1083/jcb.200507002

Boland, B., Kumar, A., Lee, S., Platt, F. M., Wegiel, J., Yu, W. H., & Nixon, R. A. (2008). Autophagy induction and autophagosome clearance in neurons: relationship to autophagic pathology in Alzheimer's disease. *The Journal of Neuroscience : The Official Journal of the Society for Neuroscience*, *28*(27), 6926–6937. https://doi.org/10.1523/JNEUROSCI.0800-08.2008

Boland, B., & Nixon, R. A. (2006). Neuronal macroautophagy: From development to

degeneration. *Molecular Aspects of Medicine*, *27*(5–6), 503–519. https://doi.org/10.1016/J.MAM.2006.08.009

Bosco, D. A., Morfini, G., Karabacak, N. M., Song, Y., Gros-Louis, F., Pasinelli, P., … Brown, R. H. (2010). Wild-type and mutant SOD1 share an aberrant conformation and a common pathogenic pathway in ALS. *Nature Neuroscience*, *13*(11), 1396–1403. https://doi.org/10.1038/nn.2660

Budovskaya, Y. V, Stephan, J. S., Deminoff, S. J., & Herman, P. K. (2005). An evolutionary proteomics approach identifies substrates of the cAMP-dependent protein kinase. *Proceedings of the National Academy of Sciences of the United States of America*, *102*(39), 13933–13938. https://doi.org/10.1073/pnas.0501046102

Budovskaya, Y. V, Stephan, J. S., Reggiori, F., Klionsky, D. J., & Herman, P. K. (2004). The Ras/cAMP-dependent protein kinase signaling pathway regulates an early step of the autophagy process in Saccharomyces cerevisiae. *The Journal of Biological Chemistry*, *279*(20), 20663–20671. https://doi.org/10.1074/jbc.M400272200

Carrì, M. T., D'Ambrosi, N., & Cozzolino, M. (2016). Pathways to mitochondrial dysfunction in ALS pathogenesis. *Biochemical and Biophysical Research Communications*. https://doi.org/10.1016/j.bbrc.2016.07.055

Chen, S., Zhang, X., Song, L., & Le, W. (2012). Autophagy Dysregulation in Amyotrophic Lateral Sclerosis. *Brain Pathology*, *22*(1), 110–116. https://doi.org/10.1111/j.1750-3639.2011.00546.x

Chen, Y., Zheng, Z.-Z., Chen, X., Huang, R., Yang, Y., Yuan, L., … Shang, H.-F. (2014). *SQSTM1 mutations in Han Chinese populations with sporadic amyotrophic*

lateral sclerosis. *Neurobiology of Aging* (Vol. 35). https://doi.org/10.1016/j.neurobiolaging.2013.09.008

Cheng, X.-T., Zhou, B., Lin, M.-Y., Cai, Q., & Sheng, Z.-H. (2015). Axonal autophagosomes recruit dynein for retrograde transport through fusion with late endosomes. *The Journal of Cell Biology*, *209*(3), 377–386. https://doi.org/10.1083/jcb.201412046

Ciuffa, R., Lamark, T., Tarafder, A. K., Guesdon, A., Rybina, S., Hagen, W. J. H., … Sachse, C. (2015). The Selective Autophagy Receptor p62 Forms a Flexible Filamentous Helical Scaffold. *Cell Reports*, *11*(5), 748–758. https://doi.org/10.1016/J.CELREP.2015.03.062

Coupé, B., Ishii, Y., Dietrich, M. O., Komatsu, M., Horvath, T. L., & Bouret, S. G. (2012). Loss of autophagy in pro-opiomelanocortin neurons perturbs axon growth and causes metabolic dysregulation. *Cell Metabolism*, *15*(2), 247–255. https://doi.org/10.1016/j.cmet.2011.12.016

Cruts, M., Engelborghs, S., Van Der Zee, J., & Van Broeckhoven, C. (1993). *C9orf72-Related Amyotrophic Lateral Sclerosis and Frontotemporal Dementia*. Retrieved from https://www.ncbi.nlm.nih.gov/books/

Dajas-Bailador, F., Bantounas, I., Jones, E. V, & Whitmarsh, A. J. (2014). Regulation of axon growth by the JIP1-AKT axis. *Journal of Cell Science*, *127*, 230–239. https://doi.org/10.1242/jcs.137208

Dal Canto, M. C., & Gurney, M. E. (1995). Neuropathological changes in two lines of mice carrying a transgene for mutant human Cu,Zn SOD, and in mice overexpressing wild type human SOD: a model of familial amyotrophic lateral sclerosis (FALS). *Brain Research*, *676*(1), 25–40. https://doi.org/10.1016/0006-

8993(95)00063-V

de Carvalho, M., & Swash, M. (2016). Lower motor neuron dysfunction in ALS. *Clinical Neurophysiology*, *127*(7), 2670–2681. https://doi.org/10.1016/j.clinph.2016.03.024

Deng, H.-X., Chen, W., Hong, S.-T., Boycott, K. M., Gorrie, G. H., Siddique, N., … Siddique, T. (2011). Mutations in UBQLN2 cause dominant X-linked juvenile and adult-onset ALS and ALS/dementia. *Nature*, *477*(7363), 211–215. https://doi.org/10.1038/nature10353

Deng, Z., Purtell, K., Lachance, V., Wold, M. S., Chen, S., & Yue, Z. (2017). Autophagy Receptors and Neurodegenerative Diseases. https://doi.org/10.1016/j.tcb.2017.01.001

Dervishi, I., Gozutok, O., Murnan, K., Gautam, M., Heller, D., Bigio, E., & Ozdinler, P. H. (2018). Protein-protein interactions reveal key canonical pathways, upstream regulators, interactome domains, and novel targets in ALS. *Scientific Reports*, *8*(1), 14732. https://doi.org/10.1038/s41598-018-32902-4

Dickson, B. J., Tessier-Lavigne, M., Goodman, C. S., Kennedy, T. E., Serafini, T., Torre, J. R. de la, … Brand, A. H. (2002). Molecular mechanisms of axon guidance. *Science (New York, N.Y.)*, *298*(5600), 1959–1964. https://doi.org/10.1126/science.1072165

Dotti, C. G., Sullivan, C. A., & Banker, G. A. (1988). The establishment of polarity by hippocampal neurons in culture. *The Journal of Neuroscience : The Official Journal of the Society for Neuroscience*, *8*(4), 1454–1468. https://doi.org/10.1523/JNEUROSCI.08-04-01454.1988

Edelstein, A. D., Tsuchida, M. A., Amodaj, N., Pinkard, H., Vale, R. D., & Stuurman, N.

(2014). *Advanced methods of microscope control using μManager software*. *Journal of Biological Methods* (Vol. 1). Retrieved from http://www.jbmethods.org/jbm/article/view/36/28

Eschbach, J., & Dupuis, L. (2011). Cytoplasmic dynein in neurodegeneration. *Pharmacology & Therapeutics*, *130*(3), 348–363. https://doi.org/10.1016/J.PHARMTHERA.2011.03.004

Evans, A. R., Euteneuer, S., Chavez, E., Mullen, L. M., Hui, E. E., Bhatia, S. N., & Ryan, A. F. (2007). Laminin and fibronectin modulate inner ear spiral ganglion neurite outgrowth in anin vitro alternate choice assay. *Developmental Neurobiology*, *67*(13), 1721–1730. https://doi.org/10.1002/dneu.20540

Fass, E., Shvets, E., Degani, I., Hirschberg, K., & Elazar, Z. (2006). Microtubules support production of starvation-induced autophagosomes but not their targeting and fusion with lysosomes. *The Journal of Biological Chemistry*, *281*(47), 36303–36316. https://doi.org/10.1074/jbc.M607031200

Fecto, F., & Siddique, T. (2011a). Making Connections: Pathology and Genetics Link Amyotrophic Lateral Sclerosis with Frontotemporal Lobe Dementia. *Journal of Molecular Neuroscience*, *45*(3), 663–675. https://doi.org/10.1007/s12031-011-9637-9

Fecto, F., & Siddique, T. (2011b). Making Connections: Pathology and Genetics Link Amyotrophic Lateral Sclerosis with Frontotemporal Lobe Dementia. *Journal of Molecular Neuroscience*, *45*(3), 663–675. https://doi.org/10.1007/s12031-011-9637-9

Fujita, E., Kouroku, Y., Isoai, A., Kumagai, H., Misutani, A., Matsuda, C., … Momoi, T. (2007). Two endoplasmic reticulum-associated degradation (ERAD) systems for

the novel variant of the mutant dysferlin: ubiquitin/proteasome ERAD(I) and autophagy/lysosome ERAD(II). *Human Molecular Genetics*, *16*(6), 618–629. https://doi.org/10.1093/hmg/ddm002

Ganley, I. G., Lam, D. H., Wang, J., Ding, X., Chen, S., & Jiang, X. (2009). ULK1.ATG13.FIP200 complex mediates mTOR signaling and is essential for autophagy. *The Journal of Biological Chemistry*, *284*(18), 12297–12305. https://doi.org/10.1074/jbc.M900573200

Gao, J., Wang, L., Yan, T., Perry, G., & Wang, X. (2019). TDP-43 proteinopathy and mitochondrial abnormalities in neurodegeneration. *Molecular and Cellular Neuroscience*, *100*, 103396. https://doi.org/10.1016/J.MCN.2019.103396

GJ, S., BW, A., & RW, A. (1999). Characteristics of the Neuron. In *Basic Neurochemistry: Molecular, Cellular and Medical Aspects* (6th ed.). Philadelphia: Lippincott-Raven. Retrieved from https://www.ncbi.nlm.nih.gov/books/NBK28209/

Goll, D. E., Thompson, V. F., Li, H., Wei, W., & Cong, J. (2003). The Calpain System. https://doi.org/10.1152/physrev.00029.2002.-The

Goode, A, Butler, K., Scott, D., Long, J., Cavey, J., Shaw, B., … Layfield, R. (2016). P55 A CELLULAR MODEL OF ALS WITH SQSTM1 MUTATIONS EXHIBITS AUTOPHAGY DEFECTS; A NEW PLATFORM FOR EVALUATING GENETIC INTERACTIONS AND DRUG SCREENING. *Theme 3 In_Vitro Experimental Models, Amyotrophic Lateral Sclerosis and Frontotemporal Degeneration*, *23*, 118. https://doi.org/10.1080/21678421.2016.1232054

Goode, Alice, Butler, K., Long, J., Cavey, J., Scott, D., Shaw, B., … Layfield, R. (2016). Defective recognition of LC3B by mutant SQSTM1/p62 implicates impairment of

autophagy as a pathogenic mechanism in ALS-FTLD. *Autophagy*, *8627*(May), 1–11. https://doi.org/10.1080/15548627.2016.1170257

Gumy, L. F., Tan, C. L., & Fawcett, J. W. (2010, May). The role of local protein synthesis and degradation in axon regeneration. *Experimental Neurology*. https://doi.org/10.1016/j.expneurol.2009.06.004

Gurney, M., Pu, H., Chiu, A., Dal Canto, M., Polchow, C., Alexander, D., … et, al. (1994). Motor neuron degeneration in mice that express a human Cu,Zn superoxide dismutase mutation. *Science*, *264*(5166), 1772–1775. https://doi.org/10.1126/science.8209258

Hand, R., Bortone, D., Mattar, P., Nguyen, L., Heng, J. I.-T., Guerrier, S., … Polleux, F. (2005). Phosphorylation of Neurogenin2 Specifies the Migration Properties and the Dendritic Morphology of Pyramidal Neurons in the Neocortex. *Neuron*, *48*(1), 45–62. https://doi.org/10.1016/J.NEURON.2005.08.032

Hara, T., Nakamura, K., Matsui, M., Yamamoto, A., Nakahara, Y., Suzuki-Migishima, R., … Mizushima, N. (2006). Suppression of basal autophagy in neural cells causes neurodegenerative disease in mice. *Nature*, *441*(7095), 885–889. https://doi.org/10.1038/nature04724

He, C., & Klionsky, D. J. (2009). Regulation mechanisms and signaling pathways of autophagy. *Annual Review of Genetics*, *43*, 67–93. https://doi.org/10.1146/annurev-genet-102808-114910

Heitman, J., Movva, N. R., & Hall, M. N. (1991). Targets for cell cycle arrest by the immunosuppressant rapamycin in yeast. *Science (New York, N.Y.)*, *253*(5022), 905–909. https://doi.org/10.1126/science.1715094

Ichikawa, M., Muramoto, K., Kobayashi, K., Kawahara, M., & Kuroda, Y. (1993).

Formation and maturation of synapses in primary cultures of rat cerebral cortical cells: an electron microscopic study. *Neuroscience Research*, *16*(2), 95–103. https://doi.org/10.1016/0168-0102(93)90076-3

Inoki, K., Zhu, T., & Guan, K.-L. (2003). TSC2 Mediates Cellular Energy Response to Control Cell Growth and Survival. *Cell*, *115*(5), 577–590. https://doi.org/10.1016/S0092-8674(03)00929-2

Ishigaki, S., Masuda, A., Fujioka, Y., Iguchi, Y., Katsuno, M., Shibata, A., … Ohno, K. (2012). Position-dependent FUS-RNA interactions regulate alternative splicing events and transcriptions. *Scientific Reports*, *2*(1), 529. https://doi.org/10.1038/srep00529

Isogai, S., Morimoto, D., Arita, K., Unzai, S., Tenno, T., Hasegawa, J., … Tochio, H. (2011). Crystal Structure of the Ubiquitin-associated (UBA) Domain of p62 and Its Interaction with Ubiquitin. *Journal of Biological Chemistry*, *286*(36), 31864–31874. https://doi.org/10.1074/jbc.M111.259630

Jäger, S., Bucci, C., Tanida, I., Ueno, T., Kominami, E., Saftig, P., & Eskelinen, E.-L. (2004). Role for Rab7 in maturation of late autophagic vacuoles. *Journal of Cell Science*, *117*(Pt 20), 4837–4848. https://doi.org/10.1242/jcs.01370

Jara, J. H., Villa, S. R., Khan, N. A., Bohn, M. C., & Özdinler, P. H. (2012). AAV2 mediated retrograde transduction of corticospinal motor neurons reveals initial and selective apical dendrite degeneration in ALS. *Neurobiology of Disease*, *47*(2), 174–183. https://doi.org/10.1016/j.nbd.2012.03.036

Johnson, J. O., Mandrioli, J., Benatar, M., Abramzon, Y., Van Deerlin, V. M., Trojanowski, J. Q., … Traynor, B. J. (2010). Exome Sequencing Reveals VCP Mutations as a Cause of Familial ALS. *Neuron*, *68*(5), 857–864.

https://doi.org/10.1016/J.NEURON.2010.11.036

Ju, J.-S., Fuentealba, R. A., Miller, S. E., Jackson, E., Piwnica-Worms, D., Baloh, R. H., & Weihl, C. C. (2009). Valosin-containing protein (VCP) is required for autophagy and is disrupted in VCP disease. *The Journal of Cell Biology*, *187*(6), 875–888. https://doi.org/10.1083/jcb.200908115

Jung, C. H., Jun, C. B., Ro, S.-H., Kim, Y.-M., Otto, N. M., Cao, J., … Kim, D.-H. (2009). ULK-Atg13-FIP200 complexes mediate mTOR signaling to the autophagy machinery. *Molecular Biology of the Cell*, *20*(7), 1992–2003. https://doi.org/10.1091/mbc.e08-12-1249

Jung, H., Yoon, B. C., & Holt, C. E. (2012). Axonal mRNA localization and local protein synthesis in nervous system assembly, maintenance and repair. *Nature Reviews Neuroscience*, (April). https://doi.org/10.1038/nrn3210

Kabeya, Y., Mizushima, N., Ueno, T., Yamamoto, A., Kirisako, T., Noda, T., … Yoshimori, T. (2000). LC3, a mammalian homologue of yeast Apg8p, is localized in autophagosome membranes after processing. *The EMBO Journal*, *19*(21), 5720–5728. https://doi.org/10.1093/emboj/19.21.5720

Kanai, Y., Dohmae, N., & Hirokawa, N. (2004). Kinesin Transports RNA: Isolation and Characterization of an RNA-Transporting Granule. *Neuron*, *43*(4), 513–525. https://doi.org/10.1016/J.NEURON.2004.07.022

Katsuragi, Y., Ichimura, Y., & Komatsu, M. (2015). p62/SQSTM1 functions as a signaling hub and an autophagy adaptor. *FEBS Journal*, *282*(24), 4672–4678. https://doi.org/10.1111/febs.13540

Kevenaar, J. T., & Hoogenraad, C. C. (2015, August 14). The axonal cytoskeleton: From organization to function. *Frontiers in Molecular Neuroscience*. Frontiers

Media S.A. https://doi.org/10.3389/fnmol.2015.00044

Kiernan, M. C., Vucic, S., Cheah, B. C., Turner, M. R., Eisen, A., Hardiman, O., … Zoing, M. C. (2011). Amyotrophic lateral sclerosis. *The Lancet*, *377*(9769), 942–955. https://doi.org/10.1016/S0140-6736(10)61156-7

Kim, Eunjin, & Jung, H. (2015). Local protein synthesis in neuronal axons: why and how we study. *BMB Rep*, *48*(3), 139–146. https://doi.org/10.5483/BMBRep.2015.48.3.010

Kim, Eunjung, Goraksha-Hicks, P., Li, L., Neufeld, T. P., & Guan, K.-L. (2008). Regulation of TORC1 by Rag GTPases in nutrient response. *Nature Cell Biology*, *10*(8), 935–945. https://doi.org/10.1038/ncb1753

Klimaschewski, L., Hausott, B., Ingorokva, S., & Pfaller, K. (2006). Constitutively expressed catalytic proteasomal subunits are up-regulated during neuronal differentiation and required for axon initiation, elongation and maintenance. *Journal of Neurochemistry*, *96*(6), 1708–1717. https://doi.org/10.1111/j.1471-4159.2006.03694.x

Klionsky, D. J., Abdelmohsen, K., Abe, A., Abedin, M. J., Abeliovich, H., Arozena, A. A., … Zughaier, S. M. (2016). Guidelines for the use and interpretation of assays for monitoring autophagy (3rd edition). *Autophagy*. https://doi.org/10.1080/15548627.2015.1100356

Komatsu, M., Wang, Q. J., Holstein, G. R., Friedrich, V. L., Iwata, J.-I., Kominami, E., … Yue, Z. (2007). No Essential role for autophagy protein Atg7 in the maintenance of axonal homeostasis and the prevention of axonal degeneration. *Proceedings of the National Academy of Sciences of the United States of America*, *104*(36), 14489–14494. https://doi.org/10.1073/pnas.0701311104

Kon, E., Cossard, A., & Jossin, Y. (2017). Neuronal polarity in the embryonic mammalian cerebral cortex. *Frontiers in Cellular Neuroscience*, *11*(June), 1–15. https://doi.org/10.3389/fncel.2017.00163

Korac, J., Schaeffer, V., Kovacevic, I., Clement, A. M., Jungblut, B., Behl, C., … Dikic, I. (2013). Ubiquitin-independent function of optineurin in autophagic clearance of protein aggregates. *Journal of Cell Science*, *126*, 580–592. https://doi.org/10.1242/jcs.114926

Kunda, P., Paglini, G., Quiroga, S., Kosik, K., & Cá Ceres, A. (2001). Evidence for the Involvement of Tiam1 in Axon Formation. *The Journal of Neuroscience*, *21*(7), :2361–2372.

Kwiatkowski, T. J., Bosco, D. A., Leclerc, A. L., Tamrazian, E., Vanderburg, C. R., Russ, C., … Brown, R. H. (2009). Mutations in the FUS/TLS gene on chromosome 16 cause familial amyotrophic lateral sclerosis. *Science (New York, N.Y.)*, *323*(5918), 1205–1208. https://doi.org/10.1126/science.1166066

Lattante, S., de Calbiac, H., Le Ber, I., Brice, A., Ciura, S., & Kabashi, E. (2015). Sqstm1 knock-down causes a locomotor phenotype ameliorated by rapamycin in a zebrafish model of ALS/FTLD. *Human Molecular Genetics*, *24*(6), 1682–1690. https://doi.org/10.1093/hmg/ddu580

Lee, D. Y., & Brown, E. J. (2012). Ubiquilins in the crosstalk among proteolytic pathways. *Biological Chemistry*, *393*(6), 441–447. https://doi.org/10.1515/hsz-2012-0120

Lee, K.-M., Hwang, S.-K., & Lee, J.-A. (2013). Neuronal Autophagy and Neurodevelopmental Disorders. *Experimental Neurobiology*, *22*(3), 133. https://doi.org/10.5607/EN.2013.22.3.133

Lee, S., Sato, Y., & Nixon, R. A. (2011). Lysosomal proteolysis inhibition selectively disrupts axonal transport of degradative organelles and causes an Alzheimer's-like axonal dystrophy. *Journal of Neuroscience*, *31*(21), 7817–7830. https://doi.org/10.1523/JNEUROSCI.6412-10.2011

Lewis, T. L., Courchet, J., & Polleux, F. (2013). Cell biology in neuroscience: Cellular and molecular mechanisms underlying axon formation, growth, and branching. *The Journal of Cell Biology*, *202*(6), 837–848. https://doi.org/10.1083/jcb.201305098

Lewis, Tommy L, Courchet, J., Polleux, F., & Polleux, F. (2013). Cell biology in neuroscience: Cellular and molecular mechanisms underlying axon formation, growth, and branching. *The Journal of Cell Biology*, *202*(6), 837–848. https://doi.org/10.1083/jcb.201305098

Li, J., Kim, S. G., & Blenis, J. (2014). Rapamycin: one drug, many effects. *Cell Metabolism*, *19*(3), 373–379. https://doi.org/10.1016/j.cmet.2014.01.001

Liang, J., Shao, S. H., Xu, Z.-X., Hennessy, B., Ding, Z., Larrea, M., … Mills, G. B. (2007). The energy sensing LKB1–AMPK pathway regulates p27kip1 phosphorylation mediating the decision to enter autophagy or apoptosis. *Nature Cell Biology*, *9*(2), 218–224. https://doi.org/10.1038/ncb1537

Ling, D., Song, H. J., Garza, D., Neufeld, T. P., & Salvaterra, P. M. (2009). Abeta42-induced neurodegeneration via an age-dependent autophagic-lysosomal injury in Drosophila. *PLoS ONE*, *4*(1). https://doi.org/10.1371/journal.pone.0004201

Lingor, P., Koch, J. C., Tönges, L., & Bähr, M. (2012). Axonal degeneration as a therapeutic target in the CNS. *Cell Tissue Research*. https://doi.org/10.1007/s00441-012-1362-3

Lipinski, M. M., Zheng, B., Lu, T., Yan, Z., Py, B. F., Ng, A., … Yuan, J. (2010). Genome-wide analysis reveals mechanisms modulating autophagy in normal brain aging and in Alzheimer's disease. *Proceedings of the National Academy of Sciences of the United States of America*, *107*(32), 14164–14169. https://doi.org/10.1073/pnas.1009485107

Lipton, J. O., Sahin, M., Abs, E., Goorden, S. M., Schreiber, J., Overwater, I. E., … Sabatini, D. M. (2014). The neurology of mTOR. *Neuron*, *84*(2), 275–291. https://doi.org/10.1016/j.neuron.2014.09.034

Long, J., Garner, T. P., Pandya, M. J., Craven, C. J., Chen, P., Shaw, B., … Searle, M. S. (2010). Dimerisation of the UBA Domain of p62 Inhibits Ubiquitin Binding and Regulates NF-κB Signalling. *Journal of Molecular Biology*, *396*(1), 178–194. https://doi.org/10.1016/J.JMB.2009.11.032

Long, X., Lin, Y., Ortiz-Vega, S., Yonezawa, K., & Avruch, J. (2005). Rheb Binds and Regulates the mTOR Kinase. *Current Biology*, *15*(8), 702–713. https://doi.org/10.1016/J.CUB.2005.02.053

Lowery, L. A., & Van Vactor, D. (2009). The trip of the tip: understanding the growth cone machinery. *Nature Reviews. Molecular Cell Biology*, *10*(5), 332–343. https://doi.org/10.1038/nrm2679

Maday, S., & Holzbaur, E. L. F. (2014). Autophagosome biogenesis in primary neurons follows an ordered and spatially regulated pathway. *Developmental Cell*, *30*(1), 71–85. https://doi.org/10.1016/j.devcel.2014.06.001

Maday, S., & Holzbaur, E. L. F. (2016). Compartment-Specific Regulation of Autophagy in Primary Neurons. https://doi.org/10.1523/JNEUROSCI.4401-15.2016

Majcher, V., Goode, A., James, V., & Layfield, R. (2015). Autophagy receptor defects and ALS-FTLD. *Molecular and Cellular Neuroscience*, *66*(PA), 43–52. https://doi.org/10.1016/j.mcn.2015.01.002

Mancall, E. L., & Brock, D. G. (2010). Gray's Clinical Neuroanatomy: The Anatomic Basis for Clinical Neuroscience - Elliott L. Mancall, MD, David G. Brock, MD, CIP - Google Books. Retrieved September 21, 2019, from https://books.google.co.uk/books?id=qgaA3rON4XcC&printsec=frontcover&dq=neuroanatomy&hl=en&sa=X&ved=0ahUKEwjAq6Xtl-LkAhVDZcAKHf1nAhkQ6AEIMjAB#v=onepage&q=neuroanatomy&f=false

Manday, S., Wallace, K. E., & Holzbaur, E. L. F. (2012). Autophagosomes initiate distally and mature during transport toward the cell soma in primary neurons, *196*(4), 407–417. https://doi.org/10.1083/jcb.201106120

Manning, B. D., Tee, A. R., Logsdon, M. N., Blenis, J., & Cantley, L. C. (2002). Identification of the Tuberous Sclerosis Complex-2 Tumor Suppressor Gene Product Tuberin as a Target of the Phosphoinositide 3-Kinase/Akt Pathway. *Molecular Cell*, *10*(1), 151–162. https://doi.org/10.1016/S1097-2765(02)00568-3

Marsh, D., & Dragich, J. M. (2019, April 1). Autophagy in mammalian neurodevelopment and implications for childhood neurological disorders. *Neuroscience Letters*. Elsevier Ireland Ltd. https://doi.org/10.1016/j.neulet.2018.04.017

Maruyama, H., Morino, H., Ito, H., Izumi, Y., Kato, H., Watanabe, Y., … Kawakami, H. (2010). Mutations of optineurin in amyotrophic lateral sclerosis. *Nature*, *465*(7295), 223–226. https://doi.org/10.1038/nature08971

Matheson, S. F., & Levine, R. B. (1999). Steroid hormone enhancement of neurite outgrowth in identified insect motor neurons involves specific effects on growth cone form and function. *Journal of Neurobiology*, *38*(1), 27–45. https://doi.org/10.1002/(SICI)1097-4695(199901)38:1<27::AID-NEU3>3.0.CO;2-U

Matsumoto, G., Wada, K., Okuno, M., Kurosawa, M., & Nukina, N. (2011). Serine 403 Phosphorylation of p62/SQSTM1 Regulates Selective Autophagic Clearance of Ubiquitinated Proteins. *Molecular Cell*, *44*(2), 279–289. https://doi.org/10.1016/J.MOLCEL.2011.07.039

Milde, S., Gilley, J., Coleman, M. P., Mosely, S., & Andrews, S. (2013). Subcellular Localization Determines the Stability and Axon Protective Capacity of Axon Survival Factor Nmnat2. *PLoS Biology*, *11*(4), e1001539. https://doi.org/10.1371/journal.pbio.1001539

Miller, J. W., Smith, B. N., Topp, S. D., Al-Chalabi, A., Shaw, C. E., & Vance, C. (2012). Mutation analysis of VCP in British familial and sporadic amyotrophic lateral sclerosis patients. *Neurobiology of Aging*, *33*(11), 2721.e1-2721.e2. https://doi.org/10.1016/J.NEUROBIOLAGING.2012.06.003

Mis, M. S. C., Brajkovic, S., Frattini, E., Di Fonzo, A., & Corti, S. (2016). Autophagy in motor neuron disease: Key pathogenetic mechanisms and therapeutic targets. *Molecular and Cellular Neuroscience*, *72*, 84–90. https://doi.org/10.1016/j.mcn.2016.01.012

Mizushima, N., Yamamoto, A., Matsui, M., Yoshimori, T., & Ohsumi, Y. (2004a). In vivo analysis of autophagy in response to nutrient starvation using transgenic mice expressing a fluorescent autophagosome marker. *Molecular Biology of the*

Cell, *15*(3), 1101–1111. https://doi.org/10.1091/mbc.e03-09-0704

Mizushima, N., Yamamoto, A., Matsui, M., Yoshimori, T., & Ohsumi, Y. (2004b). In Vivo Analysis of Autophagy in Response to Nutrient Starvation Using Transgenic Mice Expressing a Fluorescent Autophagosome Marker. *Molecular Biology of the Cell*, *15*(3), 1101–1111. https://doi.org/10.1091/mbc.E03-09-0704

Morlando, M., Ballarino, M., Greco, P., Caffarelli, E., Dichtl, B., & Bozzoni, I. (2004). Coupling between snoRNP assembly and 3' processing controls box C/D snoRNA biosynthesis in yeast. *The EMBO Journal*, *23*(12), 2392–2401. https://doi.org/10.1038/sj.emboj.7600254

Munemasa, Y., & Kitaoka, Y. (2015). Autophagy in axonal degeneration in glaucomatous optic neuropathy. *Progress in Retinal and Eye Research*, *47*, 1–18. https://doi.org/10.1016/J.PRETEYERES.2015.03.002

Myeku, N., & Figueiredo-Pereira, M. E. (2011). Dynamics of the Degradation of Ubiquitinated Proteins by Proteasomes and Autophagy ASSOCIATION WITH SEQUESTOSOME 1/p62 * □ S. https://doi.org/10.1074/jbc.M110.149252

N'Diaye, E.-N., Kajihara, K. K., Hsieh, I., Morisaki, H., Debnath, J., & Brown, E. J. (2009). PLIC proteins or ubiquilins regulate autophagy-dependent cell survival during nutrient starvation. *EMBO Reports*, *10*(2), 173–179. https://doi.org/10.1038/embor.2008.238

Neukirchen, D., & Bradke, F. (2011). Neuronal polarization and the cytoskeleton. *Seminars in Cell & Developmental Biology*, *22*(8), 825–833. https://doi.org/10.1016/j.semcdb.2011.08.007

Neumann, M., Rademakers, R., Roeber, S., Baker, M., Kretzschmar, H. A., & Mackenzie, I. R. A. (2009). A new subtype of frontotemporal lobar degeneration

with FUS pathology. *Brain*, *132*(11), 2922–2931. https://doi.org/10.1093/brain/awp214

Neumann, Manuela, Sampathu, D. M., Kwong, L. K., Truax, A. C., Micsenyi, M. C., Chou, T. T., … Lee, V. M.-Y. (2006). Ubiquitinated TDP-43 in frontotemporal lobar degeneration and amyotrophic lateral sclerosis. *Science (New York, N.Y.)*, *314*(5796), 130–133. https://doi.org/10.1126/science.1134108

Nikoletopoulou, V., Papandreou, M.-E., & Tavernarakis, N. (2015). Autophagy in the physiology and pathology of the central nervous system. *Cell Death and Differentiation*, *22*(3), 398–407. https://doi.org/10.1038/cdd.2014.204

Nishiyama, J., Miura, E., Mizushima, N., Watanabe, M., & Yuzaki, M. (2007). Aberrant Membranes and Double-Membrane Structures Accumulate in the Axons of Atg5-Null Purkinje Cells before Neuronal Death. *Autophagy*, *3*(6), 591–596. https://doi.org/10.4161/auto.4964

Nixon, R. A. (2006). Autophagy in neurodegenerative disease: friend, foe or turncoat? *Trends in Neurosciences*, *29*(9), 528–535. https://doi.org/10.1016/j.tins.2006.07.003

Nixon, R. A., & Cataldo, A. M. (2006). Lysosomal system pathways: Genes to neurodegeneration in Alzheimer's disease. *Journal of Alzheimer's Disease*, *9*(s3), 277–289. https://doi.org/10.3233/JAD-2006-9S331

Osaki, T., Shin, Y., Sivathanu, V., Campisi, M., & Kamm, R. D. (2018, January 24). In Vitro Microfluidic Models for Neurodegenerative Disorders. *Advanced Healthcare Materials*. Wiley-VCH Verlag. https://doi.org/10.1002/adhm.201700489

Padman, B. S., Bach, M., Lucarelli, G., Prescott, M., & Ramm, G. (2013). The

protonophore CCCP interferes with lysosomal degradation of autophagic cargo in yeast and mammalian cells Benjamin. *Autophagy*, *9*(11), 1862–1875. https://doi.org/10.4161/auto.26557

Pankiv, S., Clausen, T. H., Lamark, T., Brech, A., Bruun, J.-A., Outzen, H., … Johansen, T. (2007). p62/SQSTM1 Binds Directly to Atg8/LC3 to Facilitate Degradation of Ubiquitinated Protein Aggregates by Autophagy, *282*(33). https://doi.org/10.1074/jbc.M702824200

Parakh, S., & Atkin, J. D. (2016). Protein Folding Alterations in Amyotrophic Lateral Sclerosis. *Brain Research*. https://doi.org/10.1016/j.brainres.2016.04.010

Patten, S. A., Aggad, D., Martinez, J., Tremblay, E., Petrillo, J., Armstrong, G. A. B., … Drapeau, P. (2017). Neuroleptics as therapeutic compounds stabilizing neuromuscular transmission in amyotrophic lateral sclerosis, *2*(22). Retrieved from https://doi.org/10.1172/jci.insight.97152:e97152.

Paulus, J. D., & Link, B. A. (2014). Loss of Optineurin in vivo results in elevated cell death and alters axonal trafficking dynamics. *PLoS ONE*, *9*(10), e109922. https://doi.org/10.1371/journal.pone.0109922

Periyakaruppiah, A., de la Fuente, S., Arumugam, S., Bahí, N., Garcera, A., & Soler, R. M. (2016). Autophagy modulators regulate survival motor neuron protein stability in motoneurons. *Experimental Neurology*, *283*, 287–297. https://doi.org/10.1016/j.expneurol.2016.06.032

Pilli, M., Arko-Mensah, J., Ponpuak, M., Roberts, E., Master, S., Mandell, M. A., … Deretic, V. (2012). TBK-1 Promotes Autophagy-Mediated Antimicrobial Defense by Controlling Autophagosome Maturation. *Immunity*, *37*(2), 223–234. https://doi.org/10.1016/J.IMMUNI.2012.04.015

Polleux, F., & Snider, W. (2010). Initiating and growing an axon. *Cold Spring Harbor Perspectives in Biology*, *2*(4), a001925. https://doi.org/10.1101/cshperspect.a001925

Portera-Cailliau, C., Weimer, R. M., De Paola, V., Caroni, P., & Svoboda, K. (2005). Diverse Modes of Axon Elaboration in the Developing Neocortex. *PLoS Biology*, *3*(8), e272. https://doi.org/10.1371/journal.pbio.0030272

Ravikumar, B., Acevedo-Arozena, A., Imarisio, S., Berger, Z., Vacher, C., O'Kane, C. J., … Rubinsztein, D. C. (2005). Dynein mutations impair autophagic clearance of aggregate-prone proteins. *Nature Genetics*, *37*(7), 771–776. https://doi.org/10.1038/ng1591

Rea, S. L., Majcher, V., Searle, M. S., & Layfield, R. (2014). SQSTM1 mutations – Bridging Paget disease of bone and ALS/FTLD. *Experimental Cell Research*, *325*(1), 27–37. https://doi.org/10.1016/J.YEXCR.2014.01.020

Redmann, M., Benavides, G. A., Berryhill, T. F., Wani, W. Y., Ouyang, X., Johnson, M. S., … Zhang, J. (2017). Inhibition of autophagy with bafilomycin and chloroquine decreases mitochondrial quality and bioenergetic function in primary neurons. *Redox Biology*, *11*, 73. Retrieved from http://dx.doi.org/10.1016/j.redox.2016.11.004

Ritz, D., Vuk, M., Kirchner, P., Bug, M., Schütz, S., Hayer, A., … Meyer, H. (2011). Endolysosomal sorting of ubiquitylated caveolin-1 is regulated by VCP and UBXD1 and impaired by VCP disease mutations. *Nature Cell Biology*, *13*(9), 1116–1123. https://doi.org/10.1038/ncb2301

Rothenberg, C., Srinivasan, D., Mah, L., Kaushik, S., Peterhoff, C. M., Ugolino, J., … Monteiro, M. J. (2010). Ubiquilin functions in autophagy and is degraded by

chaperone-mediated autophagy. *Human Molecular Genetics*, *19*(16), 3219–3232. https://doi.org/10.1093/hmg/ddq231

Rubinsztein, D. C., DiFiglia, M., Heintz, N., Nixon, R. A., Qin, Z.-H., Ravikumar, B., … Tolkovsky, A. (2005). Autophagy and its possible roles in nervous system diseases, damage and repair. *Autophagy*, *1*(1), 11–22. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/16874045

Rudnick, N. D., Griffey, C. J., Guarnieri, P., Gerbino, V., Wang, X., Piersaint, J. A., … Maniatis, T. (2017). Distinct roles for motor neuron autophagy early and late in the SOD1G93A mouse model of ALS. *Proceedings of the National Academy of Sciences of the United States of America*, *114*(39), E8294–E8303. https://doi.org/10.1073/pnas.1704294114

Sahani, M. H., Itakura, E., & Mizushima, N. (2014). Expression of the autophagy substrate SQSTM1/p62 is restored during prolonged starvation depending on transcriptional upregulation and autophagy-derived amino acids. *Autophagy*, *10*(3), 431–441. https://doi.org/10.4161/auto.27344

Salvadores, N., Sanhueza, M., Manque, P., & Court, F. A. (2017). Axonal Degeneration during Aging and Its Functional Role in Neurodegenerative Disorders. *Frontiers in Neuroscience*, *11*, 451. https://doi.org/10.3389/fnins.2017.00451

Sancak, Y., Peterson, T. R., Shaul, Y. D., Lindquist, R. A., Thoreen, C. C., Bar-Peled, L., & Sabatini, D. M. (2008). The Rag GTPases bind raptor and mediate amino acid signaling to mTORC1. *Science (New York, N.Y.)*, *320*(5882), 1496–1501. https://doi.org/10.1126/science.1157535

Sánchez-Martín, P., & Komatsu, M. (2018). p62/SQSTM1 – steering the cell through

health and disease. *Journal of Cell Science*, *131*(21), jcs222836. https://doi.org/10.1242/JCS.222836

Sasaki, Y., Vohra, B. P. S., Lund, F. E., & Milbrandt, J. (2009). Nicotinamide Mononucleotide Adenylyl Transferase-Mediated Axonal Protection Requires Enzymatic Activity But Not Increased Levels of Neuronal Nicotinamide Adenine Dinucleotide. *J Neurosci*, *29*(17), 5525–5535. https://doi.org/10.1523/JNEUROSCI.5469-08.2009

Sasayama, H., Shimamura, M., Tokuda, T., Azuma, Y., Yoshida, T., Mizuno, T., … Yamaguchi, M. (2012). Knockdown of the Drosophila Fused in Sarcoma (FUS) Homologue Causes Deficient Locomotive Behavior and Shortening of Motoneuron Terminal Branches. *PLoS ONE*, *7*(6), e39483. https://doi.org/10.1371/journal.pone.0039483

Sato-Kusubata, K., Yajima, Y., & Kawashima, S. Persistent activation of G s α through limited proteolysis by calpain (2000). Portland Press Ltd. Retrieved from https://www.ncbi.nlm.nih.gov/pmc/articles/PMC1221010/pdf/10769177.pdf

Schindelin, J., Arganda-Carreras, I., Frise, E., Kaynig, V., Longair, M., Pietzsch, T., … Cardona, A. (2012). Fiji: an open-source platform for biological-image analysis. *Nature Methods*, *9*(7), 676–682. https://doi.org/10.1038/nmeth.2019

Schneider, J. L., Miller, A. M., & Woesner, M. E. (2016). Autophagy and Schizophrenia: A Closer Look at How Dysregulation of Neuronal Cell Homeostasis Influences the Pathogenesis of Schizophrenia. *The Einstein Journal of Biology and Medicine : EJBM*, *31*(1–2), 34–39. https://doi.org/10.23861/EJBM201631752

Shaid, S., Brandts, C. H., Serve, H., & Dikic, I. (2013). Ubiquitination and selective

autophagy. *Cell Death & Differentiation*, *20*(1), 21–30. https://doi.org/10.1038/cdd.2012.72

Shibata, M., Lu, T., Furuya, T., Degterev, A., Mizushima, N., Yoshimori, T., … Yuan, J. (2006). Regulation of intracellular accumulation of mutant Huntingtin by Beclin 1. *The Journal of Biological Chemistry*, *281*(20), 14474–14485. https://doi.org/10.1074/jbc.M600364200

Shibata, N., Hirano, A., Kobayashi, M., Siddique, T., Deng, H.-X., Hung, W.-Y., … Asayama, K. (1996). Intense Superoxide Dismutase-1 Immunoreactivity in Intracytoplasmic Hyaline Inclusions of Familial Amyotrophic Lateral Sclerosis with Posterior Column Involvement. *Journal of Neuropathology and Experimental Neurology*, *55*(4), 481–490. https://doi.org/10.1097/00005072-199604000-00011

Singh, A. K., Kashyap, M. P., Tripathi, V. K., Singh, S., Garg, G., & Rizvi, S. I. (2016). Neuroprotection Through Rapamycin-Induced Activation of Autophagy and PI3K/Akt1/mTOR/CREB Signaling Against Amyloid-β-Induced Oxidative Stress, Synaptic/Neurotransmission Dysfunction, and Neurodegeneration in Adult Rats. *Molecular Neurobiology*, 1–14. https://doi.org/10.1007/s12035-016-0129-3

Son, J. H., Hee Shim, J., Kim, K.-H., Ha, J.-Y., & Young Han, J. (2012). Neuronal autophagy and neurodegenerative diseases. *EXPERIMENTAL and MOLECULAR MEDICINE*, *44*(2), 89–98. https://doi.org/10.3858/emm.2012.44.2.031

Song, A., Wang, D., Chen, G., Li, Y., Luo, J., Duan, S., & Poo, M. (2009). A Selective Filter for Cytoplasmic Transport at the Axon Initial Segment. *Cell*, *136*(6), 1148–1160. https://doi.org/10.1016/j.cell.2009.01.016

Song, J. W., Misgeld, T., Kang, H., Knecht, S., Lu, J., Cao, Y., … Lichtman, J. W. (2008).

Lysosomal activity associated with developmental axon pruning. *The Journal of Neuroscience : The Official Journal of the Society for Neuroscience*, *28*(36), 8993–9001. https://doi.org/10.1523/JNEUROSCI.0720-08.2008

Spaulding, E. L., & Burgess, R. W. (2017). Accumulating Evidence for Axonal Translation in Neuronal Homeostasis. *Frontiers in Neuroscience*, *11*, 312. https://doi.org/10.3389/fnins.2017.00312

Staats, K. A., Hernandez, S., Schönefeldt, S., Bento-Abreu, A., Dooley, J., Van Damme, P., … Van Den Bosch, L. (2013). Rapamycin increases survival in ALS mice lacking mature lymphocytes. *Molecular Neurodegeneration*, *8*, 31. https://doi.org/10.1186/1750-1326-8-31

Stavoe, A. K. H., Hill, S. E., Hall, D. H., & Colón-Ramos, D. A. (2016). KIF1A/UNC-104 Transports ATG-9 to Regulate Neurodevelopment and Autophagy at Synapses. *Developmental Cell*, *38*(2), 171–185. https://doi.org/10.1016/j.devcel.2016.06.012

Stavoe, A. K. H., & Holzbaur, E. L. F. (2019). Axonal autophagy: Mini-review for autophagy in the CNS. *Neuroscience Letters*, *697*, 17–23. https://doi.org/10.1016/J.NEULET.2018.03.025

Stokoe, D., Stephens, L. R., Copeland, T., Gaffney, P. R., Reese, C. B., Painter, G. F., … Hawkins, P. T. (1997). Dual role of phosphatidylinositol-3,4,5-trisphosphate in the activation of protein kinase B. *Science (New York, N.Y.)*, *277*(5325), 567–570. https://doi.org/10.1126/science.277.5325.567

Takano, T., Funahashi, Y., & Kaibuchi, K. (2019). Neuronal polarity: Positive and negative feedback signals. *Frontiers in Cell and Developmental Biology*, *7*(APR), 1–10. https://doi.org/10.3389/fcell.2019.00069

Takei, N., Nawa, H., Luikart, B. W., Tarassishin, L., & Einstein, A. (2014). mTOR signaling and its roles in normal and abnormal brain development. https://doi.org/10.3389/fnmol.2014.00028

Tamura, H., & Ohkuma, S. (1991). Induction of neurite outgrowth of PC12 cells by an inhibitor of vacuolar H^+-ATPase, bafilomycin A_1. *FEBS Letters*, *294*(1–2), 51–55. https://doi.org/10.1016/0014-5793(91)81341-5

Teyssou, E., Takeda, T., Lebon, V., Boillée, S., Doukouré, B., Bataillon, G., … Millecamps, S. (2013). Mutations in SQSTM1 encoding p62 in amyotrophic lateral sclerosis: genetics and neuropathology. *Acta Neuropathologica*, *125*(4), 511–522. https://doi.org/10.1007/s00401-013-1090-0

Thomson, A. W., Turnquist, H. R., & Raimondi, G. (2009). Immunoregulatory functions of mTOR inhibition. *Nature Reviews. Immunology*, *9*(5), 324–337. https://doi.org/10.1038/nri2546

Tresse, E., Salomons, F. A., Vesa, J., Bott, L. C., Kimonis, V., Yao, T.-P., … Taylor, J. P. (2010). VCP/p97 is essential for maturation of ubiquitin-containing autophagosomes and this function is impaired by mutations that cause IBMPFD. *Autophagy*, *6*(2), 217–227. https://doi.org/10.4161/auto.6.2.11014

Tsvetkov, A. S., Miller, J., Arrasate, M., Wong, J. S., Pleiss, M. A., & Finkbeiner, S. (2010). A small-molecule scaffold induces autophagy in primary neurons and protects against toxicity in a Huntington disease model. *Proceedings of the National Academy of Sciences of the United States of America*, *107*(39), 16982–16987. https://doi.org/10.1073/pnas.1004498107

Tuck, S. P., Layfield, R., Walker, J., Mekkayil, B., & Francis, R. (2017). Adult Paget's disease of bone: a review. *Rheumatology*, *56*.

https://doi.org/10.1093/rheumatology/kew430

Udagawa, T., Fujioka, Y., Tanaka, M., Honda, D., Yokoi, S., Riku, Y., … Sobue, G. (2015). FUS regulates AMPA receptor function and FTLD/ALS-associated behaviour via GluA1 mRNA stabilization. *Nature Communications*, *6*(1), 7098. https://doi.org/10.1038/ncomms8098

Usategui-Martín, R., García-Aparicio, J., Corral-Gudino, L., Calero-Paniagua, I., Del Pino-Montes, J., & González Sarmiento, R. (2015). Polymorphisms in Autophagy Genes Are Associated with Paget Disease of Bone. *PLOS ONE*, *10*(6), e0128984. https://doi.org/10.1371/journal.pone.0128984

Van Deerlin, V. M., Leverenz, J. B., Bekris, L. M., Bird, T. D., Yuan, W., Elman, L. B., … Yu, C.-E. (2008). TARDBP mutations in amyotrophic lateral sclerosis with TDP-43 neuropathology: a genetic and histopathological analysis. *The Lancet Neurology*, *7*(5), 409–416. https://doi.org/10.1016/S1474-4422(08)70071-1

Vance, C., Rogelj, B., Hortobágyi, T., Vos, K. J. De, Nishimura, A. L., Sreedharan, J., … Shaw, C. E. (2009). Mutations in FUS, an RNA Processing Protein, Cause Familial Amyotrophic Lateral Sclerosis Type 6. *Science*, *323*(5918), 1208–1211. https://doi.org/10.1126/SCIENCE.1165942

Verma, P., Oonam, Chierzi, S., Codd, A. M., Campbell, D. S., Meyer, R. L., … Fawcett, J. W. (2005). Axonal protein synthesis and degradation are necessary for efficient growth cone regeneration. *Journal of Neuroscience*, *25*(2), 331–342. https://doi.org/10.1523/JNEUROSCI.3073-04.2005

Wang, I. F., Guo, B. S., Liu, Y. C., Wu, C. C., Yang, C. H., Tsai, K. J., & Shen, C. K. J. (2012). Autophagy activators rescue and alleviate pathogenesis of a mouse model with proteinopathies of the TAR DNA-binding protein 43. *Proceedings of*

the National Academy of Sciences of the United States of America, *109*(37), 15024–15029. https://doi.org/10.1073/pnas.1206362109

Wang, J. T., Medress, Z. A., & Barres, B. A. (2012, January). Axon degeneration: Molecular mechanisms of a self-destruction pathway. *Journal of Cell Biology*. https://doi.org/10.1083/jcb.201108111

Wang, Q. J., Ding, Y., Kohtz, S., Mizushima, N., Cristea, I. M., Rout, M. P., … Yue, Z. (2006). Induction of Autophagy in Axonal Dystrophy and Degeneration. *The Journal of Neuroscience*, *26*(31), 8057– 8068. https://doi.org/10.1523/JNEUROSCI.2261-06.2006

Wegierski, T., & Kuznicki, J. (2018, September 1). Neuronal calcium signaling via store-operated channels in health and disease. *Cell Calcium*. Elsevier Ltd. https://doi.org/10.1016/j.ceca.2018.07.001

Wei, Luo, & Chen. (2019). Roles of mTOR Signaling in Tissue Regeneration. *Cells*, *8*(9), 1075. https://doi.org/10.3390/cells8091075

Williams, A., Sarkar, S., Cuddon, P., Ttofi, E. K., Saiki, S., Siddiqi, F. H., … Rubinsztein, D. C. (2008). Novel targets for Huntington's disease in an mTOR-independent autophagy pathway. *Nature Chemical Biology*, *4*(5), 295–305. https://doi.org/10.1038/nchembio.79

Williams, K. L., Warraich, S. T., Yang, S., Solski, J. A., Fernando, R., Rouleau, G. A., … Blair, I. P. (2012). UBQLN2/ubiquilin 2 mutation and pathology in familial amyotrophic lateral sclerosis. *Neurobiology of Aging*, *33*(10), 2527.e3-2527.e10. https://doi.org/10.1016/J.NEUROBIOLAGING.2012.05.008

Winton, M. J., Van Deerlin, V. M., Kwong, L. K., Yuan, W., Wood, E. M., Yu, C.-E., … Lee, V. M.-Y. (2008). A90V TDP-43 variant results in the aberrant localization of

TDP-43 in vitro. *FEBS Letters*, *582*(15), 2252–2256. https://doi.org/10.1016/j.febslet.2008.05.024

Wong, Y. C., & Holzbaur, E. L. F. (2014). Optineurin is an autophagy receptor for damaged mitochondria in parkin-mediated mitophagy that is disrupted by an ALS-linked mutation. *Proceedings of the National Academy of Sciences of the United States of America*, *111*(42), E4439-48. https://doi.org/10.1073/pnas.1405752111

Yamamoto, A., Tagawa, Y., Yoshimori, T., Moriyama, Y., Masaki, R., & Tashiro, Y. (1998). Bafilomycin A1 Prevents Maturation of Autophagic Vacuoles by Inhibiting Fusion between Autophagosomes and Lysosomes in Rat Hepatoma Cell Line, H-4-II-E Cells. *Cell Structure and Function*, *23*(1), 33–42. https://doi.org/10.1247/csf.23.33

Yang, D.-S., Stavrides, P., Mohan, P. S., Kaushik, S., Kumar, A., Ohno, M., … Nixon, R. A. (2011). Reversal of autophagy dysfunction in the TgCRND8 mouse model of Alzheimer's disease ameliorates amyloid pathologies and memory deficits. *Brain*, *134*(1), 258. https://doi.org/10.1093/BRAIN/AWQ341

Yang, L., Gal, J., Chen, J., & Zhu, H. (2014). Self-assembled FUS binds active chromatin and regulates gene transcription, *111*(50), 17809–17814. https://doi.org/10.1073/pnas.1414004111

Yang, Y., Coleman, M., Zhang, L., Zheng, X., & Yue, Z. (2013). Autophagy in axonal and dendritic degeneration. *Trends in Neurosciences*, *36*(7), 418–428. https://doi.org/10.1016/j.tins.2013.04.001

Yao, J., Qiu, Y., Frontera, E., Jia, L., Khan, N. W., Klionsky, D. J., … Zacks, D. N. (2018). Inhibiting autophagy reduces retinal degeneration caused by protein misfolding.

Autophagy, *14*(7), 1226–1238. https://doi.org/10.1080/15548627.2018.1463121

Yin, X., Takei, Y., Kido, M. A., & Hirokawa, N. (2011). Molecular Motor KIF17 Is Fundamental for Memory and Learning via Differential Support of Synaptic NR2A/2B Levels. *Neuron*, *70*(2), 310–325. https://doi.org/10.1016/j.neuron.2011.02.049

Yoon, B. C., Zivraj, K. H., & Holt, C. E. (2013). Local Translation and mRNA Trafficking in Axon Pathfinding. https://doi.org/10.1007/400_2009_5

Yue, Z., Friedman, L., Komatsu, M., & Tanaka, K. (2009). The cellular pathways of neuronal autophagy and their implication in neurodegenerative diseases. *Biochimica et Biophysica Acta (BBA) - Molecular Cell Research*, *1793*(9), 1496–1507. https://doi.org/10.1016/j.bbamcr.2009.01.016

Yue, Z., Wang, Q. J., & Komatsu, M. (2008). Neuronal autophagy: going the distance to the axon. *Autophagy*, *4*(1), 94–96. https://doi.org/10.4161/auto.5202

Zhang, K. Y., Yang, S., Warraich, S. T., & Blair, I. P. (2014). Ubiquilin 2: A component of the ubiquitin–proteasome system with an emerging role in neurodegeneration. *The International Journal of Biochemistry & Cell Biology*, *50*, 123–126. https://doi.org/10.1016/J.BIOCEL.2014.02.018

Zhang, L., Yu, J., Pan, H., Hu, P., Hao, Y., Cai, W., … Yuan, J. (2007). Small molecule regulators of autophagy identified by an image-based high-throughput screen. *Proceedings of the National Academy of Sciences of the United States of America*, *104*(48), 19023. https://doi.org/10.1073/PNAS.0709695104

Zhang, X., Li, L., Chen, S., Yang, D., Wang, Y., Zhang, X., … Le, W. (2011). Rapamycin treatment augments motor neuron degeneration in SOD1 G93A mouse model

of amyotrophic lateral sclerosis. *Autophagy*, *7*(4), 412–425. https://doi.org/10.4161/auto.7.4.14541

Zhao, T., Hong, Y., Li, S., & Li, X.-J. (2016). Compartment-Dependent Degradation of Mutant Huntingtin Accounts for Its Preferential Accumulation in Neuronal Processes. *The Journal of Neuroscience : The Official Journal of the Society for Neuroscience*, *36*(32), 8317–8328. https://doi.org/10.1523/JNEUROSCI.0806-16.2016

Zheng, Y., Wildonger, J., Ye, B., Zhang, Y., Kita, A., Younger, S. H., … Jan, Y. N. (2008). Dynein is required for polarized dendritic transport and uniform microtubule orientation in axons. *Nature Cell Biology*, *10*(10), 1172–1180. https://doi.org/10.1038/ncb1777

Zhou, Y., Liu, S., Liu, G., Öztürk, A., & Hicks, G. G. (2013). ALS-Associated FUS Mutations Result in Compromised FUS Alternative Splicing and Autoregulation. *PLoS Genetics*, *9*(10), e1003895. https://doi.org/10.1371/journal.pgen.1003895

8. Appendix

8.1 PIPS Reflective Statement

Note to examiners:

This statement is included as an appendix to the thesis in order that the thesis accurately captures the PhD training experienced by the candidate as a BBSRC Doctoral Training Partnership student.

The Professional Internship for PhD Students is a compulsory 3-month placement which must be undertaken by DTP students. It is usually centred on a specific project and must not be related to the PhD project. This reflective statement is designed to capture the skills development which has taken place during the student's placement and the impact on their career plans it has had.

PIPS Reflective Statement

For my professional internship, I worked on 2 different, but overlapping projects in 2 separate organisations. My time was split working between the University of Nottingham (UoN) Political and Public Affairs Unit (PPAU), as Interim Public Engagement Officer (2 days a week) and with Ignite Futures as Producer of the Nottingham Festival of Science and Curiosity 2018 (3 days a week).

When joining PPAU, the team was workings towards establishing what is now known as the Institute for Policy and Engagement at the university. Related to this, my role with the team was essential to help scope out the existing public engagement activity and related networks at the university with the aim of bringing relevant parties together to a consultation event that would inform the future workings of the new Institute for Policy and Engagement. The main outcome from this project was the successful running of a university-wide networking and consultation event that brought together key staff, academics, students and external partners, all of

whom were to some extent involved in the range of public engagement activity taking place at the university and across the city. This event was used as a way to encourage networking building, gather information about the current scope of activity taking place, and what those undertaking such work would expect in terms of institutional support.

Working with Ignite Futures, my main role was to coordinate the production of the Nottingham Festival of Science & Curiosity 2018, specifically using my work with the UoN PPAU to develop the working relationship between the festival partners and the university, increase content contributions from the university, as well as to use my own skills and experience to expand the festival's 2018 program to target a wider audience base. The outcome of this project was the successful running of the 2018 festival, supported by a new funding agreement which I established with the university, and the inclusion of a new series of 'Lates' events, targeting adult audiences.

Undertaking these PIPS projects taught a me a great deal of valuable skills that I feel have already proved useful towards my career progression. Working with the PPAU, I learnt about the inner workings of the non-academic side of universities and gained a valuable understanding of how professional demands and priorities can differ between the academic and professional services. I developed skills in negotiating agreements between stakeholders and project partners, as well as how to take the lead on tasks when working with partners who have to consider their own interests and priorities. I learnt a lot about my own approach to work and my own skillset in a context outside of academia and working between one very large organisation and

another very small, third sector organisation gave me valuable insights into the inner workings of each.

I'm very glad that I had the opportunity to take part in a professional placement during my PhD. The PhD process can very quickly become very intense, and not only was taking a step out of that environment for 3 months useful in re-framing how to saw my research work, it also allowed me to contextualise my skills, priorities and experience outside of the academic bubble. Working with the UoN PPAU and Ignite Futures has most definitely influenced my future career plans. Firstly, the experience opened my eyes to potential new areas of work or roles that I may not have previously considered, particularly within universities and higher education in general. Additionally, I also found the experience extremely useful in identifying the kinds of roles and areas of work that I would now be less likely to consider going into, as being immersed in 2 starkly different professional environments has given me insights into the reality of certain tasks and roles that I would have previously not known about. In conclusion, I've found the PIPS to be of immense personal and professional value to me. I would strongly encourage anyone undertaking a PhD to engage with similar opportunities, or actively seek them out as a way to greatly enhance, compliment and contextualise the skills developed in the world of research.