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THE ROLE OF CYTOSKELETON PROTEINS IN THE DETERMINATION OF NEURONAL POLARITY

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ABSTRACT

The Par protein family and the LIM kinase-slingshot-cofilin complex of proteins are implicated in regulating neuronal development and differentiation, the study of which holds promise for applications of neurological disease pathologies and therapies. This research project focuses on proteins that affect the components of the neuronal cytoskeleton, primarily actin and tubulin, which form the cell's actin filaments and microtubules, respectively.

The main proteins of interest were the LIM kinase-slingshot-cofilin complex. Downstream of well-established polarity proteins Par-3 and Par-6, this complex impacts neurite differentiation through the stabilization or destabilization of actin and tubulin. By transfecting young neurons with an excess of a particular wild-type protein or with a knockdown plasmid of a particular protein, it was determined that cofilin wild-type and LIM kinase wild-type caused little change in polarity development while cofilin knockdown and slingshot wild-type caused moderate changes and LIM kinase knockdown dramatically reduced the normal polarity phenotype.

Rescue mechanisms were also studied. By co-transfecting proteins known to affect normal polarity expression with an excess of actin and tubulin, it was found that polarity defects were able to be rescued. Future research avenues include studying the molecular mechanisms of and identifying alternative proteins for polarity rescue.

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Thanks finally to my parents, who have supported my interests and passions with infinite patience and encouragement. Everything I do is in recognition of and with gratitude for their immeasurable gifts to me.

Chapter 1

Background and Literature Review

Neuronal polarity is directly influenced by the protein complexes that regulate actin-filament and microtubule dynamics; research in the field suggests that the various proteins involved in regulation of actin phosphorylation and stabilization of microtubules are crucial for initial neuronal differentiation and development. This review discusses the molecular mechanisms of neuronal differentiation, the known protein complexes that influence polarity, and the future directions of polarity research.

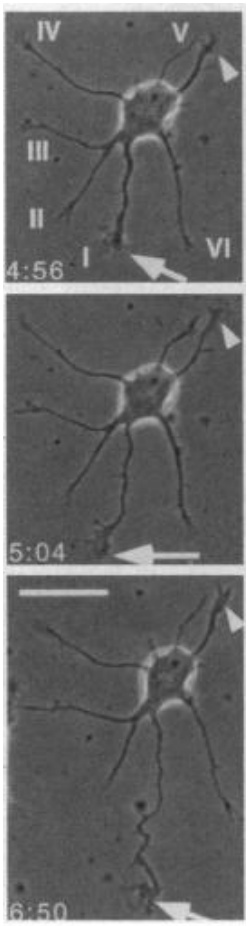

Figure 1. Axon differentiation (Bradke, 1999).

Molecular mechanisms

Actin filaments and microtubules are two components of the cytoskeleton that impact neuronal polarity. It has yet to be determined whether it is actin or microtubule dynamics that dominate neuronal polarity, but research has shown that the stability of each in an immature neurite is associated with the ultimate fate of the neurite. Actin destabilization is correlated with rapid neurite growth and axonal differentiation; multiple axons can be induced by depolymerizing the actin cytoskeleton of neurons with cytochalasin D. Figure 1 depicts the development of neurites and the growth of a

dendrite (arrowhead) and an axon (arrow) relative to each other; the neurite that developed into the axon exhibited a more active growth cone, indicating higher actin turnover in that individual process. Additionally, stable actin filaments are more prevalent in developing dendrites than in the developing axon (Bradke, 1999).

In contrast, microtubules are more stable in the axon than in the dendrites, and microtubule stabilization has been shown to generate axonal differentiation in neurons. Stable microtubules, identified through staining of acetylated alpha-tubulin, are present in high levels only in the axon shaft while dynamic microtubules, identified through staining for tyrosinated alpha-tubulin, are present in the growth cones of all neurites (Witte, 2008). Figure 2 depicts the localization of acetylated alpha-tubulin (green) and tyrosinated alpha-tubulin (red) in a neuron.

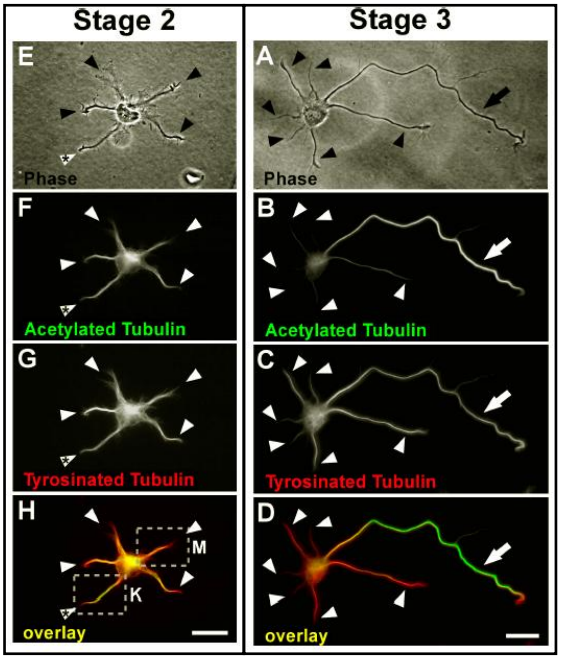

Figure 2. Microtubule stabilization in the axon (Witte, 2008).

Protein complexes

aPKC/Par-3/Par-6

Several protein complexes are associated with the polarization of neurons during early development. The atypical protein kinase C (aPKC)/Par-3/Par-6 complex is perhaps the most thoroughly studied of all the protein complexes known to be involved in neuronal polarity. In model organisms *C. elegans* and *Drosophila*, Par-3 and Par-6 homologues were observed to cause atypical symmetric cell division or to preclude proper cell-fate determination (Etemad-Moghadam, 1995; Watts, 1996; Lin, 2000). This indicated a relationship between these Par proteins and polarity; studies of hippocampal rat neurons confirmed that, along with atypical protein kinase C (aPKC), Par-3 and Par-6 form a complex to control spindle formation and mediate axonal development (Lin, 2000; Shi, 2003).

The mechanism by which Par-3 influences neuronal polarity is the prevention of cofilin phosphorylation. It has been found that levels of phosphorylated (inactive) cofilin, a protein which contributes to the tight junction assembly that partitions the apical and basolateral regions of a cell, are higher when Par-3 is depleted. Implicated in the rescue of tight junction assembly in the absence of Par-3 is the depletion of a LIM kinase protein or the expression of a cofilin mutant that cannot be phosphorylated (Chen, 2006). LIM kinase and other associated proteins will be discussed further in later paragraphs.

In normal neurons, during the process of axon specification, Par-3 and Par-6 localize in one process, which elongates to become the cell's single axon.

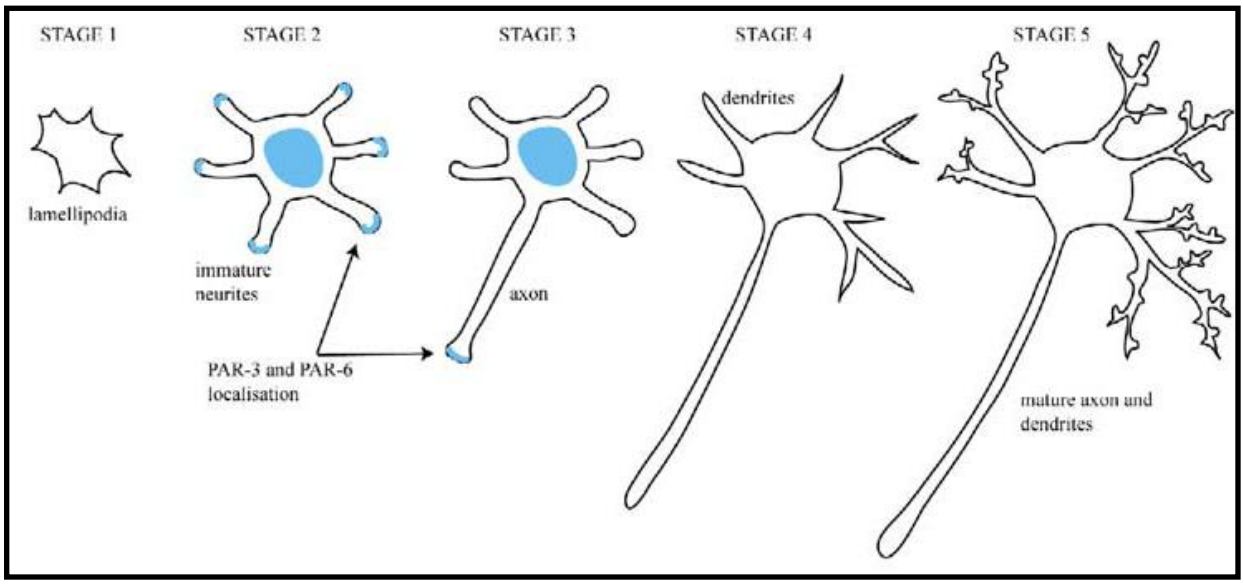

Figure 3. Par protein localization (Wiggin, 2005).

Prior to this, the two proteins are found symmetrically in all nascent neurites (Johansson, 2000; Lin, 2000). The localization of the Par proteins is shown in Figure 3; while a Stage 2 neuron exhibits Par-3 and Par-6 in the cell body and in each neurite, by Stage 3, the proteins are found in the cell body and only one process, the axon. It is known that the Par protein complex regulates actin dynamics; recall that actin filaments are dynamic in axonal growth cones but not in those of other processes.

Previous research in the Chen Lab has shown that the overexpression of Par-3 and Par-6 results in neuronal defects of either an increase in the number of axons or a reduction in axonal development. However, polarity defects can be rescued when actin and tubulin are transfected along with Par-3 and Par-6, as the higher expression of cytoskeleton proteins enables the cell to recover from the effects of Par-3 and Par-6.

aPKC likely acts in relation to both Par-3 and Par-6. It should be noted that Par-3 reduces PKC activity while Par-6 does not, demonstrating that the protein-binding between aPKC and the two Par proteins is dissimilar. Inhibition of PKC with Bisindolylmaleimide I (Bis) was found to eliminate axon growth dramatically. Specifically, when aPKC inhibitors were applied, neurons were five times as likely to fail to polarize. PI 3-kinase, thought to act upstream of aPKC/Par-3/Par-6, also affects polarity development. PI 3-kinase activity in neurons is limited to the axon tip; when it is inhibited, neurons are prevented from polarizing (Shi, 2003; Yoshimura, 2006).

Cdc42 and Rac 1

The Rho GTPase family of proteins may link the Par-3 and Par-6 proteins to other signaling pathways. Interestingly, PI 3-kinase is also believed to be an upstream regulator of Cdc42/Rac 1, two specific Rho GTPases (Yoshimura, 2006). Par-6 is bound to these two proteins, which act as switches for cytoskeleton organization. Cdc42 in its active, GTP-bound form also appears to be a regulator for Par-3/Par-6 (Lin, 2000). Both Cdc42 and Rac 1 are believed to be crucial for cell polarization in epithelia: membrane proteins were mislocalized in cells lacking Cdc42 while tight junction assembly defects were observed in cells expressing constitutively active and dominant negative Rac 1. In *C. elegans*, Cdc42 complexes with Par-3/Par-6 to regulate polarity (Welchman, 2007). In *Drosophila*, Cdc42 is necessary for normal axon growth; however, in mammalian cells, it was found not to be necessary for cell polarization or motility.

There is also a distinct relationship between Cdc42 and cofilin. Cofilin acts downstream of Cdc42, and both influence actin dynamics. Mouse embryos with depleted Cdc42 did not survive past their date of birth; brain staining showed cortex deformations, including less axonal tract development (Garvalov, 2007). For more conclusive study on the effect of Cdc42 on neuronal polarity, however, research was focused on Cdc42 knock-out in hippocampal neurons specifically.

Although knock-out Cdc42 neurons exhibited normal early development in the same culture as wild-type cells, when axonal differentiation began, 70% of the Cdc42 knock-outs did not generate axons whereas 70% of wild-type neurons did. It is important to note that other neurites of the knock-out cells developed normally and at nearly the same rate as wild-type cells. Axon formation was able to be rescued in Cdc42-deficient cells by applying actin-destabilizing drugs (Garvalov, 2007).

A study of Cdc42 in relation to signaling pathways indicated that knock-out Cdc42 inactivates cofilin by phosphorylation; this has been shown to result in reduced axon growth. Cofilin activity is associated with axonal growth cones more so than those of other neurites, and in Cdc42 knock-out cells, cofilin was between two and three times less active in growth cones (Garvalov, 2007). Cofilin will be discussed in-depth later in this section.

GSK-3β and CRMP-2

Another pair of proteins, GSK-3β and CRMP-2, is related to the aPKC and Par proteins and influence neuronal polarization. GSK-3β has been shown to phosphorylate

and inactivate CRMP-2 *in vitro* (Yoshimura, 2005). In epithelial cells, Par-6 was found to regulate GSK-3β and aPKC. Apoptosis specifically associated with Par-6 expression was prevented by GSK-3β inhibition, and when GSK-3β was actively expressed, the Par-6-specific apoptosis levels were elevated. Additionally, aPKC expression was found to rescue this apoptosis, in opposition to GSK-3β. When aPKC activity was inhibited using a peptide that prevents aPKC phosphorylation, rates of apoptosis were much higher. The results of a similar experiment in which aPKC was knocked down also demonstrated an increase in apoptosis (Kim, 2007).

The inhibition of GSK-3β, via knockdown or pharmacological mechanism, disrupts normal axonal differentiation; the expression of CRMP-2 also induces this effect. GSK-3β inhibition produces an increase in the elongation and branching of the axon while overexpression of CRMP-2 has been found to result in the development of multiple axons. The mechanism by which CRMP-2 promotes axonal elongation is its binding to tubulin and enhancing microtubule dynamics and assembly (Witte, 2008).

It has been found that expression of nonphosphorylated CRMP-2, found to localize in the axonal growth cone, is able to rescue the effects of GSK-3β inhibition, suggesting that phosphorylation is necessary for CRMP-2 to function in regulating neuronal polarity development. Additionally, inhibition of GSK-3β results in a reduction of phosphorylated CRMP-2 levels in hippocampal neurons. The phosphorylation of CRMP-2 also plays a role in tubulin binding. A CRMP-2 mutant designed to mimic phosphorylated CRMP-2 was found not to localize along the microtubules of the mitotic

spindle while nonphosphorylated CRMP-2 displayed a uniform localization along the spindle (Yoshimura, 2005).

There are several proteins upstream of GSK-3β that regulate its activity besides Par-6 and aPKC. The signaling from these upstream proteins, including Akt kinase and PTEN phosphatase, is vital for proper neuronal polarization (Jiang, 2005). GSK-3β influences microtubule dynamics by phosphorylating proteins, aside from CRMP-2, that also bind to microtubules during neurite development. Phosphorylation may either activate the microtubule-associated protein, as is the case of MAP1B, or it may inhibit it, as in the case of others, including adenomatous polyposis coli protein. Accordingly, GSK-3β may direct or impede growth in the neurites, particularly the axon. As previously mentioned, microtubule stabilization has been found to be essential in axonal differentiation (Yoshimura, 2005).

LIM kinase/slingshot/cofilin

The main proteins of interest in my researuch are those of the LIM kinase/slingshot/cofilin (LSC) group. Like several of the previously mentioned proteins, LIM kinase affects actin and microtubule dynamics and cytoskeleton changes. Inactivity of proteins of the actin-depolymerizing factor (ADF)/cofilin family has been shown to cause the loss of cell polarity (Dawe, 2003). ADF/cofilin proteins are responsible for regulating many properties of the cell, including cell size, motility, actin dynamics and cytokinesis (Hotulainen, 2005). LIM kinase inactivates cofilin through phosphorylation

while slingshot activates cofilin through dephosphorylation (Edwards, 1999; Endo, 2007). Figure 4 provides two models of the effect of LIM kinase and slingshot on cofilin.

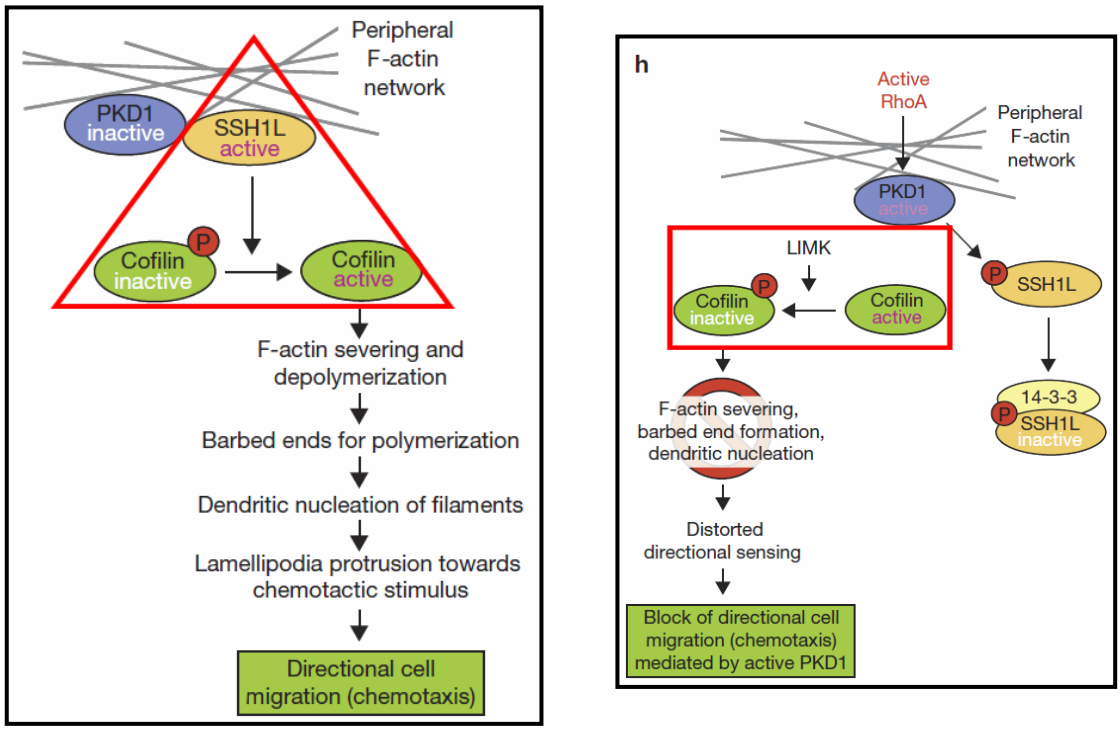

Figure 4. LIM kinase-slingshot-cofilin relationships (Eiseler, 2009).

One area in which the regulation of cofilin phosphorylation by LIM kinase and slingshot is implicated is the motility of growth cones. Research suggests that LIM kinase is dynamic in growth cones, exhibiting both anterograde and retrograde movement. Compared to control neurons not infected with a LIM kinase recombinant HSV, experimental LIM kinase-expressing neurons demonstrated considerably slower motility (Hsieh, 2006). Another experimental group, neurons infected with a kinase-inactive form of LIM kinase, D467A, further established the relationship between LIM kinase and neuronal motility: for LIM kinase (D467A) neurons, the growth cone was actually enhanced slightly, though the neuronal morphology was unusual and the filopodia and

lamellipodia were malformed. While quantification of growth cone motility using time-lapse recording also verified a reduced neurite extension rate in cells expressing active LIM kinase, there was no statistical difference in the rate of neurite extension in the kinase-inactive neurons (Endo, 2003).

Similar experiments performed using cofilin overexpression indicated that growth cone motility was enhanced by wild-type or S3A cofilin, a constitutively active form, though they displayed a reduced fan-like shape and few branches. The rate of neurite extension in these cells was also higher than in control cells. An inactive form of cofilin had no obvious effects on morphology, motility, or neurite extension rate (Meberg, 2000).

Knockdown studies of cofilin have demonstrated several substantial abnormalities. In mammalian non-muscle cells, the knockdown of ADF/cofilin results in the reduction of cell size and the increase in F-actin levels. ADF and cofilin proteins are often referred to together as one unit; in fact, they may operate by similar mechanisms, a theory that has been supported by studies that show that the knockdown effects of one protein can be rescued with overexpression of the other. The knockdown of cofilin also prevents neurite growth; the knockdown of slingshot, a protein that activates cofilin, prevents neurite growth as well. Expression of either protein enhances growth cone dynamics while LIM kinase expression represses growth cone development and motility (Hotulainen, 2005; Garvalov, 2007).

The LSC complex has been associated with myelin-associated inhibitors (MAIs), which are responsible for inhibiting axon regeneration after injury to the central nervous

system. In dorsal root ganglion neurons, LIM kinase phosphorylation of cofilin was determined to be essential to MAI function, as dominant negative LIM kinase expression reduced the level of inhibition while inactive slingshot prevented MAI function as well. Several MAIs studied, including Nogo-66, were given to cerebellar neurons, and was found to stimulate cofilin dephosphorylation (Hsieh, 2006).

Protein kinase D and Golgi bodies

A recent development in the field involves the relationship between protein kinase D and protein transport. Protein kinase D regulates neuronal polarity neither through the activation nor inhibition of proteins in the cytoskeleton but rather through the trafficking of neuronal proteins in the Golgi apparatus. This protein trafficking plays a crucial role in the proper polarization of hippocampal neurons. PKD expression rescues the disruption of membrane trafficking and abnormal polarity caused by F-actin depolymerization (Bisbal, 2008).

While all three isoforms of PKD (1, 2, and 3) are expressed in primary cortical neurons, PKD1 is the form of PKD that is most prevalent in hippocampal neurons, with demonstrably lower levels of PKD3 and no expression of PKD2. PKD1 and PKD2 are essential in polarity regulation. In the absence of PKD1 and PKD2, whether by knockdown, dominant negative, or inhibition, multiple axons are formed. PKD expression can rescue the disruption of membrane trafficking and abnormal polarity caused by F-actin depolymerization (Yin, 2008). PKD1 alone is involved in the regulation of two proteins, the transferrin receptor TfR and low-density receptor-related

protein LRP, which are found almost exclusively in dendrites. While the expression of PKD1 changed the distribution of these proteins within the neuron, expression of an inactive mutant form of PKD1 did not (Bisbal, 2008).

When PKD1 expression is decreased, these dendritic proteins are carried to both axon and dendrites. Furthermore, in the absence of PKD1 and one of its mammalian homologues, PKD2, multiple axons are formed. The research implies that PKD is necessary for normal dendrite arborization as well as polarity regulation. Also, dominant negative PKD1 limits dendritic expansion while constitutively active PKD1 increases it. DN PKD1 also affects normal Golgi apparatus function in neurons (Czondor, 2009).

Summary

The implications for a greater understanding of neuronal polarity are considerable and far-reaching. My research focused primarily on the LSC complex and its effects on neuronal polarity. I hypothesized that any abnormal expression of the proteins in the LSC complex, either overexpression or knockdown, would have a clear negative effect on neuronal polarity. Additionally, I attempted to determine a mechanism of polarity rescue through co-expression of actin and tubulin.

I found that overexpression of cofilin wild-type and LIM kinase wild-type caused little change in polarity development. Cofilin knockdown and slingshot wild-type overexpression caused moderate changes while LIM kinase knockdown demonstrated the most dramatic negative effect on polarity.

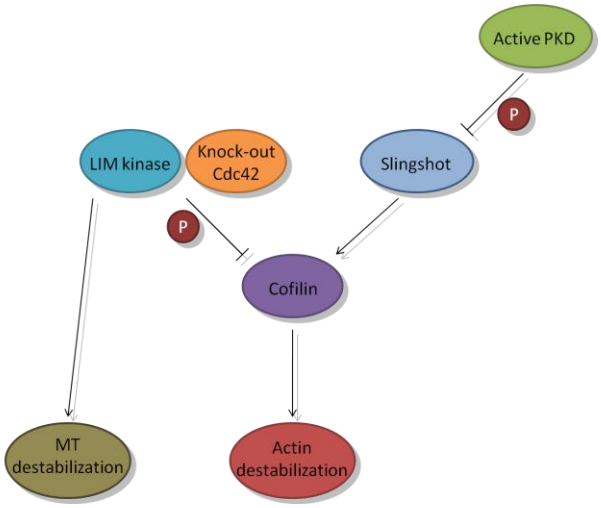

Figure 5. Model of protein interactions involved in polarity development.

Based on a review of the existing literature and my own research, I constructed a model of protein interactions that illustrates various activation and inhibition mechanisms (Figure 5). Though certainly not a comprehensive diagram, the central proteins of interest, LIM kinase, slingshot, and cofilin, are implicated as having a direct effect on actin and microtubule destabilization and, subsequently, on polarity development.

By recognizing the mechanisms of neurite differentiation and discovering ways in which to rescue abnormal development, potential treatments for neurodegenerative disease can be uncovered. Cofilin, for example, along with regulating actin dynamics in the cytoskeleton, has also been found to aggregate in older, senile rat brains, suggesting a either a causal or correlative relationship with Alzheimer's disease or dementia (Bamburg, 1999; Heredia, 2006). Through greater research, the connections between protein expression and physiological disease expression can be further clarified and understood.

Chapter 2

Materials and Methods

Transfection

The transfection protocol I followed was developed in the Chen Lab (Jiang, 2006). The plasmid(s) in question is mixed with water, a calcium chloride solution, and a salt solution (HBS), forming a calcium phosphate precipitate after a brief incubation period. Once added to a coverslip of neurons, the precipitate is taken up by the cells via endocytosis. Typically, the plasmid(s) is co-transfected with a fluorescent protein such as GFP. After incubation for 24-48 hours, neurons that have endocytosed the precipitate will be able to be identified under an epifluorescent microscope. Figure 6 provides a detailed visual reference for this procedure.

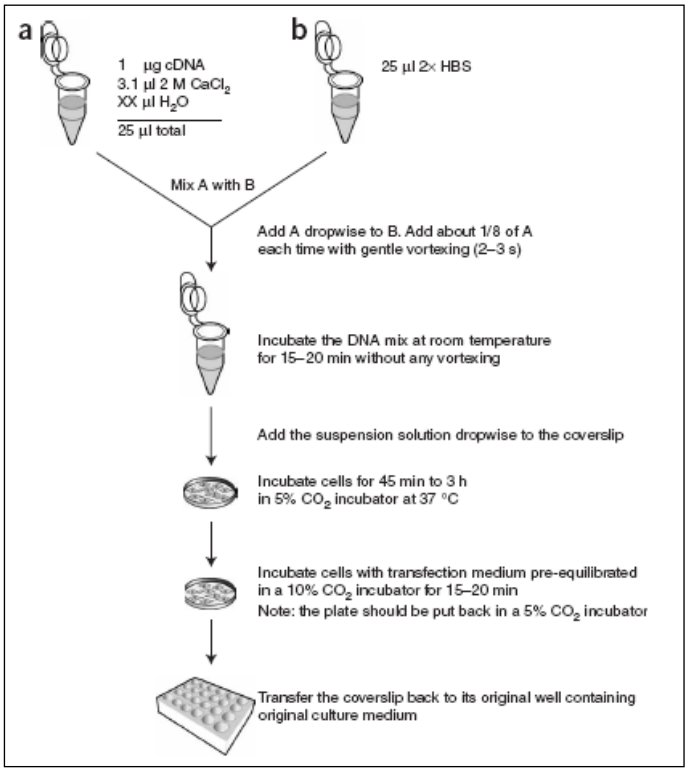

Figure 6. Flowchart of central transfection procedure (Jiang, 2006).

Certain timing and procedural modifications have been made to the protocol since publication, which I followed for all of my transfection experiments:

1. Vortexing of solutions A and B after they have been combined was limited to lightly tapping the Eppendorf tube on the vortex at 600 rpm two to three times.
2. The DNA mix was incubated for eight minutes at room temperature in the culture hood.
3. Upon adding the suspension solution to the coverslip, the cells were incubated for 25 minutes in the 5% CO_2 incubator.
4. After washing the cells with a transfection medium from the 10% CO_2 incubator, the cells were incubated again in the 5% CO_2 incubator for ten minutes.
5. When GFP was co-transfected with a protein or proteins of interest, only 0.3-0.4 micrograms per coverslip were necessary.

These modifications were made based on ongoing experimentation in the Chen Lab. Most were preventative measures to avoid excessive precipitate formation, which can lead to cell death, or to avoid too-high transfection efficiency, which, for the purposes of cell polarity study, is detrimental to quantification of neuronal processes. This was further

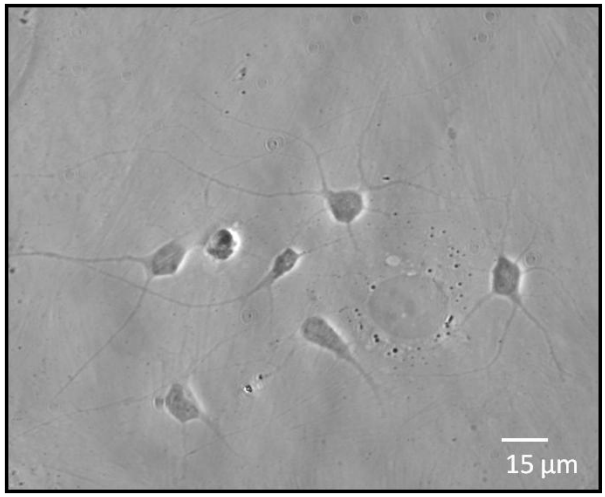

Figure 7. Neurons in culture, phase image.

avoided by plating cultures with very low initial neuronal density. Figure 7 provides a representative image of neurons in a low density culture.

Because neuronal polarity is established very early on, I typically transfected cells the day after they are cultured, or one day *in vitro* (1 DIV). Transfection later in the lifespan would likely not yield suitable data for determining protein effects on initial development because the neurites would have already differentiated, but the data could still be useful in studying other developmental mechanisms, such as the formation of dendritic spines or synaptic activation. Additionally, one limitation was that transfection becomes progressively more difficult as cells age past a few days *in vitro*.

Immunocytochemical staining

Two days following transfection, when the cells are three days *in vitro* (3 DIV), immunocytochemical staining was performed to prepare slides of the coverslips for quantification. The coverslips were first transferred from the culture wells to small Petri dishes and washed once in bath solution and twice in PBS. Then, the cells were fixed using a 4% paraformaldehyde solution for twelve minutes and washed three times with PBS. The cell membranes were permeabilized using a solution of PBS, normal donkey serum or normal goat serum, and 0.5% Triton X-100, a surfactant, applied for five minutes. The coverslips were washed three times again with PBS before the primary antibody solution was applied and the coverslips placed on a shaker in a 4°C cold room to incubate overnight.

The primary antibodies are a monoclonal Tau antibody at a 1:200 dilution with PBS and neuronal donkey serum or normal goat serum to identify axons and a polyclonal MAP2 antibody at a 1:1000 dilution to identify dendrites. The Tau antibody is specific to the Tau protein, present in axonal but not dendritic processes; the MAP2 antibody is specific to MAP2, a protein whose expression is strong in dendrites and weak in axons. The next day, at 4 DIV, the secondary antibodies, Alexa 647 goat-anti-mouse and Alexa 546 goat-anti-rabbit, corresponding to the Tau and MAP2 primary antibodies, respectively, were applied to the cells. Both these antibodies were diluted at a ratio of 1:300. These antibodies were tagged with fluorophors so that they would be visible under specific epifluorescent microscope filters.

Imaging and quantification

The slides were imaged using the software program SimplePCI. This program allows fluorescent and phase images of the neurons to be taken for future use in quantification. Using the GFP filter first, transfected neurons were identified. A methodical top-to-bottom and side-to-side sweep of each coverslip

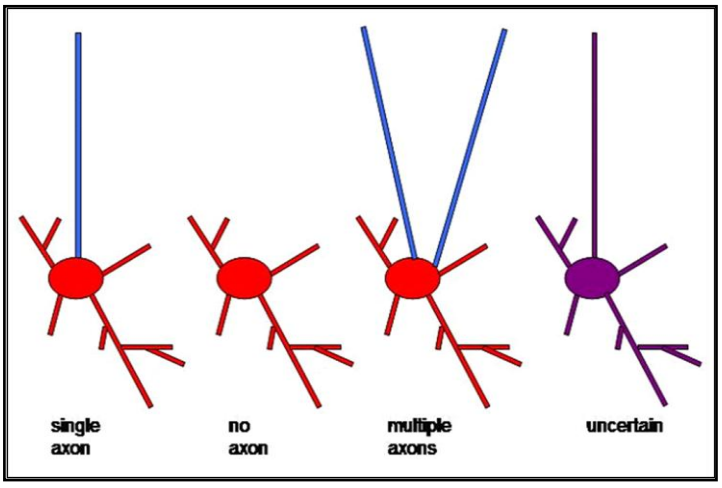

Figure 8. Quantification categories (Cichon, 2008).

generally ensured that each suitable neuron was imaged only once. Appropriate filters (Cy5 and TRITC) were used for the antibodies of interest, and a phase image was also taken to establish the condition of the environment around the neuron in question. Images were taken at 40X magnification.

Based on what is known about the microtubule-associated proteins, processes in which Tau appeared strong and in which the MAP2 signal was weak were determined to be axonal. Processes in which the opposite was true – strong MAP2, weak Tau – were determined to be dendritic. Figure 8 demonstrates the four quantification categories used: neurons were identified as "single axon" if one and only one process demonstrated a strong Tau signal; "no axon" if only a MAP2 signal was strong; "multiple axon" if more than one process demonstrated strong Tau; or "uncertain" if a neuron had both weak Tau and MAP2 signals or if the two signals appeared with the same intensity and were thusly inconclusive regarding which protein was the dominant one in the processes.

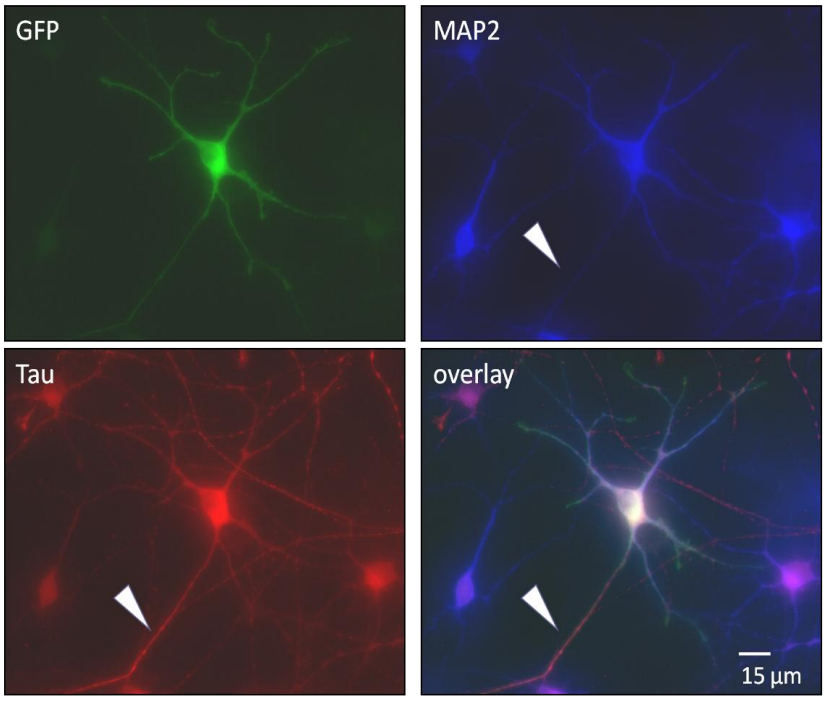

Figure 9. Images of a neuron colored to represent fluorescent stainings.

The figures of the neurons in this

thesis were enhanced and colored to show the respective stainings using Adobe Photoshop. As seen in Figure 9, for all enhanced images of neurons, green represents a transfected neuron under the GFP filter; blue represents the neuron as seen under the MAP2 filter; and red represents the neuron as seen under the Tau filter. Arrowheads mark the axonal process.

Chapter 3

Results

The questions I wanted to answer with my research were:

1) Which proteins have the greatest effect on the development of neuronal polarity?
2) Is the overexpression or knockdown of a particular protein more detrimental to polarity development?
3) What molecular mechanisms – phosphorylation or dephosphorylation of cofilin, for example – are responsible for polarity defects?

Through experimentation with various constructs of Par-3, LIM kinase, slingshot, and cofilin, it was determined that the greatest effects on neuronal polarity were observed following transfection with LIMK-shRNA, slingshot wild-type, and cofilin-shRNA. Also, shRNA (knockdown) constructs demonstrated greater effect on abnormal polarity than their respective wild-type (WT) constructs, the transfection of which results in overexpression of the protein in the neuron. Additionally, it was not evident from the data whether one mechanism in particular was responsible for polarity defects.

Figures 10 and 11 collectively show one representative neuron for each of the six transfection categories I studied. The colored GFP, MAP2, and Tau images are included for each neuron, along with an overlay image to assist with differentiation of relative fluorescent signal intensities.

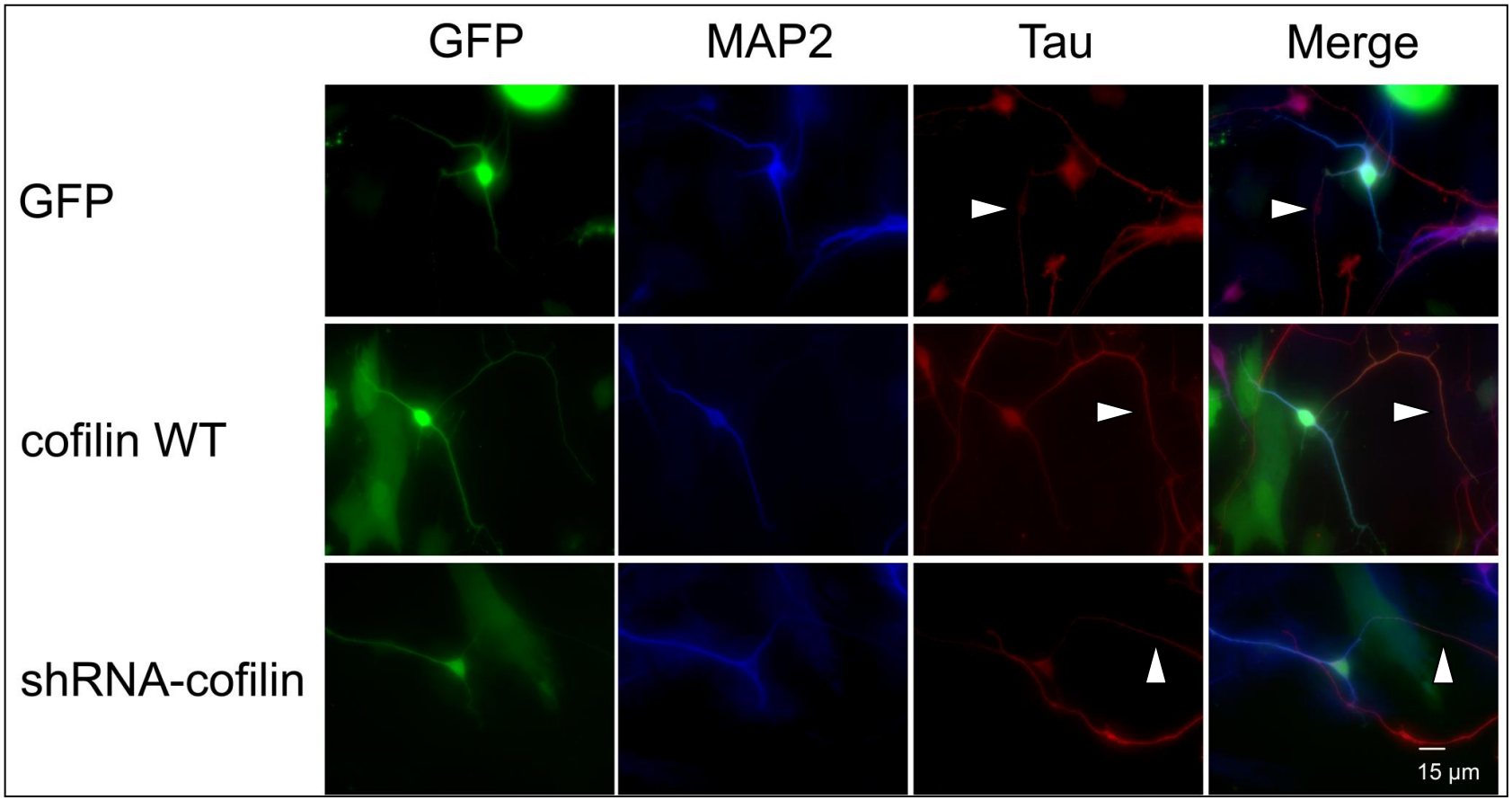

Figure 10. Representative images of GFP- , cofilin WT- and cofilin-shRNA transfected neurons. The arrowheads point to the axonal process.

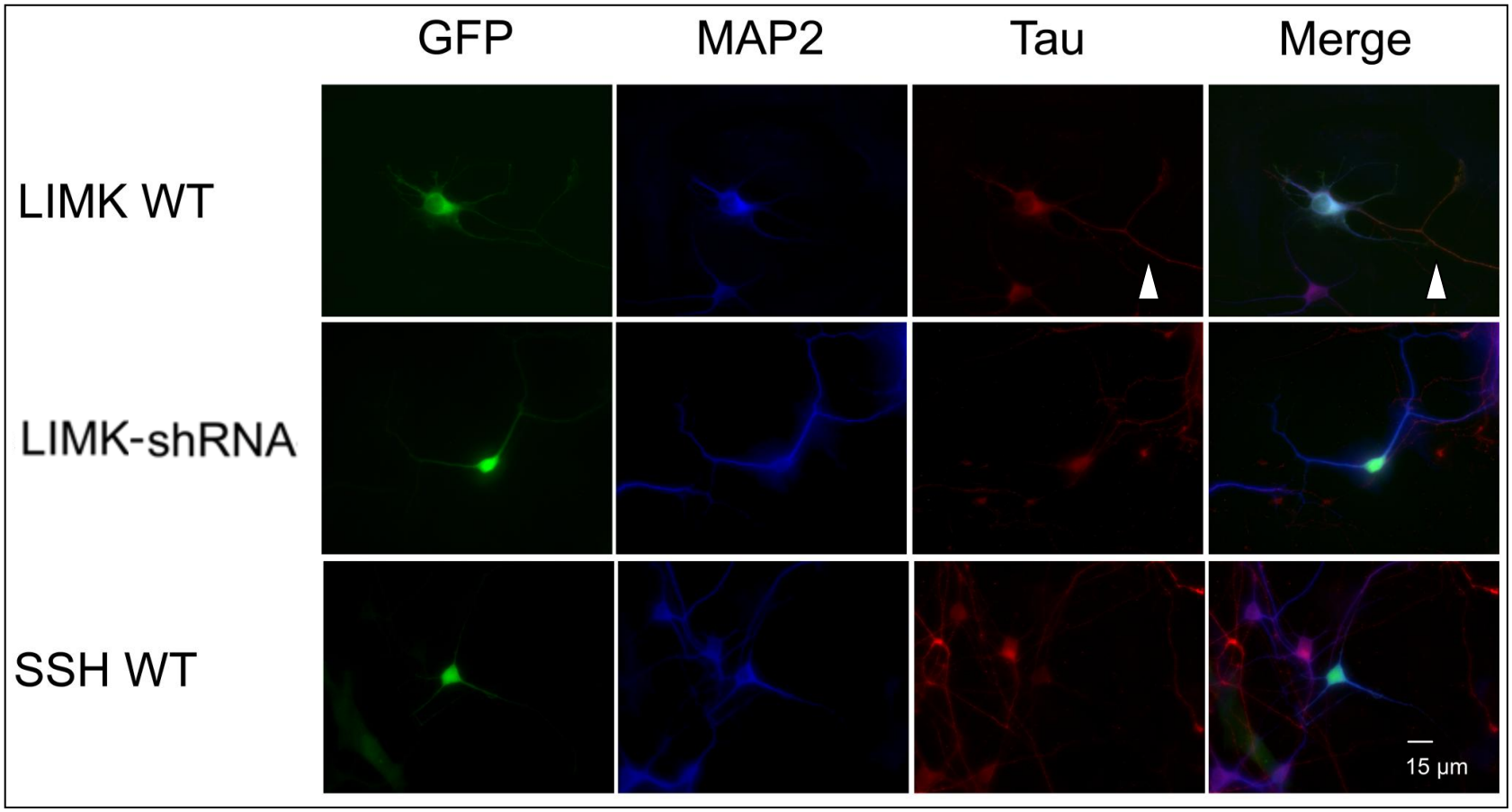

Figure 11. Representative images of LIMK WT- , LIMK-shRNA- and SSH WT-transfected neurons.

Figure 12 provides the results of three GFP transfection trials. The data indicates that normal, single-axon neurons develop approximately 70% of the time. The polarity percentages are consistent with previous data collected by Joseph Cichon, a former member of the Chen Lab. The overall GFP data (right-most bar) is used as the GFP control to which to compare all other transfection variables.

The graphical data for the other transfection variables are available in the appendices. It should be noted that, for each transfection variable, to ensure the accuracy of the quantification, at least three sets of data were collected from different cultures.

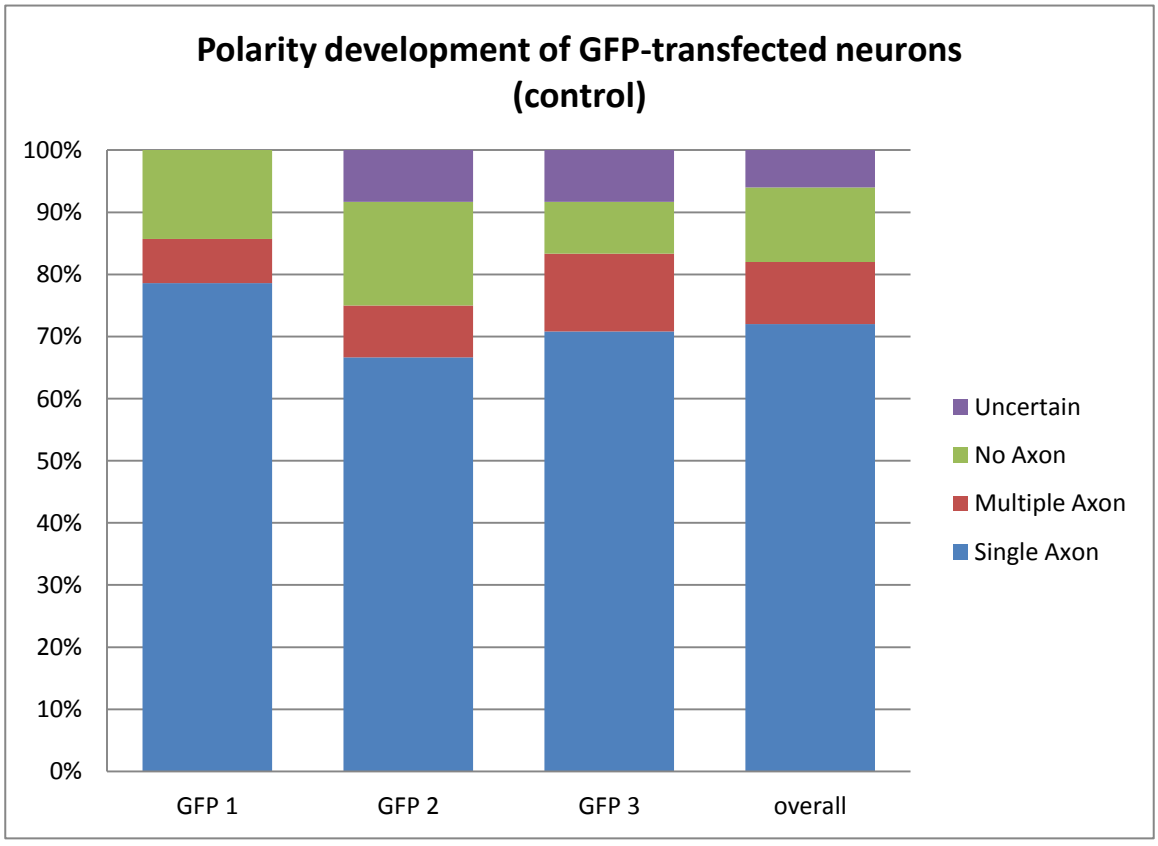

Figure 12. Polarity ratios for three control trials with GFP and an average of the trials (n=50).

Included as Appendix A are the results of preliminary experimentation conducted with Par-3-shRNA constructs. The two different Par-3-shRNA constructs produced

substantially different results. This was found to be due to the initial use of an older construct along with an excessive amount of GFP. (GFP is always either co-transfected with or already tagged to the protein of interest.) Once it was determined that overuse of GFP was limiting the expression of the protein of interest and that the Par-3-shRNA was not fresh, the experiments were repeated using a lower concentration of GFP and a new Par-3-shRNA plasmid. As expected, the polarity defects increased after the experimental parameters were adjusted for optimal Par-3-shRNA expression.

Similar transfection issues were encountered using LIMK-shRNA, as demonstrated in Appendix B. The same procedures were followed as for the Par-3-shRNA trials, but with an additional set of experiments using a limited amount of GFP but the same, older LIMK-shRNA plasmid. The results align with the expectation that normal polarity would be reduced with each sequential adjustment to the transfection procedure for eventual optimization.

Once the optimal LIMK-shRNA transfection procedure was determined, two additional trials with the newest plasmid were completed to ensure accuracy of results with a larger sample size. Appendix C reveals that transfection of LIMK-shRNA has a substantial negative effect on normal polarity development. Nearly half of all transfected neurons demonstrated no axonal differentiation.

In contrast, LIMK WT-transfected neurons and control neurons transfected with GFP demonstrated very similar polarity development (Appendix D). The difference in the percentages between the three separate trials of LIMK WT can be explained by the relatively small sample sizes of approximately twelve neurons each. Thus, one additional

neuron in a category has a weight of over 8%, producing a contrast that is not as dramatic as it appears at first glance.

The two cofilin transfections demonstrated a similarity to the two transfections with LIM kinase. In both cases, the knockdown transfections produced a higher percentage of abnormally polarized neurons while the wild-type overexpression of the proteins did not cause much deviation from the control phenotypes. Although not as striking of a decrease in normal polarity was observed for the cofilin-shRNA transfections compared to the LIMK-shRNA experiments, it is clear that the cofilin knockdown also disrupts neuronal development (Appendix E). The opposite was observed for the cofilin WT-transfected neurons: they demonstrated very similar polarity ratios to the GFP control neurons, indicating that polarity development was not noticeably disrupted (Appendix F).

SSH WT transfection was also found to have a strong effect in producing abnormal polarity phenotypes (Appendix G). The difference in the percentages between the three separate trials of SSH WT transfection, like the LIMK WT transfection, can be explained by the relatively small sample sizes of approximately twelve neurons each.

Figure 13 is a synthesis of the six transfection categories. While cofilin WT and LIMK WT appear to have little effect on normal polarity development, the knockdowns of those proteins appear to interfere considerably with axonal specification. Additionally, the transfection of SSH WT appears to have a similarly disruptive effect.

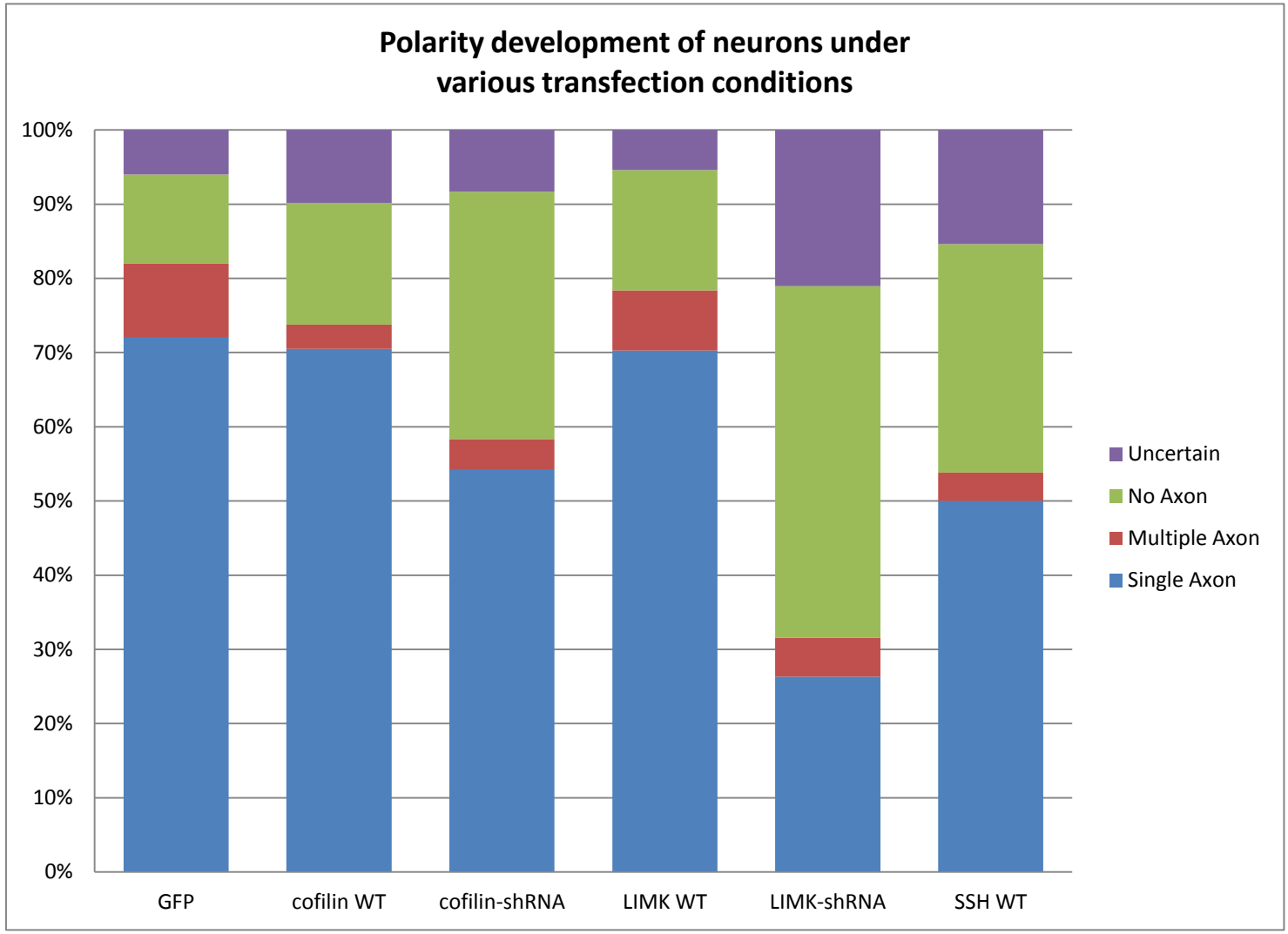

Figure 13. Polarity ratios for GFP- , cofilin WT- , cofilin-shRNA- , LIMK WT- , LIMK-shRNA- , and SSH WT-transfected neurons.

Chapter 4

Discussion

Regarding the question of which proteins have the greatest effect on polarity development, the results do not provide a clear answer. However, regarding the question of whether overexpression or knocking down a protein has the greater effect, the results suggest that protein knockdown is more powerful. While the results support the hypothesis that the knockdown of LIM kinase or cofilin has a noticeable negative impact on neuronal polarity, the overexpression of both these proteins' wild-types appears to produce little polarity difference compared to the control tests. The slingshot transfection does show a negative effect on polarity from overexpression of the protein; knockdown tests performed in the future would be able to confirm whether silencing that protein has a greater impact on polarity also.

The data also indicates that knocking down LIMK expression has a more profound impact than simply prevention of the inactivation of cofilin. Were the impact straightforward, the results of the LIMK-shRNA transfection should have resembled the results of the transfection with overexpressed cofilin. Similarly, the data indicates that slingshot overexpression also has a more profound impact on polarity than increased cofilin activation. The results of the SSH WT transfection correspond more, in fact, to the results seen following cofilin-shRNA transfection. It appears that LIM kinase and slingshot have an effect on neuronal polarity outside of their respective inactivation (phosphorylation) and activation (dephosphorylation) of cofilin.

The data supports previous cofilin research which demonstrated that the knockdown of cofilin caused severe abnormalities in neuronal development. Cofilin also appears to be crucial to neurite extension (Hotulainen, 2005; Garvalov, 2007). Additionally, the data supports the previous research that demonstrated that cofilin inactivation resulted in a loss of polarity (Dawe, 2003).

The co-transfection of actin and tubulin with proteins that negatively affect polarity has been shown previously in the Chen Lab to rescue polarity defects, resulting in single-axon development at a higher percentage than even the control. Based on this potential rescue mechanism, I performed co-transfection of actin and tubulin with LIMK-shRNA in an effort to counteract the polarity defects observed from the transfection of LIMK-shRNA alone. Although not enough data was collected to draw any conclusions about polarity, the area of polarity rescue is one that has great clinical implications and the exploration of rescue mechanisms is one of the next steps of polarity research in the Chen Lab.

The knockdown of Par-3 results are also worth exploring further. These experiments were performed early on in my time in the Chen Lab and were largely to familiarize myself with the transfection and immunostaining processes. Because Par-3 and Par-6 have already been studied extensively, it was decided that I would pursue the LSC complex as my main area of research. In addition, because the LSC complex is downstream of the Par-3/Par-6 signaling pathway, it is likely that it has a more direct effect on actin and microtubule dynamics (Barnes, 2009). (This was also illustrated in my model in the Background and Literature Review section.) Still, the effect of knocking

down polarity proteins such as Par-3 has not been explored in as much depth as overexpression has been.

It was noted during quantification that even when polarity ratios were normal in regards to axonal development for the experimental transfections, the dendritic processes of the transfected neurons did not always resemble those of control neurons. Stunted growth as well as the growth of short, filopodia-like structures along the dendrites was observed. This appears to indicate that dynamic microtubules, present in dendrites but not the axon, are disrupted by the proteins of interest. The atypical morphology of the dendrites can also be quantified in the future; whether these abnormalities have any effect on synaptic connections and communication can be determined through electrophysiology.

The growth of these structures was most conspicuous among neurons transfected with LIMK-shRNA, actin, and tubulin in the rescue experiments; there were noticeably more of these short extensions from the cell soma and dendrites than were observed in any other transfection category. While it is suspected that the overabundance of actin and tubulin following transfection was responsible for these extensions, it is unclear what effect, if any, they have on the neuron's polarity development or synaptic connections, but further experimentation with actin and tubulin rescue can help to shed light on the mechanisms behind this extra growth and its function.

The development of multiple axons is another avenue of research that is noteworthy because of the potential for multiple routes of neuron signaling. SV2 staining was performed for some experiments to determine the functionality of observed axonal processes; however, the staining was very weak and resulted in much ambiguity during

quantification. Continued experimentation using SV2 staining and future experimentation using electrophysiology can also be used to study the possibility of multiple axons' all being functional.

One limitation of this research is the potential inconsistency of the DNA plasmids used for transfection. As noted in the Results section, there were cases early in my research of using relatively out-of-date plasmids, which resulted in distorted polarity ratios. This misrepresentation appeared suspicious because the ratios did not match previously obtained results that had been thoroughly repeated and confirmed; it was only after comparison to these previous results that the issues of excess GFP and outdated DNA were realized.

Thus, despite repeated tests of each of my experimental groups, it may be that the results are still skewed because of the use of only one plasmid for each protein over all experiments. Since there were no previous results to which to compare most of them, further experimentation should include tests using plasmids from separate preparations to verify my results.

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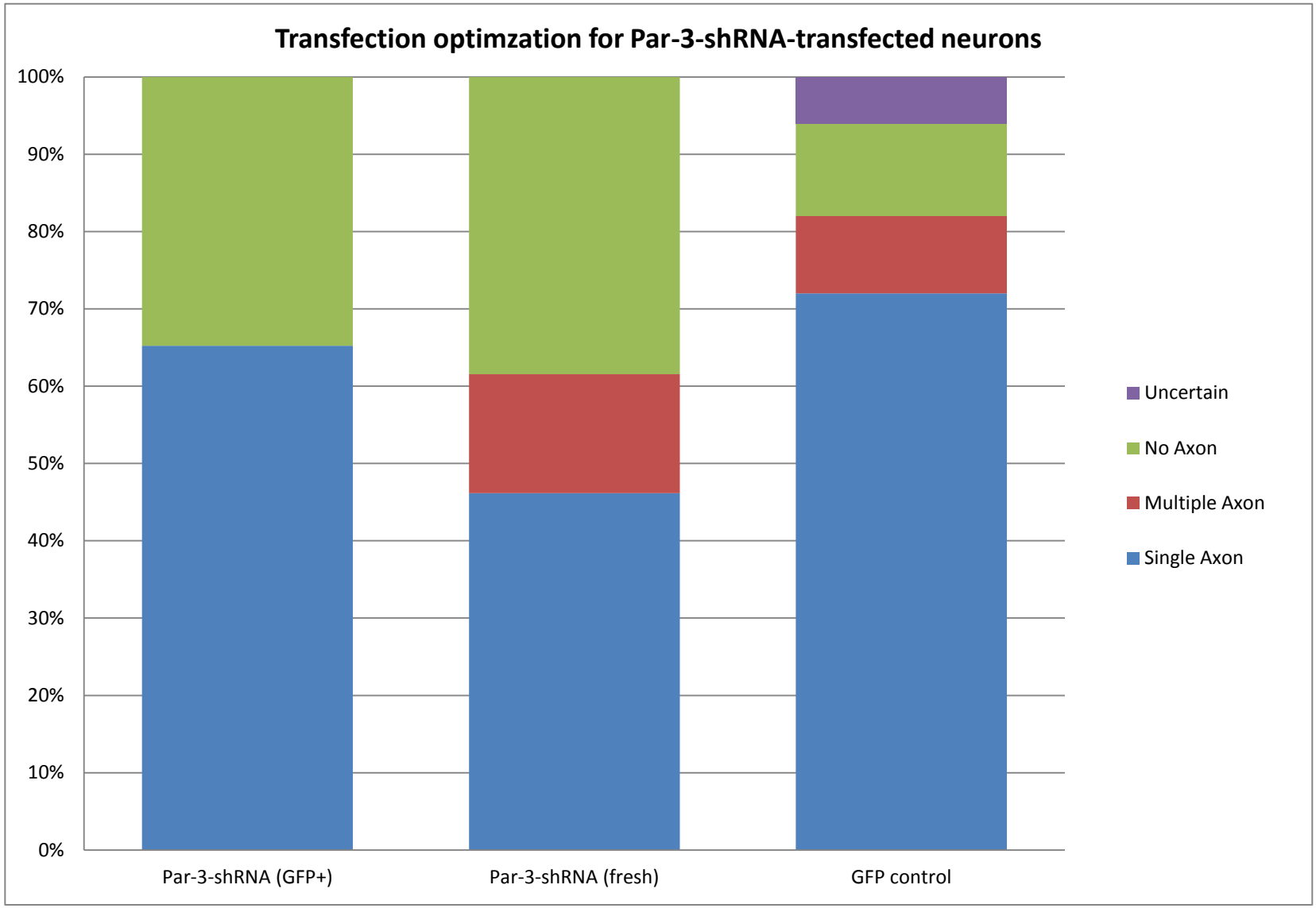
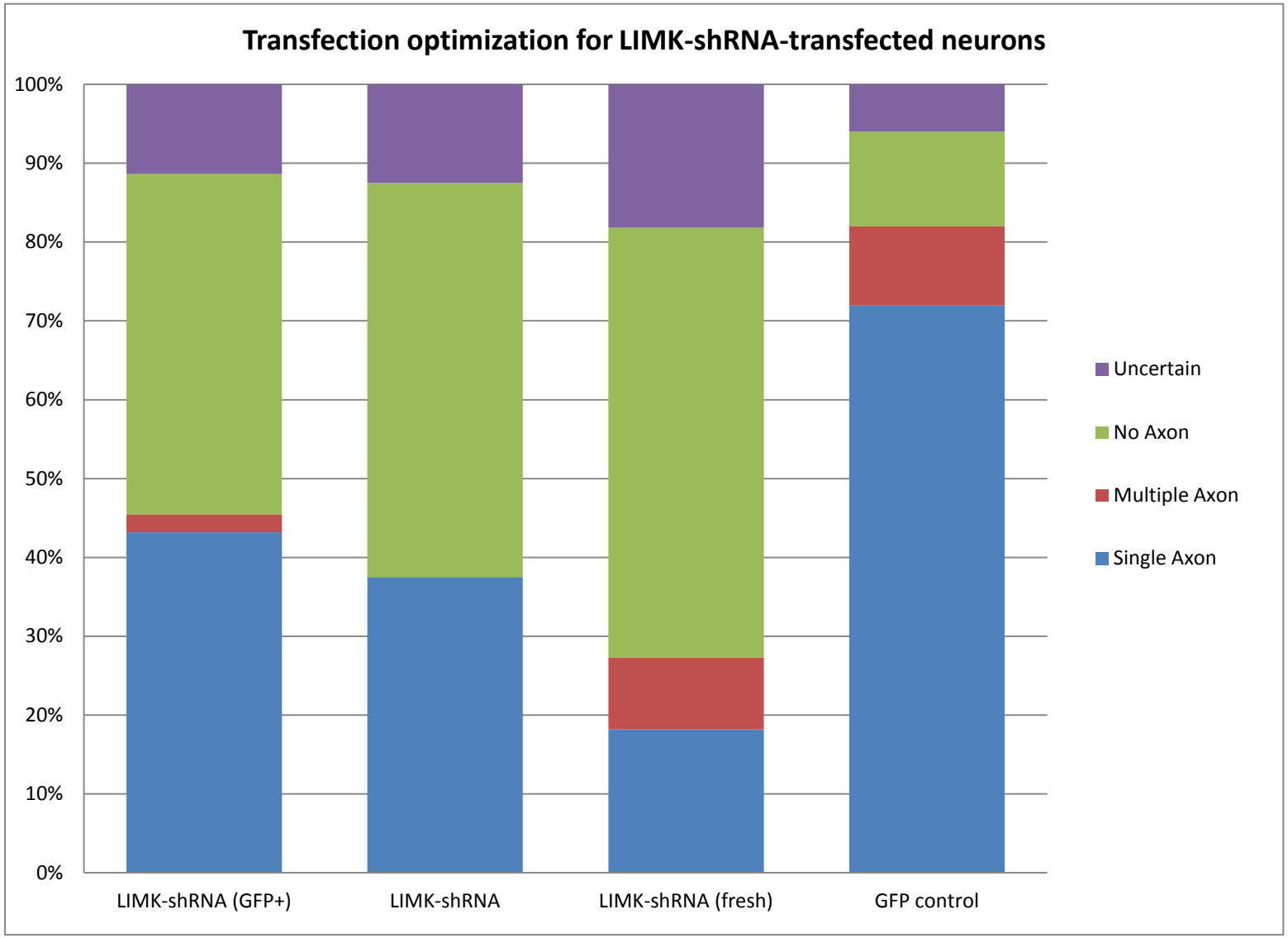
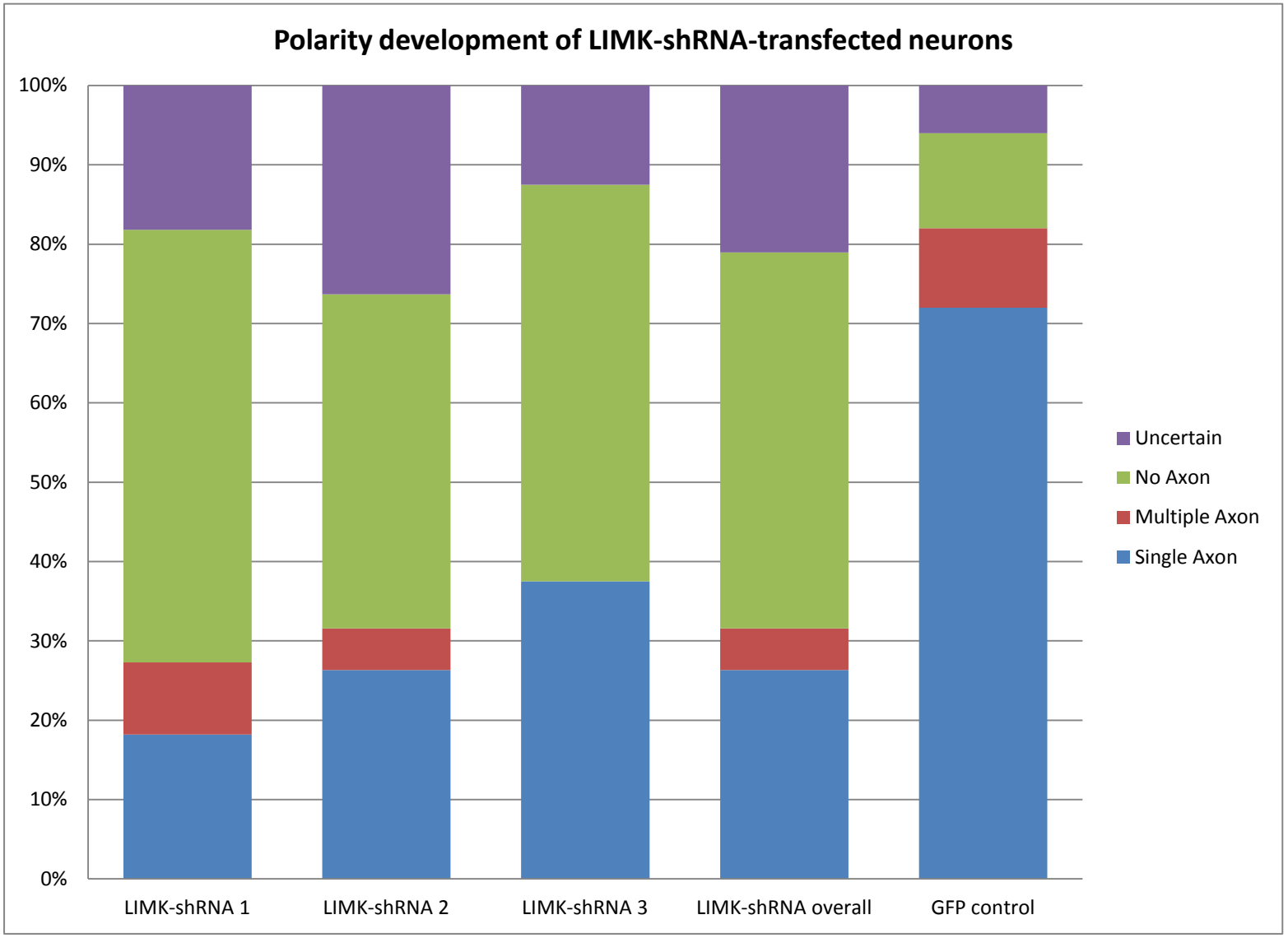
Appendix A: Comparison of polarity ratios between varied transfection conditions for Par-3-shRNA- and GFP-transfected neurons.



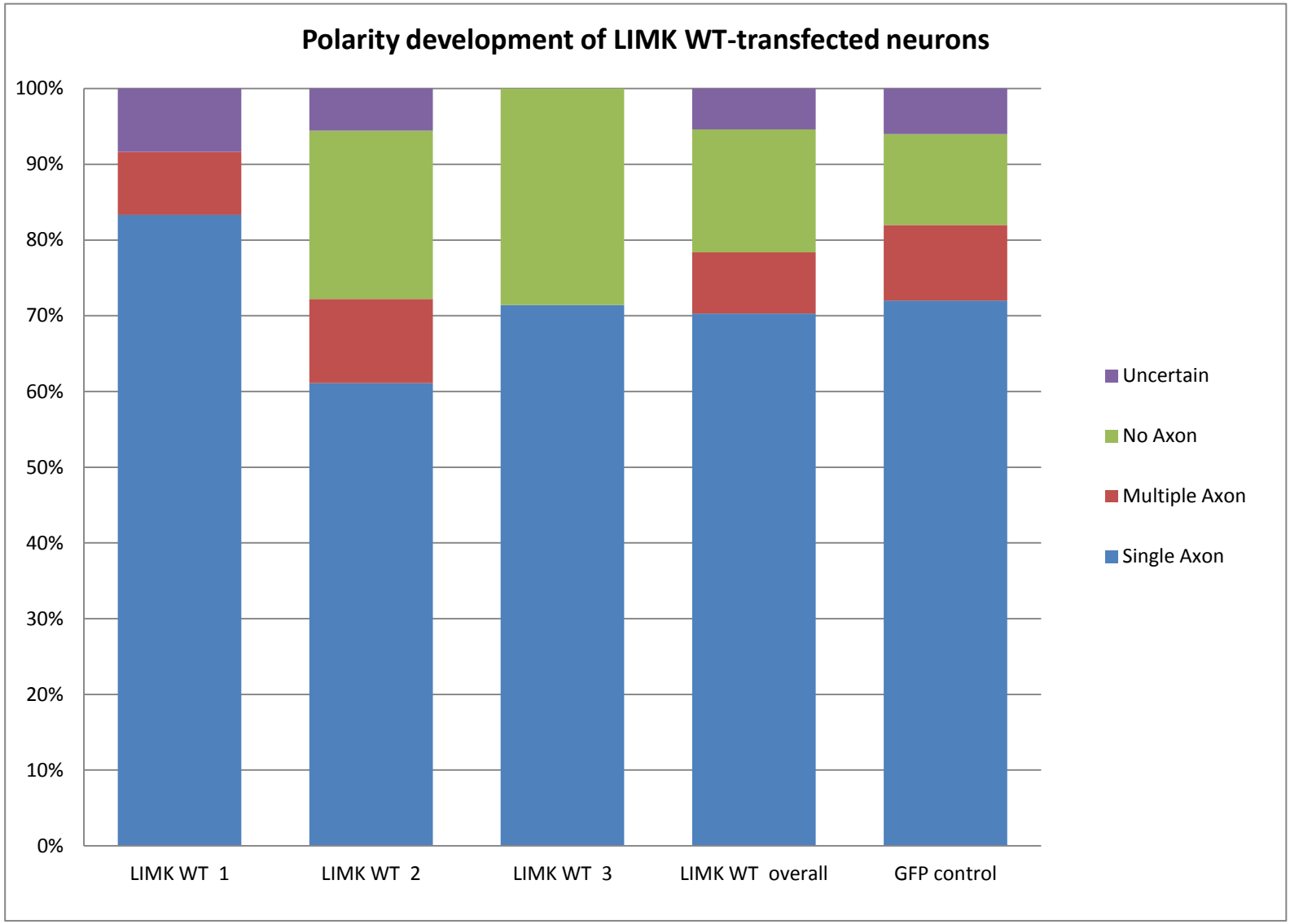
Appendix B: Comparison of polarity ratios between varied transfection conditions for LIMK-shRNA- and GFP-transfected neurons.



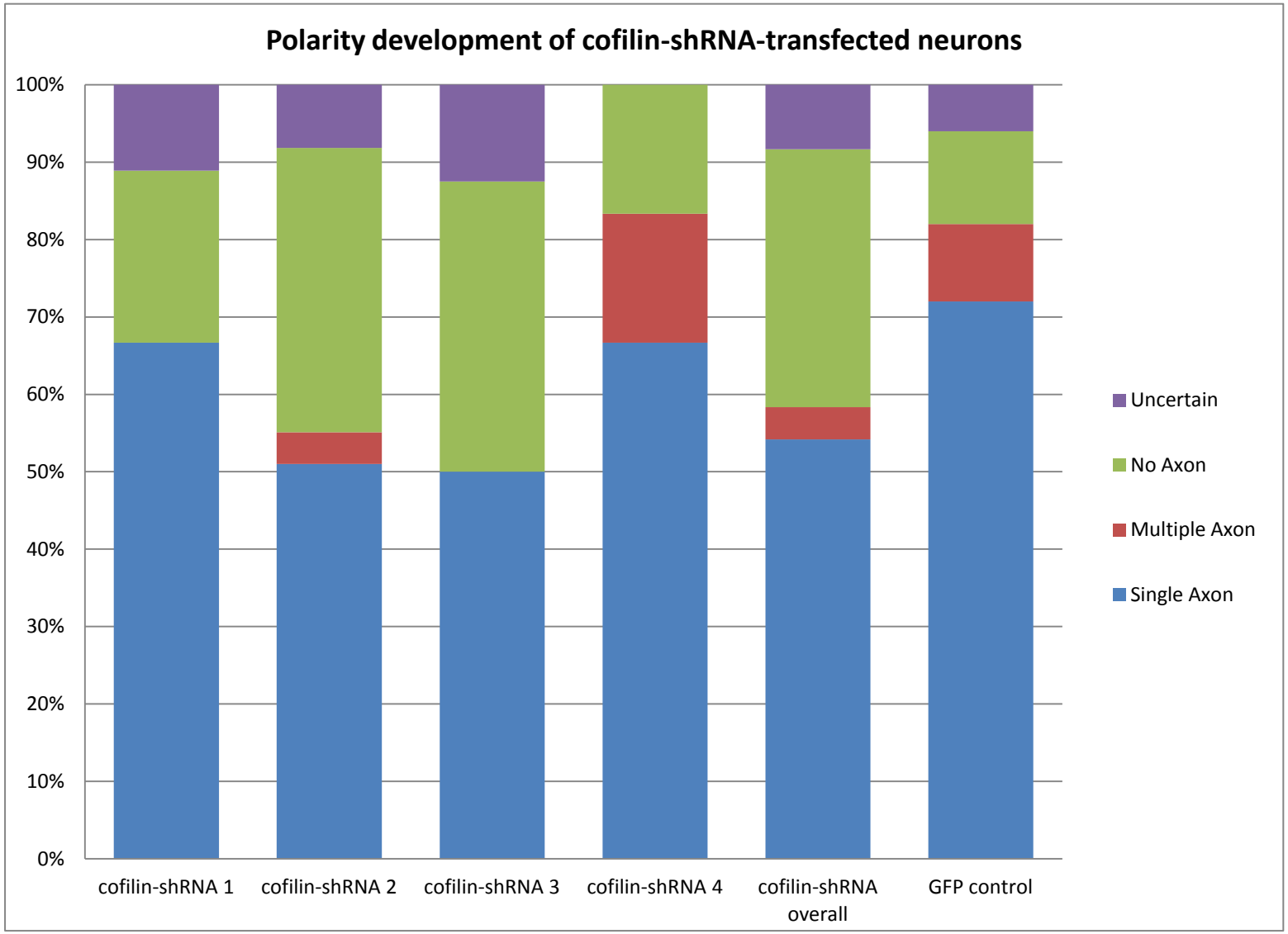
Appendix C: Polarity ratios for three trials of LIMK-shRNA-transfected neurons, an average of the trials (n=38), and the GFP control.



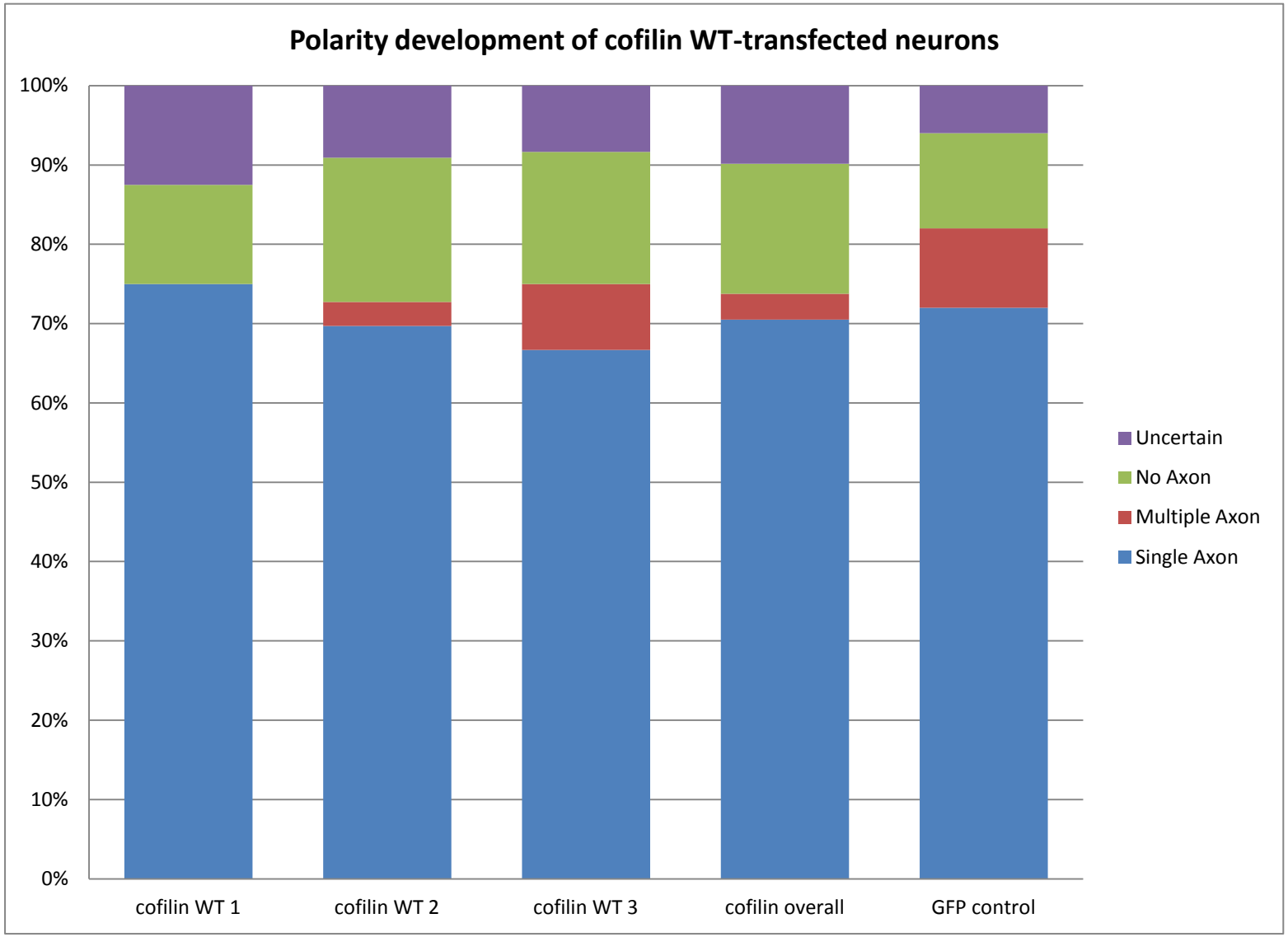
Appendix D: Polarity ratios for three trials of LIMK WT-transfected neurons, an average of the trials (n=37), and the GFP control.



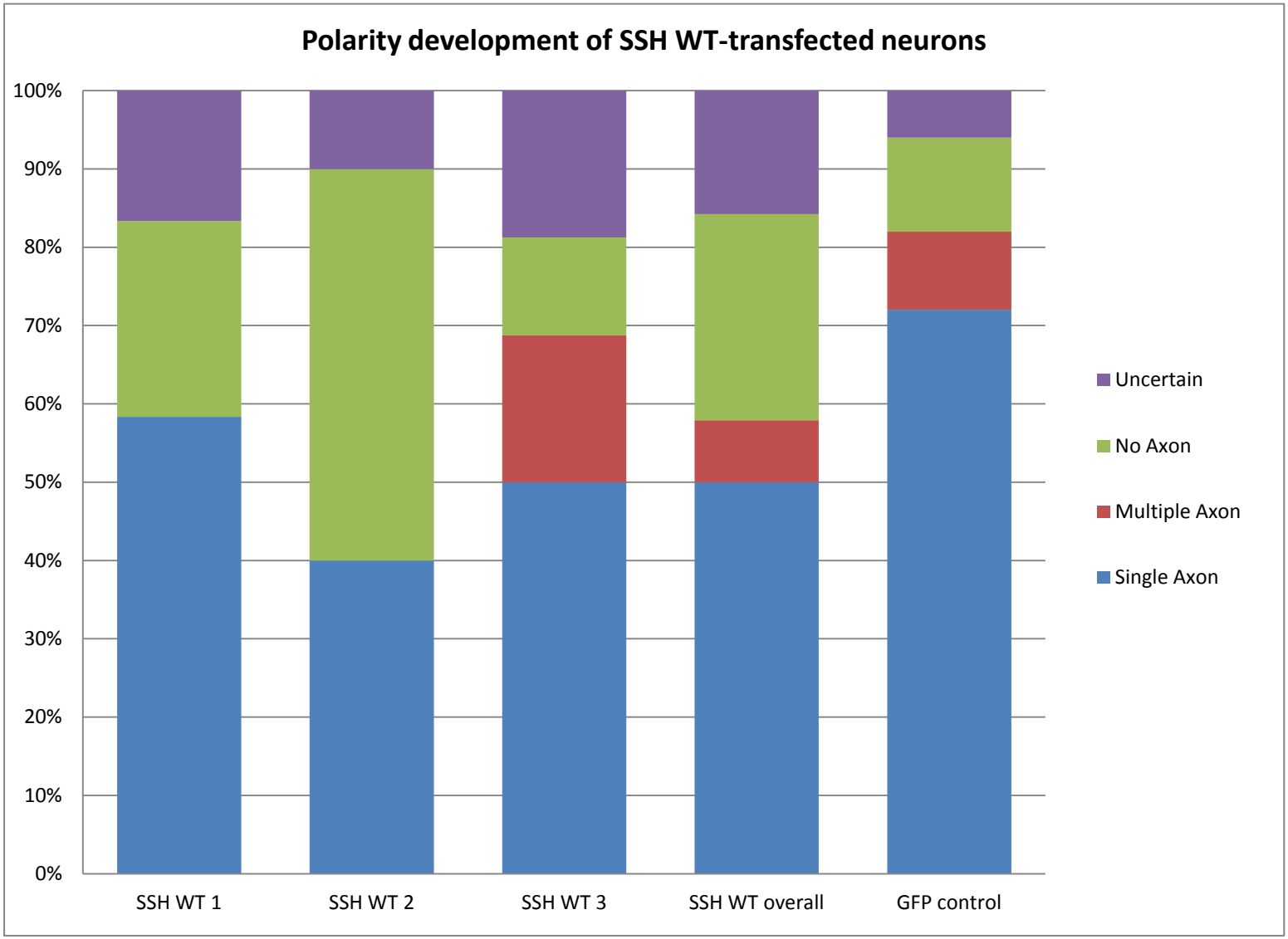
Appendix E: Polarity ratios for four trials of cofilin-shRNA-transfected neurons, an average of the trials (n=72), and the GFP control.



Appendix F: Polarity ratios for three trials of cofilin WT-transfected neurons, an average of the trials (n=61), and the GFP control.



Appendix G: Polarity ratios for three trials of SSH WT-transfected neurons, an average of the trials (n=36), and the GFP control.



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