UNIVERSITY OF CALIFORNIA SAN DIEGO

Transient sublethal excitotoxic stress and its connections to long term neurodegeneration in primary neurons.

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in

Biology

by

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2019

The Thesis of Jay Chitale is approved, and it is acceptable in quality and form for publication on microfilm and electronically:

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University of California San Diego

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Supplemental Figure 1 is coauthored with Jay Chitale and Huanqiu Zhang, and is used in this work with the permission of the coauthor.

ABSTRACT OF THE THESIS

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Master of Science in Biology

University of California San Diego, 2019

Professor Shelley Halpain, Chair
Professor Gulcin Pekkurnaz, Co-Chair

Short term brain injuries such as ischemic stroke and concussions have been identified as risk factors for the long-term development of neurodegenerative pathologies such as Alzheimer's Disease and chronic traumatic encephalopathy. These brain injuries are known to trigger a substantial release of glutamate, an excitatory neurotransmitter, into the synaptic cleft between neurons. The highly concentrated extracellular glutamate repeatedly activates post-synaptic receptors and induces an influx of cations into the post-synaptic neuron. This influx triggers a disruption of normal neuronal morphology and cytoskeletal structure, which may potentiate cell

death. We observe that dendritic microtubule dynamics are rapidly disrupted by repetitive rapid glutamate receptor activation, and the disruption is negatively correlated to previously described aberrant F-actin reorganization. We also observe that neurons exposed to a transient excitotoxic stress can recover certain cytoskeletal features following an extended period of recovery, but may develop pathological microtubule-associated-protein tau aggregates that are associated with the aforementioned long term neurodegenerative pathologies. This suggests that transient excitotoxic glutamate stress may be a link between traumatic brain injuries and the development of long term neurodegenerative disease.

I:

Introduction

An increase in extracellular glutamate occurs in several neuropathological states, such as stroke, epilepsy, and concussions, which are considered risk factors for developing late stage neurodegenerative pathologies including Alzheimer's disease and chronic traumatic encephalopathy (CTE) (Schaffert et. al., 2018) (Stern et. al., 2011). Glutamate is the primary excitatory neurotransmitter in the central nervous system that targets different types of post-synaptic receptors, which primarily fall under three categories: one group of G-protein coupled metabotropic glutamate receptors (mGluRs), and two main groups of ionotropic glutamate receptors called the AMPA (α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid) and NMDA (N-methyl D-aspartate) receptor subgroups (Meldrum, 2000). During normal stimulation events, the synaptic cleft usually contains 0.6 μM of glutamate, but significant trauma events can fill the synaptic cleft with upwards of 10 μM of glutamate, causing significant repetitive activation of glutamate receptors on the post-synaptic neuron and promoting neuronal death (Mark et. al., 2001).

Glutamate excitotoxicity is caused by an excessive release of glutamate from pre-synaptic neurons coupled with a failure of regulatory glutamate uptake channels in surrounding support cells. Glutamate can be released in excessive amounts due to rapid overexcitation or through the loss of membrane integrity of the pre-synaptic neuron (Mark et. al., 2001). Regulatory glutamate transporters on associated astrocytes may become dysfunctional in response to brain injury, which will maintain the excitotoxic concentration of extracellular glutamate (Danbolt, 2001) (Yi & Hazell, 2006). The AMPA and NMDA glutamate receptors contain ion channels that promote the influx of cations, such as sodium and calcium, when the receptors are activated. The continuous

ion influx creates a series of intracellular responses that promote cytoskeletal disruption and ultimately neuronal death (Hara & Snyder, 2007).

The effect of acute exposures to excitotoxic concentrations of glutamate can be studied *in vitro* by exposing cultured neurons to bath applied NMDA, a synthetic agonist specific for NMDA receptors, or glutamate, the endogenous neurotransmitter that activates both AMPA and NMDA receptors, as well as the mGluRs. Even short exposures have been shown to induce a loss of dendritic spines and mitochondrial dysfunction (Greenwood & Connolly, 2007) (Schinder et. al., 1996).

Furthermore, NMDA has been shown to disrupt the microtubules and microtubule associated proteins found in the dendrite (Hoskison & Shuttleworth, 2006) (Kapitein et al., 2011). Microtubules are hollow tube-like cytoskeletal elements comprised of several tubulin subunits, and undergo cycles of growth and depolymerization. Microtubules are polarized, and contain a dynamic region that is the site of growth and depolymerization, as well as a stable region that is less susceptible to these mechanics (Baas et. al., 2016). In neural dendrites, microtubules fill the dendritic shaft and provide structure and a platform for bidirectional transport mechanisms (Baas et. al., 2016). The disruption of dendritic microtubules is a major contributor to the dysfunction of the dendrite and ultimately neurodegeneration (Hoskison & Shuttleworth, 2006).

When stressed, cells are capable of mounting various protective responses to promote survival. Previously the Halpain lab has described a novel and rapid glutamate-induced filamentous actin (F-actin) accumulation in the dendritic shaft of dissociated hippocampal neurons, which we have dubbed “actinification” (Calabrese & Halpain, 2013; Calabrese et al., unpublished). Normally F-actin is enriched in dendritic spines and almost absent in the dendritic shaft. However, following glutamate stimulation, it polymerizes into straight bundles, with little to no branching,

in the somatodendritic region of neurons. The accumulation of F-actin in the dendritic shaft is formin mediated. Formins are a family of proteins which are known to interact with both F-actin and microtubules (Goode & Eck, 2007) (DeWard & Alberts, 2008). If specific formins are inhibited, actinification does not occur and this results in increased susceptibility to cell death, suggesting a pro-survival role of this early cytoskeletal response to stress (Calabrese & Halpain, unpublished).

When excitotoxic stress is removed, neurons have been shown to recover key cytoskeletal features. Previous studies, both by members of the Halpain lab and others, have demonstrated that neurons are able to recover certain key features following an acute excitotoxic stress. Studies by our lab have demonstrated that after an extended period of recovery, neurons are able to reverse actinification and restore a normal F-actin distribution (Calabrese & Halpain, unpublished). We have also observed microtubule associated proteins reappear in dendrites following an extended period of recovery (Supplementary Figure 1). Other studies have shown that dendritic swelling and mitochondrial dysfunction can be reversed following a transient excitotoxic stress as well (Greenwood & Connolly, 2007).

As stated above, a history of glutamate excitotoxicity-based pathologies such as ischemic stroke, epilepsy, and traumatic brain injuries are risk factors for developing neurodegenerative diseases such as CTE and Alzheimer's later in life. CTE and Alzheimer's disease are classified as tauopathies, because of the aberrant self-aggregation of microtubule associated protein (MAP) tau into soluble oligomers and insoluble filaments (Arendt et al., 2016). In tauopathies, tau monomers lose their affinity for axonal microtubules, and are hyperphosphorylated and aggregate into oligomers (Albayram et al., 2016). Oligomers are missorted into the somatodendritic compartment, and will continue to aggregate to form insoluble neurofibrillary tangles (NFTs). While NFTs were

originally considered to be the indicator of pathological tau, recent evidence has suggested that oligomeric tau is the driver of tau pathology (Gerson et. al., 2016). Aggregated tau is known to induce mitochondrial damage, inhibit axonal transport mechanisms, and cause synaptic dysfunction, which all contribute to cytotoxicity (Lasagna-Reeves et al., 2011) (Ward et al., 2012) (Lasagna-Reeves et al., 2011). In mechanisms that are still being studied, tau oligomers are able to induce neurodegeneration throughout the brain through prion-like transmission from cell to cell (Goedert et al., 2017). The connection between glutamate excitotoxicity and the development of these tauopathies is still unknown, but studies have shown that simulated traumatic brain injuries can induce tau oligomerization and long-term neurodegeneration *in vivo* (Hawkins et al., 2013) (Mouzon et al., 2014).

The goal of this thesis is to characterize the early and late stage cytoskeletal changes following a transient excitotoxic stress event. We explored the early changes to the dynamics of dendritic microtubules following an acute stress, and if these changes are connected to the previously described actinification. Our results indicate that dendritic microtubule dynamics are rapidly disrupted following an acute excitotoxic stress. We did observe that the microtubule dynamics of neurons that were rapidly actinified are less susceptible to disruption for a short time, although the nature of this relationship is still unknown. We also investigated the recovery of neuronal cytoskeletal features following an acute excitotoxic stress and determined if such a stress will promote tau aggregation. We determined that recovery of cytoskeletal features such as the distribution of F-actin was likely dependent on the intensity of the transient excitotoxic stress, even following an extended recovery period. We also observed that sufficiently intense excitotoxic stress events promoted substantial tau aggregation events. These findings may provide new

insights into the connection between transient brain traumas and long term neurodegenerative diseases.

II:

Results

All the experiments referred to below were either conducted with a U2OS (human osteosarcoma) cell line or primary rat hippocampal cultures. Rat hippocampi were dissected from embryos and plated on glass coverslips and allowed to grow to 21 days in vitro (DIV) to allow neurons to establish a mature morphology and functional synapses. For stress experiments, 50 μM NMDA was added to the cultures and left for the indicated duration of time before cells were treated with other drugs or fixed and processed for immunocytochemistry.

Microtubule dynamics are rapidly disrupted by NMDA-induced excitotoxicity

To evaluate the effect of glutamate excitotoxicity on the dynamics of dendritic microtubules, we treated dissociated hippocampal cultures with sublethal concentrations of NMDA for 5 minutes, 30 minutes, 1 hour, or 3 hours. In order to evaluate changes to the dynamic state of microtubules, cultures were then fixed and stained with antibodies against tyrosinated, detyrosinated and total tubulin. Tyrosinated tubulin is the original translated state of alpha-tubulin and is commonly associated with the dynamic region of microtubules that repeatedly undergo cycles of growth and depolymerization (Wloga & Gaertig, 2010). Post-translational modifications are usually made after tubulin subunits are incorporated into a microtubule. Detyrosinated tubulin is formed when the C-terminal tyrosine residue on tyrosinated α-tubulin is stripped by the detyrosinating enzymes vasohibin 1 and 2 (Alilaud C. et. al., 2017) (Nieuwenhuis J. et. al., 2017). Tubulin detyrosination is associated with stability, as the post-translational modification only occurs to tubulin subunits that have remained incorporated into sufficiently long-lived microtubules (Nieuwenhuis J. et. al., 2017). Using ImageJ, we quantified the changes to the

intensity of each tubulin staining in the proximal dendrites of neurons. The intensity measurements from each isoform staining were normalized to control and analyzed for statistical significance.

Our analysis revealed that tyrosinated tubulin intensity was significantly decreased within a mere 5 minutes of the application of NMDA (Fig. 1A). Tyrosinated tubulin was further depleted as NMDA stress continued (Fig. 1A). Detyrosinated tubulin and total tubulin intensity were affected by a comparatively smaller magnitude, compared to tyrosinated tubulin intensity at 5 minutes post-NMDA (Fig 1A). The intensity of both detyrosinated tubulin and total tubulin decreased at a similar rate in response to longer NMDA stress (Fig 1A). All tubulin forms continued to be depleted with longer exposure to NMDA, eventually reaching almost complete depletion after 3 hours of NMDA exposure (Fig 1A). Our results reveal that dendritic microtubule dynamics are rapidly disrupted after an acute excitotoxic stress, and that microtubules are then almost completely lost after an extended stress.

F-actin reorganization delays the rapid disruption of microtubule dynamics

Cultures were also stained with fluorescently tagged phalloidin to detect filamentous actin. Because we observed that microtubule dynamics are disrupted in a time frame similar to actinification following NMDA-induced stress (Calabrese & Halpain, 2014), we decided to investigate if there was a relationship between the two changes.

We analyzed the changes in intensity of tyrosinated tubulin in the context of whether the neuron was actinified or not. The analysis revealed that, after 5 minutes following the application of NMDA, actinified neurons suffered significantly less tyrosinated tubulin depletion following compared to those that were not actinified (Fig 2C). This correlation between actinification and decreased tyrosinated tubulin loss was not observed following longer NMDA stress events (Fig

2C). We did not observe any significant difference in the change to detyrosinated or total tubulin intensity based on whether or not the neuron was actinified or not (data not shown). These results indicate that neurons that rapidly actinify do not experience the same depletion of dynamic tubulin as those that do. This result shows that neurons that rapidly actinify display a delayed disruption of dynamic microtubules compared to neurons that do not rapidly actinify after a short excitotoxic stress.

Partial recovery of normal F-actin distribution following a transient excitotoxic stress

Now that we had further characterized the short term effects of NMDA on microtubules and actin filaments, we wanted to investigate the *recovery* of normal neuronal morphology after an acute excitotoxic stress event. Other studies have shown that neurons are able to recover various aspects of their cellular morphology following an acute excitotoxic stress (Loo & McNamara, 2006) (Hasbani et al., 2001). We have also observed that neurons are able to reverse actinification when allowed to recover following a transient stress (Calabrese B. & Halpain S., unpublished).

We exposed hippocampal neuron cultures to two different short NMDA exposure times (5 and 15 minutes) to mimic transient excitotoxic stress, and treated the cultures with a cocktail of NMDAR antagonists AP5, MK801 and CGP to block the activated receptors and halt the excitotoxic stress. For half of the treated cultures, we washed out both the NMDA and NMDAR antagonist with multiple washes using conditioned media from same-age hippocampal cultures. For the other half of the treated cultures, we did not wash cultures with conditioned media and allowed the NMDA receptor antagonists to remain. After an extended period of recovery (48 hours), cells were fixed and stained using fluorescently tagged phalloidin to label F-actin.

As expected, we saw that longer exposure to NMDA, on average, resulted in less actinification reversal than the shorter exposure to NMDA in both recovery treatments (Fig 3). We observed that the unwashed cultures contained a greater fraction of neurons that displayed normal F-actin distributions for both exposures to NMDA (Fig 3). Conditioned media washes did not induce actinification, but it did reduce the fraction of neurons that recovered normal F-actin distributions following a short NMDA stress and an extended period of recovery (Fig 3).

This experiment demonstrated the ability of neurons to reverse actinification and reacquire a punctated filamentous actin distribution that resembles the normal spine-enrichment seen in control conditions. This experiment also suggests that longer exposures to NMDA results in slower actinification reversal even following an extended period of recovery. No stressed condition fully recovered a normal F-actin distribution despite the extended period of recovery. These results may pave the way for new investigations on the potential of neurons to regain key cytoskeletal features following different intensities of a transient excitotoxic stress.

Tau Bimolecular Fluorescence Complementation is not a functional means of detecting tau aggregation following short excitotoxic stresses in neurons

We hypothesized that short excitotoxic stress events would promote tau aggregation after a long period of recovery. These tau oligomers are known to contribute to cell toxicity and can spread from cell to cell through prion-like transmission (Gerson J & Kayed R., 2014) (Shafiei et. al., 2017). Previous experiments have revealed that tau becomes hyperphosphorylated and missorted following a transient excitotoxic stress, suggesting that we may be able to detect self-aggregated tau under similar conditions (Supp Fig 1). We attempted to use a Tau Bimolecular Fluorescence Complementation (Tau BiFC) construct described by Tak and colleagues (2013) to

try to visualize the formation of tau aggregates. The system utilizes two full length tau constructs that each have half of a split-Venus protein. When the tau proteins are driven to aggregate, the constructs should align such that the split-Venus becomes whole and fluoresces (Tak et. al., 2013). We transfected hippocampal cultures with both Tau constructs, and stressed cultures using different short NMDA exposure times (5 and 15 minutes). We then allowed cultures to recover by treating them with the NMDA receptor antagonists AP5 and MK801 for 1 hour. We then removed both the NMDA and NMDA receptor antagonists by washing cultures with conditioned media, and fixed them after 24 hours. We did not observe any distinct aggregation after a transient NMDA stress followed by recovery using NMDA receptor antagonists and conditioned media washes (Fig 4). We did not observe the substantial change to the intensity of the construct in transfected neurons between stressed and unstressed conditions as predicted by Tak & colleagues (2013) (Fig 4).

We hypothesized that the lack of conclusive aggregation may have been due to potential interference by an unknown mechanism during cell fixation. The paper that described the construct did not use neurons and instead used HEK293 cells transfected with the constructs and the authors observed the aggregation using live imaging (Tak et. al., 2013). When we transfected human osteosarcoma (U2OS) cells and imaged them live, we observed rapid and noticeable aggregation after applying 3 μM vinblastine, a drug that induces tubulin precipitation and was shown in the original paper to induce distinct Tau BiFC aggregation (Fig. 5A), thereby confirming the observations of (Tak et. al., 2013) using non-neuronal cells. However, upon attempting the same live-cell imaging procedure in neurons, we were unable to demonstrate any aggregation of the Tau-BiFC constructs after exposure to vinblastine (Fig 5B) or NMDA (Fig 5C). Though some blebbing was seen, we observed no evidence of Tau BiFC aggregation in neurons. The transfected proteins appear to mislocalize into the dendrite in some neurons even with no stress applied,

suggesting that the regulatory mechanisms in neurons that normally prevent tau proteins from remaining in dendrites were not acting upon these recombinant tau proteins (Fig 5B, C). We concluded that Tau-BiFC is not a viable means of assessing tau aggregation in neurons, or that further assay optimization would be required.

Tau Oligomer Monoclonal Antibody reveals tau oligomerization in response to transient excitotoxic stress

Due to difficulties with our attempts to visualize the formation of pathogenic tau, we decided to use an antibody generously provided by the Kayed Lab of the University of Texas Medical Branch, which specifically binds to tau oligomers (Castillo-Carranza et. al., 2014). We assessed the formation of tau oligomers using this tau oligomer monoclonal antibody (TOMA) following a short excitotoxic stress.

We stressed hippocampal cultures with two different short NMDA exposure times (5 and 15 minutes), and allowed cultures to recover for an extended period (48 hours) following the application of NMDA receptor antagonists. The tau oligomer antibody generally appeared dim or lightly punctated in control neurons, but became bright after the stress-and-recovery treatment (Fig 6A). The analysis of the TOMA immunostaining indicated that short-term NMDA exposure induced an increase in tau oligomerization. Control neurons were nearly always negative for TOMA staining, while in NMDA treated cultures some neurons displayed a bright signal in the cell body. We observed variable staining patterns in cultures treated with NMDA, as some neurons contained several bright puncta throughout the cell body, while others displayed a more uniform appearance of densely packed puncta (Fig 6A). Though not statistically significant, there was a substantial increase in the fraction of neurons (approximately 30 percent) that displayed tau oligomers following the longer NMDA stress condition (Fig 6B). When we sorted neurons that

displayed tau oligomers based on the coefficient of variation, we did not observe any pattern in the prevalence of either aforementioned pattern across the tested conditions (Fig 6C). Further repeats of the experiment will be needed to confirm the increase in tau oligomerization following a short excitotoxic stress. However, these results suggest that transient but sufficiently intense excitotoxic stress can promote the delayed emergence of potentially pathological tau oligomers.

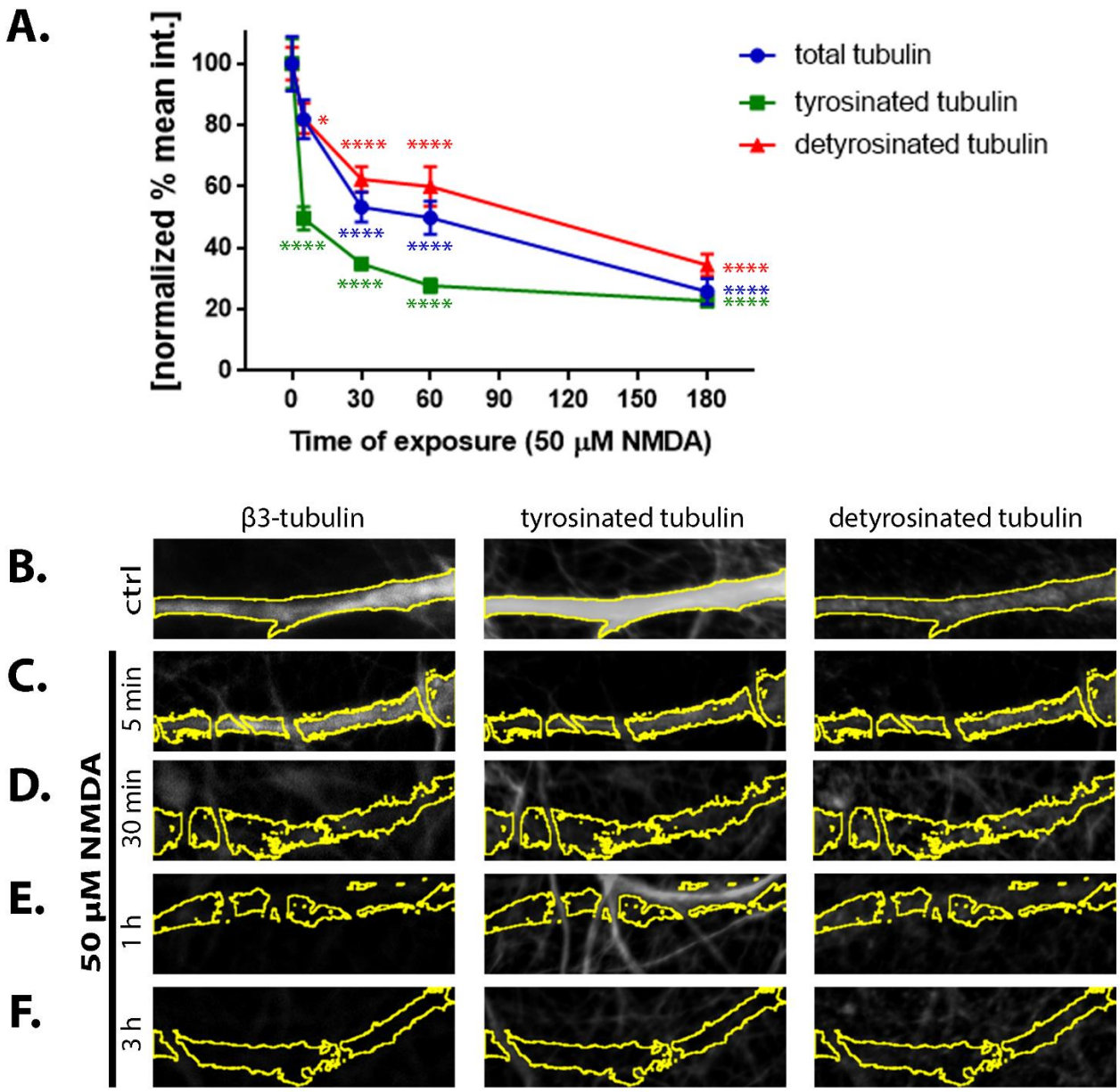


Figure 1: NMDA induces rapid decrease of dynamic tubulin followed by a slower depletion of stable tubulin. A. *Mean intensities were calculated and normalized to control values. Dynamic tyrosinated tubulin intensity decreased by 50% after only 5 minutes of while total tubulin and stable detyrosinated tubulin intensity decreased much slower.* ***B-F.*** *Representative images of proximal dendrite regions of interest in control (B), and after exposure to 50 μM NMDA for 5 min (C), 30 min (D), 1 h (E), and 3 h (F) demonstrate rapid loss of dynamic tubulin and slower loss of stable and total tubulin. Statistical analysis was conducted using ANOVA with Tukey's Post-hoc Test. * = $p < 0.05$. **** = $p < 0.0001$. n=3.*

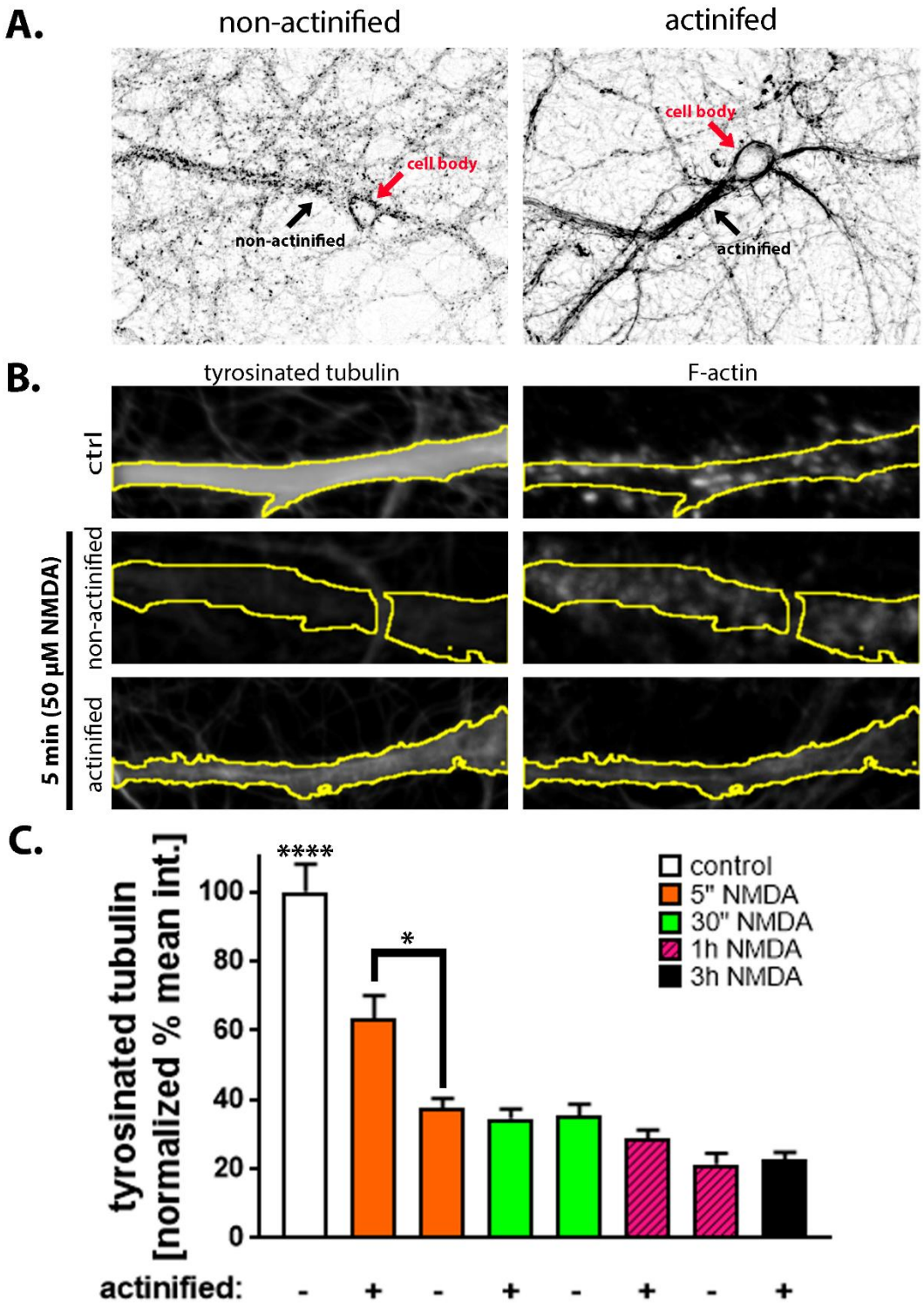


Figure 2: Actinification following NMDA exposure partly delays rapid loss of dynamic tubulin. A. *Images of actinified and non-actinified neurons provided for context.* ***B.*** *Representative images display delayed tyrosinated tubulin depletion* ***C.*** *Tyrosinated tubulin intensity values from Fig. 1A were sorted based on if the neuron was actinified or not actinified. Non-actinified neurons displayed a greater early depletion of dendritic dynamic tubulin compared to actinified neurons after 5 min exposure to 50 µM NMDA. Statistical analysis was conducted using ANOVA with Tukey's Post-hoc Test. * = $p < 0.05$. **** = $p < 0.0001$. n=3.*

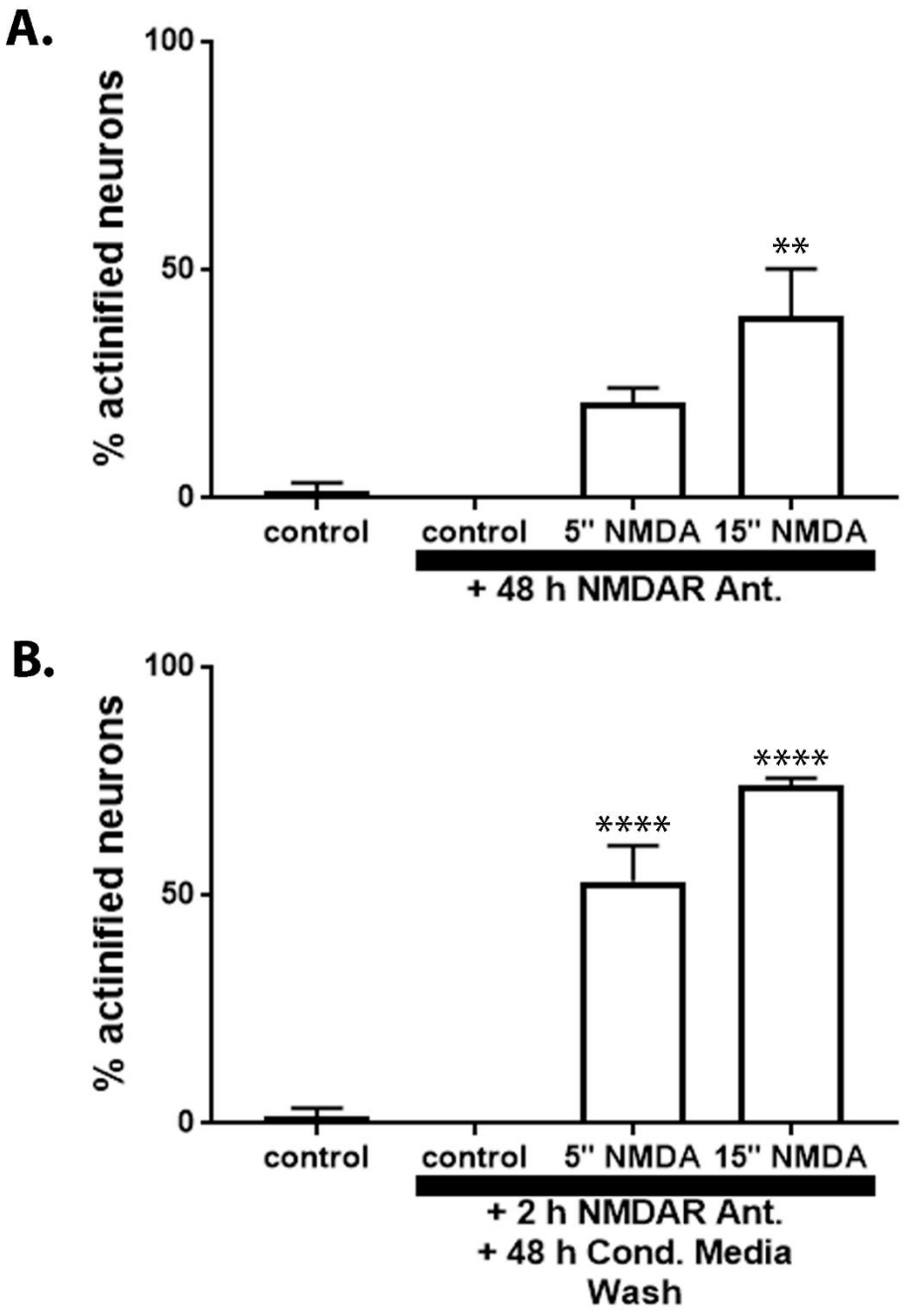


Figure 3: Actinification is persistent following a transient excitotoxic stress and extended period of recovery. **A.** *Neuron cultures were treated with 50 μM NMDA for 5 or 15 minutes. Cultures were then treated with NMDA Receptor Antagonists for 48 hours (A.) or for two hours before media was replaced with conditioned media from same-age cultures (B.). Cultures were fixed and stained for F-actin using phalloidin to evaluate the fraction of neurons that were actinified. Statistical analysis was conducted using a one-way ANOVA ($p<0.05$) with Tukey's Post-hoc Test. ** = $p < 0.01$, **** = $p < 0.0001$. n=2-4.*

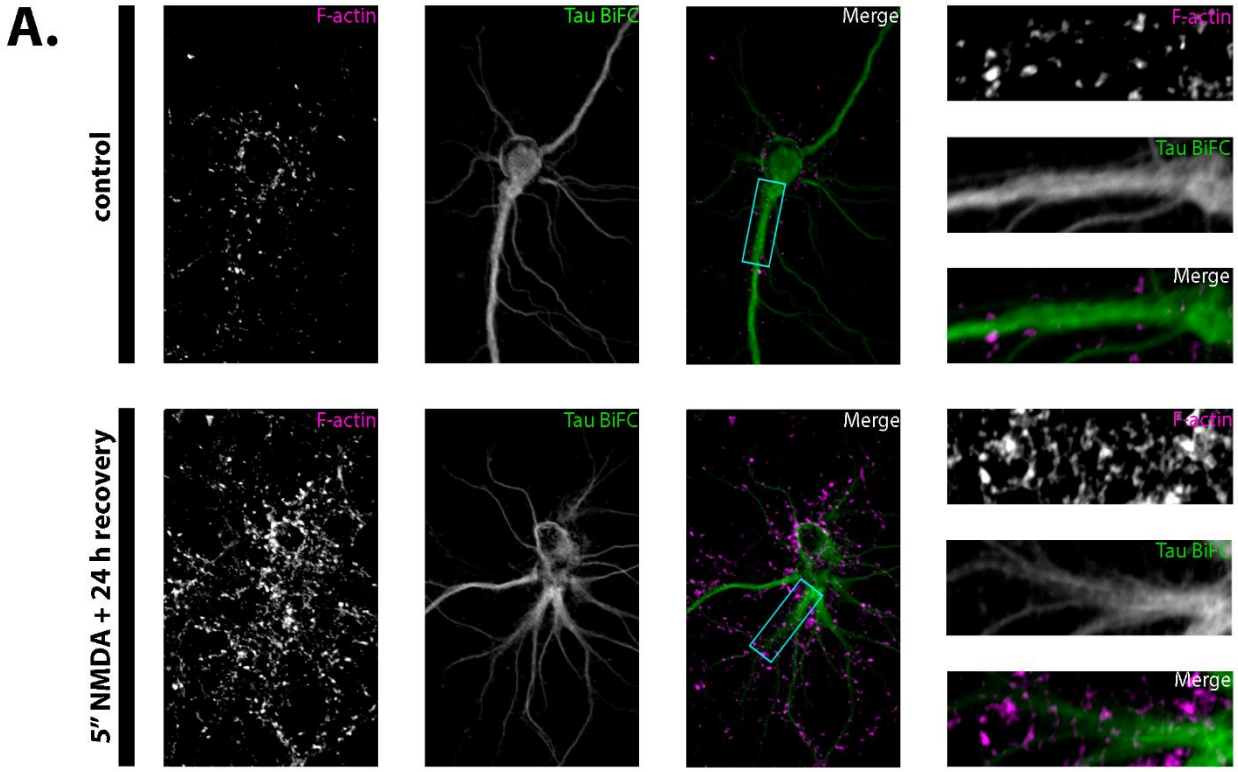


Figure 4: Tau BiFC is not a practical tool for assessing tauopathy in fixed neuronal cultures. A. *Hippocampal cultures were transfected with Tau BiFC constructs and then subjected to short NMDA stresses. Cultures were then treated with NMDAR antagonists (50 μM APV & 25 μM MK801) for 1 hour and washed with conditioned media and allowed to recover for 24 hours. The tau construct showed no significant changes to brightness and did not demonstrate aggregation as claimed by Tak and colleagues. The dendritic regions of interest demonstrate that Tau BiFC did not enter dendritic spines. n=1.*

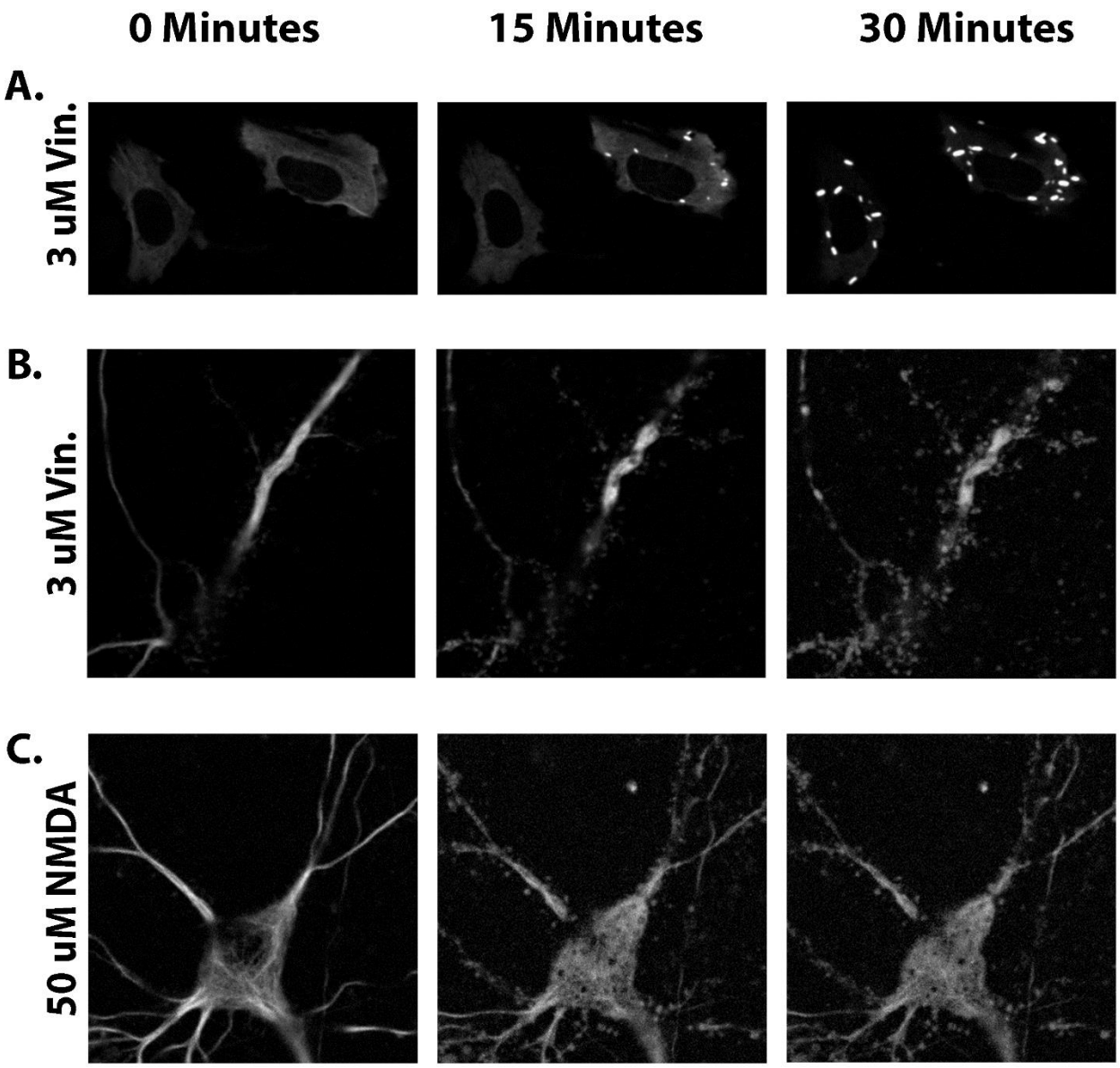
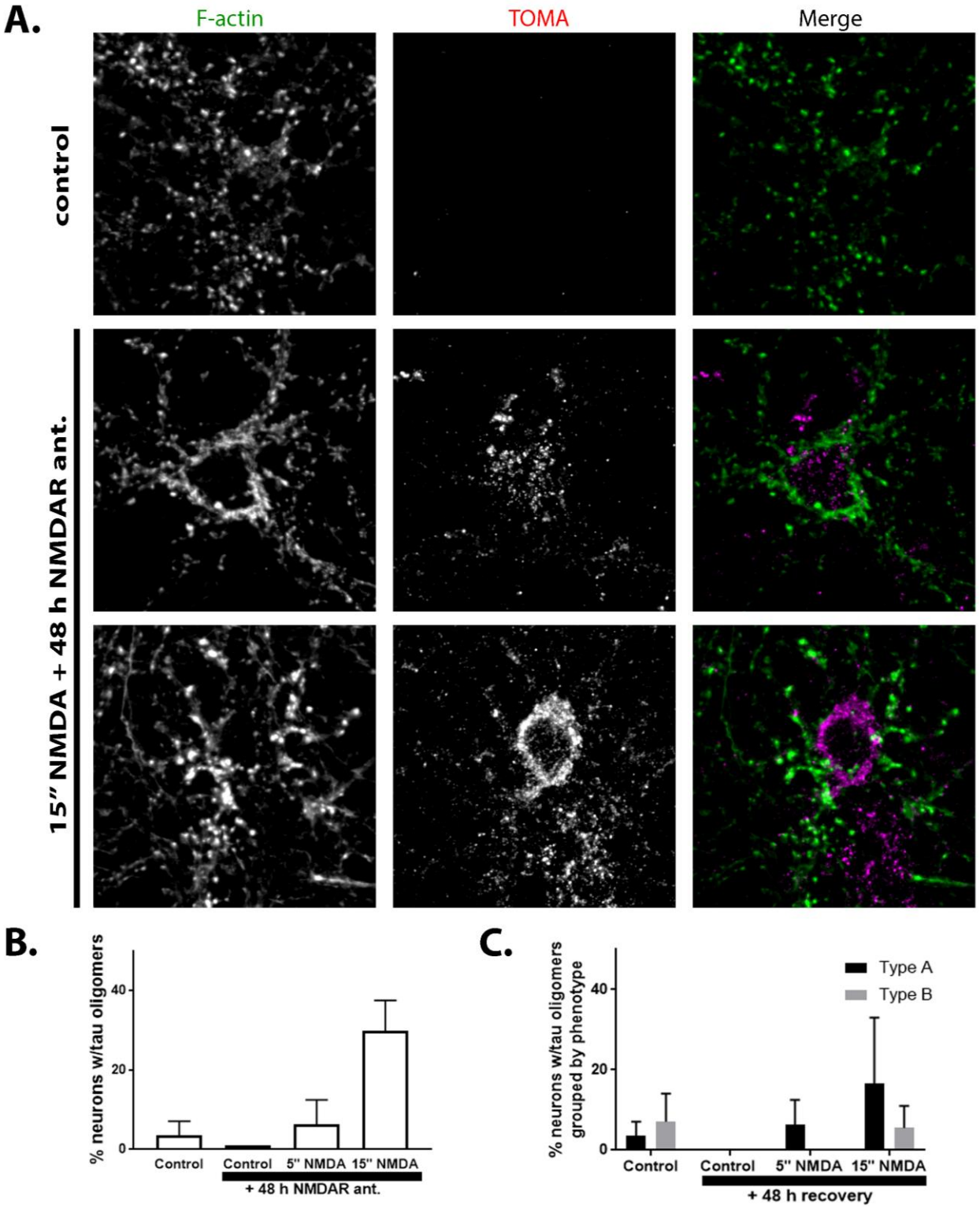


Figure 5: Live Imaging of Tau BiFC cells doesn't show expected aggregation events in neurons. A. *Tau BiFC was transfected into U2OS cells, and then treated with 3 μM Vinblastine. Within 30 minutes of treatment, Tau BiFC showed distinct aggregates.* ***B.*** *Tau BiFC was transfected into neurons, and then treated with 3 μM Vinblastine. Tau BiFC became cytosolic and blebs formed in the dendrite, but no distinct aggregation events seen in* ***(A)*** *were observed.* ***C.*** *Tau BiFC was transfected into neurons, and then treated with 50 μM NMDA. Tau BiFC became cytosolic, but no distinct aggregation events similar to* ***(A)*** *were observed.* ***n=1.***

Figure 6: Tau Oligomer Monoclonal Antibody (TOMA) reveals two distinct aggregation phenotypes following an acute excitotoxic stress. *Hippocampal neurons were stressed with 5 or 15 minutes of 50 μM NMDA and then allowed to recover for 48 h using NMDAR Antagonists, and then fixed and stained using Phalloidin (F-actin) and TOMA.* ***A.*** *Images representing the shift from a minimal TOMA signal to two distinct phenotypes post-stress and recovery. Type A (middle) was defined as highly punctated signal in the cell body, while Type B (bottom) was defined as having denser flatter signal around the cell body.* ***B.*** *Quantification of neurons that had tau oligomers present in their cell bodies. No significant pattern was detected, although the highest stress condition (15 min NMDA with 48h Recovery) showed the highest rate of TOMA aggregation.* ***C.*** *Quantification from (B) separated by subtype. Neurons displaying tau oligomers were sorted as Type A or Type B based on coefficient of variation. No significant pattern on the emergence of either phenotype was detected. Statistical analysis was conducted using a one-way ANOVA ($p<0.05$) with Tukey's Post-hoc Test. n=2.*


A.
F-actin
TOMA
Merge
control
15″ NMDA + 48 h NMDAR ant.
B.
% neurons w/tau oligomers
40
20
0
Control
Control
5" NMDA
15" NMDA
+ 48 h NMDAR ant.
C.
% neurons w/tau oligomers grouped by phenotype
Type A
Type B
40
20
0
Control
Control
5" NMDA
15" NMDA
+ 48 h recovery

IV

Discussion:

This study had two primary objectives. The first was to examine the short term changes to the dynamics of microtubule in response to an acute excitotoxic stress, and explore a potential relationship between changes to microtubule dynamics and actinification. We observed that the rapid disruption of dynamic microtubules following an acute excitotoxic stress is potentially attenuated by actinification. The second was to explore the effects of different durations of transient excitotoxic stress on the potential for neurons to recover cytoskeletal features and to promote the generation of pathological tau oligomers after a long recovery period. We have witnessed that, despite the recovery of other cytoskeletal elements, sufficient durations of transient NMDA exposure promotes tau oligomerization, which may provide new insight into the connection between brain injury-induced glutamate excitotoxicity and long term neurodegenerative disease.

Dendritic microtubule dynamics are rapidly disrupted by NMDA receptor overactivation

Numerous studies have demonstrated the rapid disruption or redistribution of several cytoskeletal elements following an acute glutamate excitotoxic stress. These substantial changes to the cytoskeleton of afflicted neurons are largely attributed to the influx of calcium ions through activated NMDA receptors. Calcium is known to disrupt microtubules, and has been shown to be a driver of dendritic disruption following an acute excitotoxic stress (O'Brien et al., 1997) (Hoskinson & Shuttleworth, 2007). To investigate changes to microtubule dynamics, we bath-applied NMDA for various durations before fixation. In order to assess the microtubules and microtubule dynamics, we stained the cultures using antibodies against tyrosinated (dynamic),

detyrosinated (stable), and β3 (total) tubulin (Wloga & Gaertig, 2010). Our experiments regarding the short term changes to the dynamics of microtubules following an acute excitotoxic stress reveal that microtubule dynamics are rapidly disrupted within 5 minutes of the application of NMDA. This rapid depletion of dynamic tubulin is not reflected by a similar depletion of total tubulin, suggesting that dendritic dynamic tubulin makes up a small fraction of the total mass of dendritic microtubules. The rapid disruption of the dynamic fraction of microtubules is supported by studies that show a disruption of microtubule associated proteins associated with the dynamic end of microtubules in a similar timeframe following an acute excitotoxic stress (Kapitein et al., 2011) (Halpain & Greengard, 1990).

Our analysis of the changes to microtubule dynamics in the context of changes to the actin cytoskeleton revealed that neurons that actinified within 5 minutes of bath applied NMDA experienced significantly less dynamic microtubule depletion. We did not observe a significant difference in the total microtubule or stable microtubule intensity between actinified and non-actinified neurons. We cannot yet conclude that actinification is somehow able to protect dynamic microtubules against rapid NMDA-induced disruption. It may be possible that neurons that have more dynamic microtubules are more likely to rapidly actinify in response to an acute excitotoxic stress.

However, it should be noted that these experiments were performed on fixed cultures, and we have not yet developed or found an effective assay to detect the dynamic state of microtubules during live imaging for us to conclusively determine the order in which these changes occur. As there have not been other studies investigating a relationship between actinification and microtubule dynamics, the nature of the relationship between them remains unknown. Further

experiments will need to be conducted in order to understand any potential connections between the two phenomena.

Neurons display tau aggregation events despite the recovery of other cytoskeletal features.

Although glutamate excitotoxicity is considered to be a risk factor for the development of long term neurodegenerative tauopathies, the mechanistic link between these two pathologies remains largely unknown. Studies have demonstrated the development of tau oligomers *in vivo* following simulated traumatic brain injuries (Hawkins et al., 2013), but the molecular driver of tau oligomerization is not known. While we have not determined the molecular driver of this tau oligomerization in traumatic brain injuries, this work has shown preliminary results that suggest that sufficient durations of NMDA receptor overactivation promote tau oligomerization. This simplified model will assist us in investigating the molecular basis to the formation of pathological tau oligomers following a transient excitotoxic stress. The development of oligomeric tau following a transient excitotoxic stress may be the currently unknown link between brain injuries and long term neurodegeneration.

We have observed that some neurons are able to partially recover certain cytoskeletal elements following a transient excitotoxic stress. In particular, we observed that some, but not all, neurons recover a punctated F-actin distribution, suggesting the recovery of dendritic spines and potentially synaptic connections. Other studies have shown that neurons are capable of recovering dendritic spines and synaptic connections following a transient excitotoxic stress (Hasbani et al., 2001), but the basis by which some neurons fail to recover a punctated F-actin distribution while others do is not yet known. We did not identify any correlation between the recovery of F-actin distributions and the formation of tau oligomers, although other studies have shown that tau

interferes with synaptic recovery (Bi et al., 2017). Further experiments may reveal that oligomerized and missorted tau decreases the rate of actinification reversal.

Looking Forward

We would like to continue to investigate the potential connection between actinification and changes to the dynamics of microtubules following a transient excitotoxic stress. Formins, which promote the conversion of soluble G-actin to F-actin, have been shown to regulate microtubule stability by preventing microtubule turnover and promoting the addition of stabilizing post-translational modifications (Fernandez-Barrera & Alonso, 2018). INF2, a formin that our lab has determined to be necessary for actinification, also binds to and stabilizes microtubules. INF2 converts G-actin into F-actin, and through the work of an associated complex of other proteins, promotes microtubule stabilization by capping the microtubule's dynamic (+) end and preventing tubulin turnover. Depletion of the G-actin population also promotes the addition of stabilizing post-translational modifications to the incorporated tubulin, such as lysine-40 acetylation (Fernandez-Barrera & Alonso, 2018). Actinification may reduce the depletion of dynamic tubulin through the secondary action of formins. Further experiments may reveal formins as the mechanistic link between actinification and decreased dynamic microtubule loss following an acute excitotoxic stress.

To investigate the long term changes to neurons following a transient excitotoxic stress, we will continue to assess tau oligomerization and actinification reversal following different durations of transient excitotoxic stress events and different durations of recovery. To assess the true potential of neuronal recovery, we will also examine synaptic potential and cell death following both singular and repeated transient excitotoxic stress events. Many studies investigating

the connection between brain injuries and long term neurodegeneration have suggested that mild repeated traumatic brain injuries can also contribute to the development of long term neurodegeneration (Prins et al., 2013) (Mouzon et al., 2014). A comprehensive study could enhance our understanding of the resilience of neurons following various excitotoxic stress events and the likelihood that they will develop potentially toxic tau oligomers. This information could lead to new treatments and preventative measures to attenuate the long term effects of short term brain injury.

V:

Materials and Methods:

Ethics Statement

This study was performed in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. The Institutional Animal Care and Use Committee (IACUC) of the University of California San Diego specifically approved this study under protocol #S07290. All surgical procedures were terminal, and deep anesthesia with isoflurane was used to prevent any pain or discomfort.

Cell Cultures

Hippocampi were removed from embryonic day 18 (E18) Sprague Dawley rat brains, then treated with 9U/ml papain (Worthington) for 20 min, followed by trituration with a pipette. Dissociated neurons were plated on Poly-L-lysine-coated 12-mm cover glasses at a density of 100 cells/mm2 and maintained in neurobasal medium (NB; 21103-049, GIBCO), supplemented with B27 (17504-044, Invitrogen) and 0.5 mM L-glutamine (G7513, Sigma). U2OS human cells were maintained in high glucose DMEM media with 10% fetal bovine serum in Petri dishes, and were split regularly when cells became 70% confluent. For experiments, U2OS cells were plated on glass coverslips.

Cell Culture Transfection

Hippocampal cultures were transfected at 21 days in-vitro, while the U2OS cells were transfected before reaching 100% confluency. Cultures were transfected with 1.5 μg of the two Tau BiFC constructs (a gift from Kim Lab, Korea Institute of Science and Technology) by treating

cultures with Lipofectamine 2000. Cultures were transfected two days before pharmacological treatment and live-imaging or fixation.

Drug Treatments

All drugs were purchased from Tocris Bioscience. For cell stress experiments, 50 μM NMDA was bath-applied to hippocampal cultures (21 DIV) for various times before fixation. 3 μM vinblastine was applied to test the efficacy of the tau Split-Venus reporter from Tak et al., 2013. For experiments examining the recovery of cytoskeletal features following an acute excitotoxic stress, cultures were treated with a cocktail of NMDA receptor antagonists: 100 or 50 μM APV, 25 μM MK801, and 1 μM CGP (Tau oligomer and actinification reversal experiments only). Some cultures were washed after NMDA receptor antagonist treatment using the conditioned media from sacrificed hippocampal cultures of the same age.

Immunocytochemistry

For experiments investigating changes to microtubule dynamics and actinification, hippocampal cultures were fixed and permeabalized in a solution of 3.7% paraformaldehyde, 0.15% glutaraldehyde, 2% sucrose and 0.1% Triton X-100. Cultures were then rehydrated in 1 mg/mL sodium borohydride and then blocked in a solution of 2% BSA, 2% FBS, and 0.2% fish gelatin in 1X PBS.

For other experiments, hippocampal cultures were fixed in 3.7% formaldehyde in phosphate buffered saline (PBS) with 120 mM sucrose for 15 minutes at room temperature. The hippocampal cultures were permeabalized with 0.1% Triton X-100 in PBS and blocked with 2% bovine serum albumin (BSA) in 1X PBS.

Neurons were incubated with rat anti-Tyrosinated Tubulin (1:200; BIO-RAD), rabbit anti-Detyrosinated Tubulin (1:100; EMD Millipore), K9JA (1:300, Dako), chicken anti-MAP2 (1:500, Abcam), mouse TuJ1 (1:100; Neuromics), AT100 (1:100, Invitrogen), or TOMA1 (1:300; gift from the Kayed Lab) overnight at 4° C. Cultures were then washed 3 times with PBS and incubated with Goat anti-Rat Alexa Fluor 488, 568, 647 (1:300; Invitrogen), Goat anti-Rabbit Alexa Fluor 405, 488, 568, or 647 (1:300; Invitrogen), Goat anti-Mouse Alexa Fluor 405, 488, 568, or 647 (1:300; Invitrogen), as well as Phalloidin 405, 488, 568, or 647 (1:250; Biotium) for 45 minutes at 37° C.

Cultures were then mounted on Twin Frost™ glass slides using Lerner Laboratories Aqua-Mount™, and imaged or preserved at 4° C.

Image Acquisition

Images were acquired as a z-series (11 sections at 0.2 μm each) with an Olympus IX70 confocal optical microscope using a 60 ×1.4 NA Plan APO oil immersion objective. Coverslips were imaged in a grid without overlapping fields of view. Images were taken and reconstructed using Metamorph (Molecular Devices).

Time lapse imaging of transfected cultures was acquired as a z-series in fixed time intervals on a Nikon Ti-E microscope with perfect focus system (Nikon) and an iXon X3 DU897 EM-CCD camera (Andor Technology plc) using a 60 ×1.4 NA Plan APO oil immersion objective. Cultures were maintained at 37 degrees and 5% CO_2 during live imaging. Time lapse imaging was captured using Metamorph (Molecular Devices).

Image Analysis

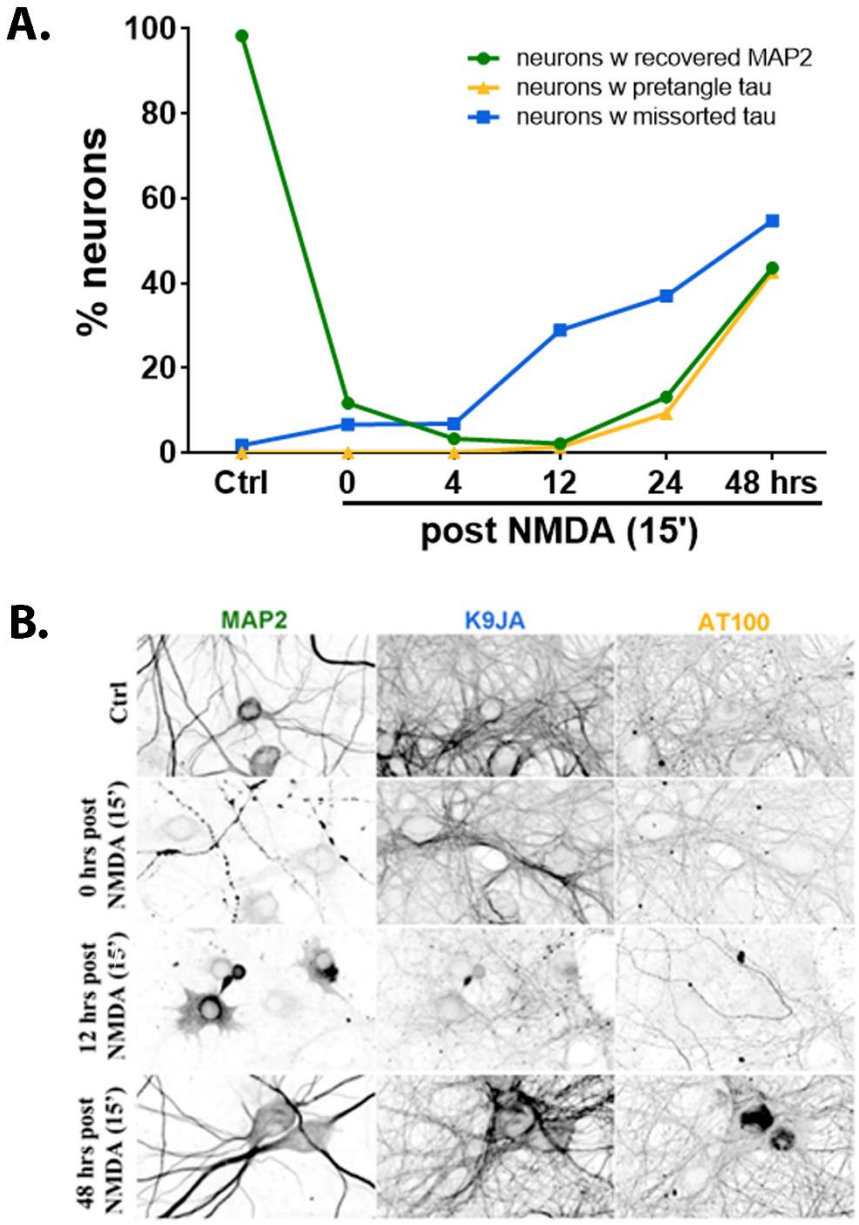
Images were analyzed using NCBI's ImageJ (Fiji). For experiments investigating changes to tubulin intensity, fixed size regions of interest of the proximal dendrite that excluded the cell body were analyzed. Masks were generated to select the dendrite, and the intensity of all tubulin subtypes were measured.

For experiments investigating actinification, neurons were classified as actinified or not actinified based on the signal displayed by the phalloidin staining.

For experiments involving the formation of tau oligomers, neurons that displayed a bright TOMA signal within their cell body above a preset threshold were counted as having tau oligomers. Neurons containing tau oligomers were sorted by phenotype based on the coefficient of variation. The coefficient of variation was calculated as the standard deviation of the intensity divided by the mean intensity in the cell body.

Graphs and Statistics

Graphs were generated using GraphPad Prism 7. Statistical analysis was done using an ANOVA ($p<0.05$) with a Tukey post-hoc test as calculated by GraphPad Prism 7. Values have been presented as mean ± SEM. Specific statistical test results are detailed in figure legends.



Supplemental Figure 1: Neurons recover certain cytoskeletal features but show hyperphosphorylated and missorted tau following a transient excitotoxic stress. *Neurons were treated with a transient (15 min) exposure to 50 μM NMDA and allowed to recover for a various amount of time in 100 μM AP5 (NMDAR antagonist) before fixation.* ***A.*** *Line graph demonstrates the fraction of neurons that show somatodendritic MAP2 immunoreactivity, as well as fraction that show missorted tau (K9JA antibody) and hyperphosphorylated tau (AT100 antibody).* ***B.*** *Representative images demonstrate the recovery of MAP2, the missorting of tau (K9JA) and the hyperphosphorylation of tau (AT100). n=1. This figure was coauthored by Jay Chitale and Huanqiu Zhang, and is used with the co-author's permission.*

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