Title

Functional Consequences of Stress : Impact of in vitro Stress Exposure on Tau Phosphorylation, Aggregation, and Axonal Transport

Permalink

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Publication Date

2014

Peer reviewed|Thesis/dissertation

UNIVERSITY OF CALIFORNIA, SAN DIEGO

Functional Consequences of Stress: Impact of *in vitro* Stress Exposure on Tau
Phosphorylation, Aggregation, and Axonal Transport

A thesis submitted in partial satisfaction of the requirements for the degree Master of
Science

in

Biology

by

Michelle H. Le

Committee in charge:

Professor Robert A. Rissman, Chair
Professor Roberto Malinow, Co-Chair
Professor Brenda Bloodgood

2014

The thesis of Michelle H. Le is approved, and it is acceptable in quality and form for publication on microfilm and electronically:

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Co-Chair

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Chair

University of California, San Diego

2014

DEDICATION

To my parents: without them, none of this would have been possible.

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ABSTRACT OF THE THESIS

Functional Consequences of Stress: Impact of *in vitro* Stress Exposure on Tau
Phosphorylation, Aggregation, and Axonal Transport

by

Michelle H. Le

Master of Science in Biology

University of California, San Diego

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Stress is implicated as a contributing factor in Alzheimer's Disease (AD).

Individuals prone to psychological distress as a consequence of stress exposure are at

three times greater risk of AD than age-matched controls. Hyperphosphorylation of tau is

a key event leading to AD neurofibrillary tangles, which positively correlates with

cognitive decline. Because tau is the primary stabilizing protein of microtubules (MTs), it

is thought that tau phosphorylation (tau-P) and aggregation causes dissociation of tau on

MTs, leading to MT destabilization and disruption in axonal transport. We have shown

that restraint stress induces hippocampal tau-P in rodents, a process that involves

corticotropin-releasing factor receptor type 1 (CRFR1), with repeated exposure showing

increased hippocampal tau-P in insoluble aggregates. Although these data provide a

potential link between chronic stress and AD tauopathy, the impact of stress-induced tau-

P on neuronal function is still unclear. To test the hypothesis that stress-induced tau-P

causes dissociation of tau from MTs and dysfunction in axonal transport, we developed a

neuronal *in vitro* system to analyze how modulation of tau by stress hormones impact cell

viability and axonal transport of cargoes. Mouse hippocampal neurons were exposed to

stress-system intermediates: CRF, corticosterone (cort), or control for 0-24 hours. CRF

and cort showed increased tau-P over the timecourse examined. We also observed

activation of specific tau kinases. We examined characteristics of axonal transport of

mitochondria and BDNF. Collectively, our results suggest that stress alters cellular

processes and CRFR1 antagonism may be a useful therapeutic strategy for AD tauopathy

and neuronal dysfunction.

INTRODUCTION

Stress has been associated as a contributing factor in the development of Alzheimer's disease (AD), a neurodegenerative disorder that results in dementia and cognitive decline. With growing population and increase in average life-expectancy, dementia is leading to a greater dependence and disability burden worldwide. In 2010, 35.6 million people worldwide are affected with dementia, with numbers expected to double in 2020 (Prince et al., 2013). With increasing life-expectancy, the social and financial impact on families affected is prominent. Healthcare cost for Americans was $203 billion in 2013 and is projected to increase 500% to reach $1.2 trillion by 2050 (Assoc, 2012). Current treatments for AD only provide temporal relief, with no clear method to attack the underlying cause of the disease. With only a small portion of AD cases is age-related, much is to be discerned about environment as a contributing factor.

Hallmarks of AD: Amyloid Precursor Plaques and Neurofibrillary Tangles

AD is not entirely understood, however, molecular and cellular changes associated with the disease pathology have been described. AD is characterized by the presence of extracellular deposits of amyloid plaques and intracellular neurofibrillary tangles. Extracellular amyloid deposits arise from cleavage of amyloid precursor protein (APP) to form beta amyloid (AB) and are believed to be neurotoxic to the surrounding neurons.

Tau is a microtubule associated protein (MAP) involved in stabilizing microtubules. Tau plays a critical role in establishing and maintaining neuronal structure

polarity, and transport. Normal phosphorylated tau is present at 2-3moles/mole of protein, but hyperphosphorylation in AD is at 7-10 molar ratio (Kenessey & Yen, 1993; Kopke et al., 1993). Interneuronal neurofibrillary tangles (NTFs) are caused by the hyperphosphorylation of the microtubule-associated protein tau. Hypersphosphorylated tau is thought to have reduced ability to stabilize microtubules and thus form insoluble paired helical filaments (PHFs) that comprise NFTs. The occurrence of NFT have a positive correlation with cognitive deficit and neuronal loss (Arriagada, Growdon, Hedley-Whyte, & Hyman, 1992; Gomez-Isla et al., 1997). Although the general function of tau is established, the functional impact of hyperphosphorylated tau on neuronal function is still unclear.

Stress and Tau Phosphorylation

AD is acquired both in a sporadic and familial form, with sporadic thought to be a result of environmental factors, age, and gender. With less than 1% of cases is a result of genetics, other contributing factors to AD pathology are being explored. Although gender and age are primary risk factors for developing AD, environmental factors also play a critical role. Of the environmental factors being investigated, stress has been indicated as a potential contributor to AD pathology. Epidemiological studies have shown that individuals more susceptible to "psychological stress" have are at a three time greater risk of AD (Wilson et al., 2003). In rodents, acute exposure to potential stressors has shown to induce rapid and reversible tau phosphorylation (Rissman, Lee, Vale, & Sawchenko, 2007). The site of stress-induced tau phosphorylation has been reported consistently

within the hippocampus, a site important for learning and memory and the initial site of tau pathology seen in AD (Rissman, 2009).

CRF Signaling Pathway

Corticotropin releasing factor (CRF) is a hypothalamic neuropeptide that governs the endocrine arm of the stress response. CRF primarily acts on the anterior pituitary, binding to G protein-coupled receptors (CRFRs) that activate adenylate cyclase (Chang, Pearse, O'Connell, & Rosenfeld, 1993; Chen, Lewis, Perrin, & Vale, 1993; Potter et al., 1994; G. W. Smith et al., 1998; Vita et al., 1993). CRFR1 is also expressed dispersedly in the brain, specifically in AD-related regions such as the isocortex, hippocampus, and amygdala (Van Pett et al., 2000). Signaling of the anterior pituitary by CRF results in the secretion of glucocorticoids, another important component of the stress response.

Cortical CRF immunoreactivity is reduced and hypothalamic expression is increased early in AD progression (Davis et al., 1999). Glucocorticoids have been linked increased levels with age to neuronal vulnerability in the hippocampus (Sapolsky, Krey, & McEwen, 1985). These levels in AD patients are elevated compared to age-matched controls (Landfield, Blalock, Chen, & Porter, 2007) and those treated with glucocorticoids show increased cognitive dysfunction (Aisen et al., 2000). While glucocorticoids are implicated in AD (Sapolsky, et al., 1985), stress-induced tau phosphorylation can occur in the absence of steroid increase, indicating that other components of the stress axis, namely CRF and signaling through CRFRs, play a integral role (Korneyev, Binder, & Bernardis, 1995; Rissman, et al., 2007). In rodent studies, acute stress exposure induces hippocampal tau phosphorylation and activation of tau

kinases, a response dependent on signaling through CRFR1 (Rissman, et al., 2007). Repeated stress led to increases in tau phosphorylation and a shift towards more insoluble forms (Rissman et al., 2012). Although these changes indicate the impact of stress exposure on tau phosphorylation, it is unclear the consequences of the changes in tau solubility on neuron functionality.

In this study we developed an in vitro platform to explore and elucidate how the modulation of tau by stress impacts axon transport and its relevance to tau pathology in AD. To understand *in vitro* stress exposure, a timecourse of stress-induced tau phosphorylation and tau kinase activation was established. We hypothesized that CRF will activate tau kinases and induce tau phosphorylation in the system. Transport of mitochondria and brain-derived neurotropic factor (BDNF) was examined under stress conditions. We also hypothesized that stress-induced tau phosphorylation leads to dissociation of tau from microtubules and subsequent destabilization of microtubules and impairment of axonal transport.

This thesis, in part, is currently being prepared for submission for publication of the materials. Le, Michelle; Wu, Chengbiao; Rissman, Robert. The thesis author was the primary researcher and author of this material.

MATERIALS AND METHODS

Cultures

Wild type timed-pregnant C57BL/6J mice were purchased from Charles River Labs. Primary hippocampal cultures were generated from embryonic day 18.5 pups. Dissection buffer contained HBSS, HEPES, and antibiotics. Isolated hippocampi were incubated in dissection buffer and 1X Trypsin (Life Technologies) for 20 minutes at 37° and 5% CO_2. Hippocampi were titrated with 0.05% DNAse I (Roche) in plating media cells of Neurobasal (Life Technologies) supplemented with 10% heat-inactivated FBS, 1% glutamax (Life Technologies), 2% B27, and 1% Penicillin Streptomycin (Life Technologies). Dissociated neurons were plated in pre-coated poly-D-lysine plates at a density of 1-1.5 $\times$ 10^6 cells mL^{-1} for mass cultures, and 200,000 cells mL^{-1} for immunocytochemistry. Cells were grown for approximately 7 days in culture before treatment to maintain maximum viability. Plating media was replaced by maintenance media (neurobasal, 1% glutamax, 2% B27) 2 hours after plating. Two-thirds of the medium was replaced every other day.

In vitro Stress

Stress ligands corticotropin-releasing factor (CRF; Bachem), corticosterone (cort; Sigma-Aldrich) or appropriate vehicles were reconstituted in a water and ethanol, and administered to the cultures in low (1uM CRF, 200ng cort) or high(10uM CRF, 400ng cort) concentrations. Upon treatment, half of the media was replaced with fresh maintenance media and treatments. Cells were treated for 0, 0.5, 2, 4, 6, 8 and 24hrs for initial characterization experiments.

Western Analysis

After cultures were grown for approximately 7 days, cells were treated with the high or low concentrations of stress hormones at interval timepoints. After treatment, cultures were put on ice, washed with cold phosphate buffered saline (PBS), collected and centrifuged at 1000g for 5 minutes at 4°C. Cells were homogenized in radioimmunoprecipitation assay (RIPA) buffer and protease inhibitors PMSF, NAF, and a protease inhibitor cocktail (Thermoscientific). Protein was boiled in sample buffer containing LDS and BME for 10 minutes at 70°C. Protein was electrophoretically separated on 10% bis-tris polyacrylamide gel and transferred to 0.2 μm PVDF membrane (Bio-rad). Membranes were incubated in primary antibody overnight at 4°C followed by HRP-linked secondary antibodies (1:1000) for 1 hour and developed with an enhanced chemiluminescence Western blot detection kit (Thermoscientific). Intensity readings from Western blots were acquired using ImageJ and analyzed using GraphPad Prism.

Immunocytochemistry

Hippocampal neurons were grown on poly-D-lysine coated coverslips with a density of 200,000 cells mL^{-1} as previously described. After about 5-7 days, cultures were treated with the respective stress ligand. Cultures were then washed with cold PBS and fixed with 4% paraformaldehyde for 10 min, quenched with 0.1M ammonium chloride, permeabilized with 0.1% triton-100 in PBS for 10 minutes, and blocked with 1% bovine serum albumin in PBS for 30 minutes. Cultures were incubated with the primary antibody overnight in 4°. Coverslips were then washed and incubated with anti-Mouse-igG Alexa conjugates (1:600) for 60 minutes at room temperature. After washing with PBS, the

coverslips were incubated with DAPI (1:10000) for 10 minutes at room temperature. Coverslips were then air-dried and mounted with CC/mount for imaging. Images were acquired using microscope (Leica DFC310 FX).

Antibodies

Quantitative Western analyses and immunocytochemistry were performed to analyze tau-P at the N- and C-terminal sites as a function of treatment—S^{202}/T^{203} (AT8; 1:1000; Thermo Scientific) and $S^{396/404}$ (1:1000; PHF-1; gift from Dr. P. Davies, Albert Einstein College of Medicine, Bronx, NY). Activation states of kinases involved in tau phosphorylation were also studied. Antibodies with specific phosphorylation sites or activator proteins of tau kinases were used. Kinases assessed include glycogen synthase-3β (GSK3 β, 1:1000, Santa Cruz Biotechnology, Santa Cruz, CA), activated GSK-3β (GSK-3β, 1:1000, BD Biosciences), inactive GSK-3β (pS^9, 1:1000, Cell Signaling Technology, Danvers, VA), phosphorylated c-Jun-*N*-terminal kinase (JNK, 1:1000, Cell Signaling Technology), mitogen-activated protein (MAP) kinases [extracellular signal-regulated kinases 1 and 2(ERK1/2); 1:500; Cell Signaling Technology], cyclin-dependent kinase 5(cdk5; 1:1000; Santa Cruz Biotechnology), and cdk5 activator proteins, p25 and p35 (1:1000; Santa Cruz Biotechnology). β-actin (1:1000; Sigma-Aldrich) was used as a protein loading control.

Axonal Transport Studies

Neurons were cultured according to the protocol previously described. 450nm microfluidic chambers (Xona Microfluidics) were used to separate axons from cell bodies in hippocampal cultures. Axons were imaged using an inverted epifluorescent microscope (Leica). During imaging, cultures were kept in a 37° chamber with 5% CO_2.

Time-lapsed images were collected using Metamorph 7.0 (Universal Imaging Corporation). Phase and fluorescent images were acquired to ensure consistent neuron health from before and after time-lapse images were taken. ImageJ software (NIH) and Metamorph 7.0 were used to generate movies and kymographs for analysis.

For mitochondria studies, cells were treated with stress ligands and subsequently with 2.5uM Mitotracker (Invitrogen) for 30 minutes. 60 second, 1 frame per second videos of mitochondria in the distal axons were collected. For BDNF studies, maintenance media was replaced with fresh Neurobasal for one hour prior to treatment. Cell bodies were treated and subsequently with 8mg/ml of BDNF conjugated with QD655 (Invitrogen) for 45 minutes and subsequently administered to the axon side. 60 second, 2 frames per second videos of BDNF in the distal axons were collected.

This thesis, in part, is currently being prepared for submission for publication of the materials. Le, Michelle; Wu, Chengbiao; Rissman, Robert. The thesis author was the primary researcher and author of this material.

RESULTS

Timecourse of *in vitro* Stress-Induced Tau Phosphorylation

To understand *in vitro* stress response, a timecourse of stress-induced tau phosphorylation was examined (Figure 1). Western analysis was used to examine tau phosphorylation at the C- terminal site, PHF-1, in extracts cultured from hippocampal neurons (Figure 1A). Compared to control, CRF and cort-treated neurons show significant spikes in phosphorylation in early timepoints (0, 0.5h) and stabilized from 4-24h (Figure 1B). From timepoints and concentrations selected from preliminary studies, fluorescent immunostaining of cells were acquired to examine the distribution of phosphorylated tau at PHF-1 in neurites. Control cultures showed consistent distribution of PHF-1 along neuron projections(Figure 1C). However, increased globular accumulations localized in neurites were seen in cells treated with CRF or cort (Figure 1D, Figure 1E). These results indicate that stress-ligands induces tau phosphorylation.

Upstream Mediators: Impact of Stress Peptides on Tau Kinases

Several tau kinases were examined to distinguish potential mediators of stress-induced tau phosphorylation. Insoluble RIPA extracts were used to investigate the activity of these tau kinases over a timecourse of 0-24h treated with low and high CRF (1uM, 10uM) or cort (200ng, 400ng).

At low treatments of CRF, only the β-subunit of total GSK showed a significant increase concurrently with tau phosphorylation (Figure 2E, Figure 2F). The α-subunit of active GSK-3 (pY216) significantly increased compared to control, however, a decrease

across the timecourse (Figure 2A, Figure 2B). Inactive GSK-3 was only significantly increased at 0.5h for the β-subunit (Figure 2C, 2D). At high treatments of CRF, both subunits of total GSK-3 was significantly increased at 0h, where tau phosphorylation was also increased (Figure 2E, Figure 2F). Active GSK-3 was significantly greater than control for both isoforms at 8h and for only the α-subunit at 4h(Figure 2A, Figure 2B). Inactive GSK-3 had significant increases for the α-subunit at 2h and the β-subunit at 0h(Figure 2C, 2D).

At low treatments of cort, the α-subunit of total GSK-3 showed significant increase at 0h and 2h, but not the β-subunit (Figure 2E, Figure 2F). However, both isoforms of active GSK-3 did not show any significance (Figure 2A, Figure 2B). Inactive GSK-3 showed increased levels of the α-subunit at 8h and the β-subunit at 2h and 4h(Figure 2C, Figure 2D). At high treatments of cort, both isoforms of active GSK-3 was significantly increased at 0h. Both isoforms of active GSK-3 were significantly increased at 2h (Figure 2A, Figure 2B). Inactive GSK-3 did not show any significance (Figure 2C, Figure 2D). Overall significant changes in GSK-3 levels paralleled with the tau phosphorylation timecourse (Figure 1) indicate GSK-3 plays a role in tau phosphorylation.

At low treatments of CRF, both subunits of JNK (pJNK54 and pJNK46) showed significant levels only at 24h (Figure 3A, Figure 3B). At high treatments of CRF, only pJNK46 showed significant levels at 4h and 24h. With significant increases at 24h for both low and high treatments, this suggests that JNK plays a role in stress induced tau phosphorylation.

At low treatments of cort, both isoforms of JNK showed significant levels at 4h and 24h. High treatment of cort resulted in both isoforms of JNK to be significant at 2h. With significant increases in tau low treatments of cort are seen at 0h and 0.5h, and high treatment of cort at 8h, these results also suggests that JNK plays a role in stress induced tau phosphorylation.

At low treatments of CRF, ERK1 was significant at 0h, 4h, and 24h, and ERK2 was significant only at 2h (Figure 4A, Figure 4B). At high treatments of CRF, both ERK1 and ERk2 showed significant levels at 0h, 0.5h and 24h. However, only the α-subunit showed increase at 2h. At low treatments off cort, only ERK2 was significant at 2h. However, at high treatments of cort, both isoforms showed significant increase at 0.5h. Additionally, ERK1 showed significant levels at 0h and 24h, and ERK2 at 4h.

Changes in cdk5 and its activator, P35, were also examined (Figure 5, Figure 6 respectively). cdk5 only showed significant levels at low treatments of CRF(Figure 5A, Figure 5B). cdk5 levels were significant at 0h for both low and high treatments of cort. P35 levels of low CRF-treated showed significance only at 24h, and at high treated only at 4h (Figure 6A, Figure 6B). Low cort treatment was significant at 4h. High cort treatment was significant at 0.5h and 2h. These data does not show consistency with P35/cdk5 relationship or tau phosphorylation timecourse.

<u>Impact of Stress-induced Tau Phosphorylation on Mitochondrial Transport</u>

Previously showing that stress induced phosphorylated tau can accumulate in neurites as globular aggregates (Figure 1D, Figure 1E), we examined the effects that this

might have on the movement of mitochondria (Figure 7). Movement of mitochondria was examined under conditions selected from preliminary studies (0.5h and 2h at 10uM CRF and 400ng cort). At 0.5h treatments, CRF treated neurons showed significant increase of average velocity of mitochondria, whereas cort-treated decreased. However, 2h treatments showed the opposite effect, with CRF treated showing decreased mitochondrial velocity and cort-treated showing increased velocity. Contradictory to the decreased velocity, 2h CRF had significant increase of the average distance the mitochondria travelled. No significance changes were found in density or movement of mitochondria for 0.5h and 2h treatments. These results indicate a stress effect on the movement of mitochondria, with longer exposure having more of a slowing effect on transport.

Impact of Stress-induced Tau Phosphorylation on BDNF Transport

The crucial roll BDNF plays in neuronal development, functional support, and neuronal plasticity leads us to examine the effects of stress-induced tau-P on BDNF transport (Figure 8). Movement of QD-BDNF was recorded for 2frames/sec and was plotted on kymographs for analysis. W measured distance travelled, velocity, and directionality, and further organized the results into direction relative to the cell body (anterograde, retrograde, stationary). QD-BDNF was examined in neurons treated with 10uM CRF, 400ng cort, or vehicle control for 0.5h and 2h.

In CRF-treated neurons, distance travelled was significantly greater than control at 0.5h, showing to be significant in retrograde moving QD-BDNF (Figure 8A). Consequently, retrograde velocity was significantly increased at 0.5h treatment. However,

2h treatment with CRF showed an opposite effect. Significant decreases in average velocity and distance travelled of QD-BDNF was apparent, with retrograde velocity significantly reduced. Changes in direction were also clear, with increased anterograde transport, decreased retrograde transport, and increased stationary QD-BDNF.

In cort-treated neurons, significant increases in distance travelled, retrograde distance travelled, and velocity were examined at 0.5h treatments (Figure 8B). At 2h treatments, significance was shown in average distance travelled; however only anterograde distance travelled was significantly increased. These results suggest an immediate response (0.5h) and long term effect (2h) of stress-induced tau phosphorylation.

This thesis, in part, is currently being prepared for submission for publication of the materials. Le, Michelle; Wu, Chengbiao; Rissman, Robert. The thesis author was the primary researcher and author of this material.

DISCUSSION

We developed an in vitro neuronal model system to analyze stress-induced tau phosphorylation on axon transport. We established a timecourse of tau phosphorylation for two stress ligands integral to the stress response. Although tau kinase activity was apparent upon stress treatments, inconsistencies in these levels made it difficult to identify an intermediate that could be responsible for the stress response. Establishing that stress ligands induce sustained elevation of phosphorylated tau and globular accumulations within neurites, we demonstrated the functional impact of transport of mitochondria and BDNF.

Timecourse of Tau Phosphorylation

Tau serves to stabilize microtubules to maintain structure, polarity, and support in neuronal transport. Hyperphosphorylated has reduced ability to stabilize microtubules (Iqbal et al., 1986; Yoshida & Ihara, 1993). Many studies indicate that hyperphosphorylated tau could lead to axon transport defects (Morfini et al., 2009). Our previous work established that repeated stress exposure resulted in elevated phosphorylated tau levels and the presence of insoluble tau aggregates. This finding suggests that under chronic conditions, hyperphosphorylated tau forms aggregates, and under long-term stress, forms filaments and possibly contributes to tangles we see in AD(Rissman, et al., 2007; Rissman, et al., 2012). Similar to our previous study, our results demonstrate that stress induced tau phosphorylation occurs in our *in vitro* model in a similar manner in rodents (in vivo). Both CRF-treated and cort-treated neurons were observed to have significant tau phosphorylation for both low and high treatments,

indicating that CRFR1 is a critical component to the stress response. The occurrence of apparent globular accumulations of phosphorylated tau found in stressed neurons may indicate that tau aggregates cause physical barriers and subsequent dysfunction in transport.

Timecourse of Upstream Mediators

Along with tau phosphorylation, tau kinases were concurrently monitored to identify potential key effectors. GSK3 is known for phosphorylating tau at several sites and reducing its ability to bind microtubules (Jope, Yuskaitis, & Beurel, 2007). GSK3B over-expression has shown to induce tau hyperphosphorylation seen in AD(Lucas et al., 2001). It is activated and inhibited by phosphorylation at Y216 or S9, respectively (Frame & Cohen, 2001). In our previous study, GSK was identified as an intermediate involved in stress induced tau phosphorylation, however it is unclear if GSK-3 can be identified as a key mediator of this *in vitro* system. Similarly, all other kinases examined (JNK, ERK, cdk5, P35) showed robust activity to stress treatments but inconsistencies compared to the tau timecourse. The inconsistency renders it unclear what upstream kinase mediates *in vitro* tau phosphorylation. Although our biochemical data did not distinguish a clear modulator, we believe that this is potentially due to the types of assays used. We hypothesize that more sensitive methods that involve protein arrays and/or document activity of kinases may prove more reliable for these types of analyses.

Axon transport of Mitochondria and BDNF

Beading of phosphorylated tau and varicosities along axons in stress-induced mice were observed in our previous work, which is similar to that seen in dystrophic neurites pathology in AD, suggesting structural alterations (i.e. blockages) and transport dysfunction. It is believed that defects in transport of important vesicles contribute towards neurodegeneration in some diseases (Stokin & Goldstein, 2006). Phosphorylated tau accumulations have been seen to block transportation of organelles, mitochondria, and other vesicles in neurons (Stamer, Vogel, Thies, Mandelkow, & Mandelkow, 2002). In our study, we examined the transport of mitochondria and BDNF in hippocampal axons. From the established tau-stress response, we chose optimal conditions from preliminary studies to evaluate the impact of stress on axon transport.

Mitochondria are concentrated in areas of high demand of ATP—pre and post synaptic terminals and growth cones of neurons. With the dynamic nature of neurons, mitochondria must be able to rapidly redistribute to different areas in order to meet increased energy demands. Neuronal function have high energy demands to support mobilization of synaptic vesicles, actin assembly and disassembly, and generation of membrane potentials. Mitochondria are transported to activated synapses in response to levels of ATP: velocity increases in areas of high ATP and decrease in areas of depleted ATP (Sheng & Cai, 2012). Earlier changes in velocity seen under stress indicate neurons eliciting an immediate stress response. Movement of mitochondria likely increased due to higher energy demands in the cell body and synaptic terminal. Mitochondria velocity later decreased likely because of the increase in accumulation of phosphorylated tau, resulting in blockages within axons. Models of overexpressed tau have also illustrated

blockages of microtubule tracks and reduction of transport of vesicles and cell organelles (Mandelkow, Stamer, Vogel, Thies, & Mandelkow, 2003). Blockage of vesicles and cell organelles implies depletion of crucial supplies to the cellular processes. Although not examined in this study, tau has also shown to inhibit movement of motor proteins (Ebneth et al., 1998; Trinczek, Ebneth, Mandelkow, & Mandelkow, 1999). The regulation of these motor proteins by tau could also have a substantial impact on changes in mitochondria movement. Ultimately, it is clear that physical blockage by tau aggregation and dysfunction of motor proteins by tau would likely also decrease mitochondrial velocity, results in the depletion of local ATP, oxidative stress, and interruption of neuronal function.

BDNF, an important neurotropic factor critical to neuronal survival, development, network connectivity, and formation of synaptic connections, is found concentrated in the cerebral cortex and hippocampus (Dugich-Djordjevic et al., 1995). BDNF has neuroprotective benefits demonstrated in many animal models of AD (Nagahara et al., 2009). Chronic stress conditions and AD have shown decreases BDNF expression levels in the dentate gyrus and hippocampus (M. A. Smith, Makino, Kvetnansky, & Post, 1995). Tau pathology is common component of chronic stress and AD, but it is unclear what effects these have on BDNF sequestration.

In this study, we investigated the effects of stress-induced tau phosphorylation on BDNF transport. At the same parameters as the mitochondria study, changes in the direction of movement were apparent in early and later stress responses. Early treatments showed increased velocity, travel distance, and number of mitochondria moving in the

anterograde movement, suggesting the need for BDNF in the soma as a response to stress. However, in the later stress response, an opposite effect is seen. The impact of the stress response on decreased velocity, travel, distance, and number of mitochondria moving in the retrograde direction suggests impairment of transport along the axon. With the accumulation of tau protein due to acute stress, these results have suggested that the phosphorylated tau destabilizes the cytoskeleton and affects axonal transport by blocking the trafficking of important cargoes. The impact on axon transport could impact BDNF availability, resulting in decreased BDNF seen in chronic stress and AD studies.

Our study shows that stress-induced tau phosphorylation occurs in a similar manner to that seen in our *in vivo* study and form phosphorylated tau aggregates along neurites. Although many kinases showed increased levels, we could not identify a specific upstream kinase mediator and suggest alternate detection methods to assess kinase activity. Stress-induced tau phosphorylation ultimately interfered with axon transport of mitochondria and BDNF, which lead to impairments of transportation of cargoes, depleted energy supply, and oxidative stress that contribute to AD. Continued exploration of the circuits and mechanism of stress induced tau phosphorylation would allow us to further uncover the relationship between stress and AD tau pathology.

This thesis, in part, is currently being prepared for submission for publication of the materials. Le, Michelle; Wu, Chengbiao; Rissman, Robert. The thesis author was the primary researcher and author of this material.

Figure 1. Timecourse of Stress-induced Tau Phosphorylation

(A) Western blot analysis of PHF-1 in cultured mouse hippocampal cells exposed to low or high concentrations of stress hormones CRF(1uM or 10uM), or cort (200ng or 400ng) over a period of 0, 0.5, 2, 4, 8, or 24 hours. B-actin was used as loading control. (B) Quantitative analysis of tau phosphorylation at PHF-1 of CRF treated (top) and cort treated (bottom) cells. *Low treatments differs significantly from untreated control ($p < 0.01$); ** ($p < 0.001$); *** ($p < 0.0001$). † High treatments differ significantly from vehicle control ($p < 0.01$); ††($p < 0.001$); ††† ($p < 0.0001$). Immunostaining of mouse hippocampal cell cultures for tau-P in (C) vehicle controls and cells treated with (D) 1uM CRF or (E) 200ng cort at 0.5h. Tau-P and nuclei were immunostained using PHF-1antibody (green) and DAPI (blue), respectively.

A.
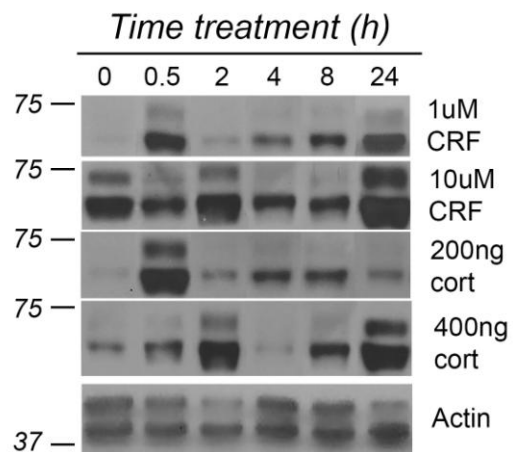
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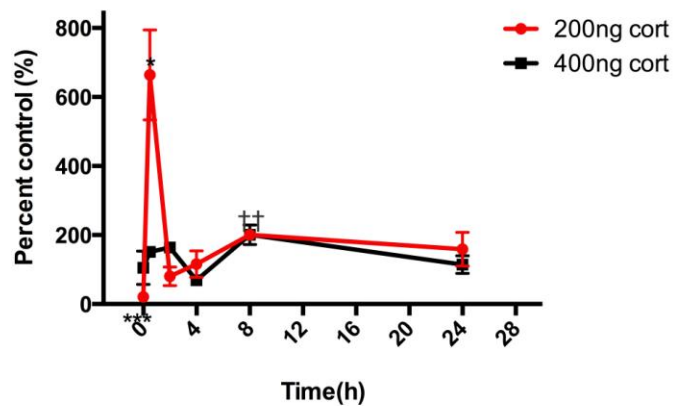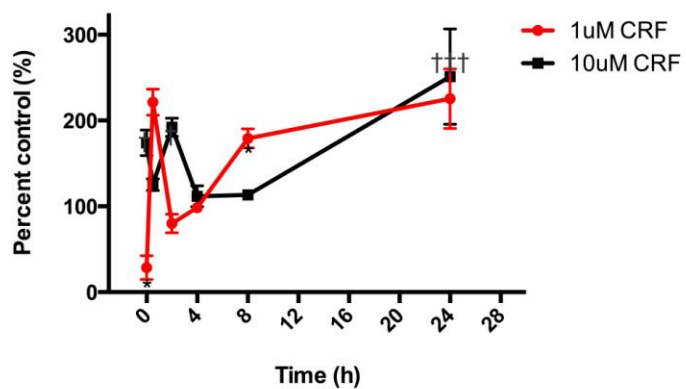
C. 0.5h Veh Control
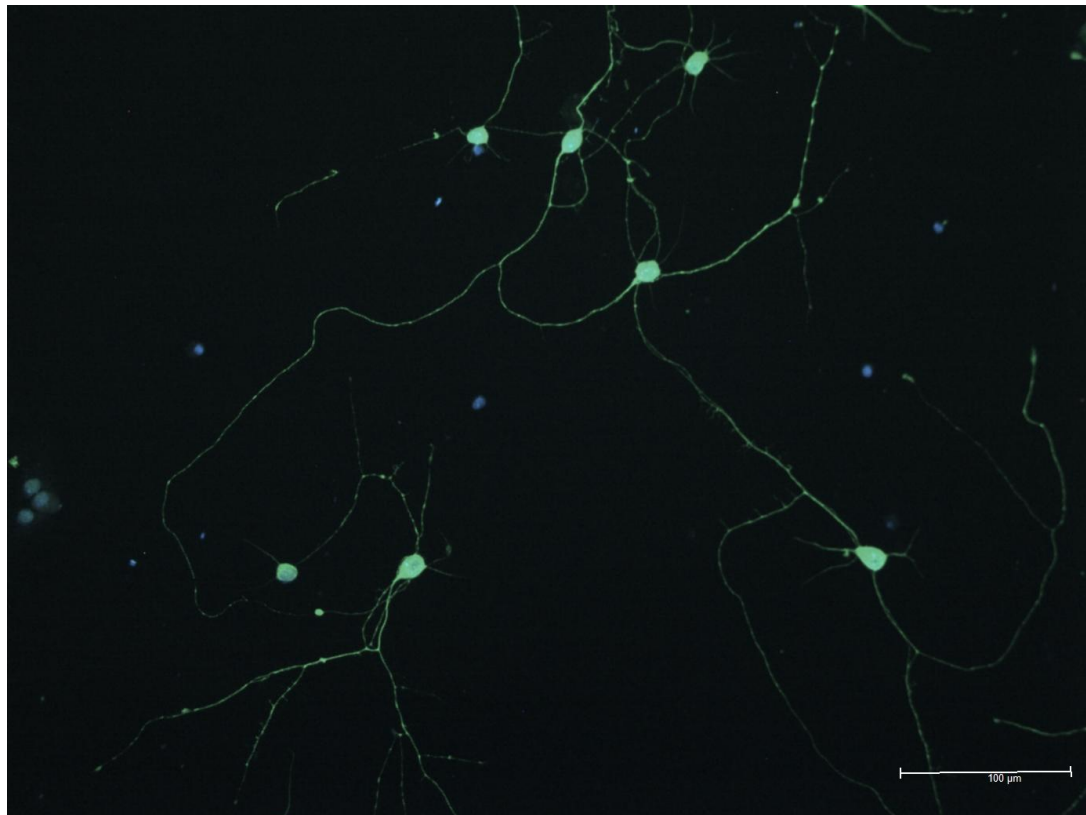
Figure 1: Continued

D. 0.5h 1uM CRF
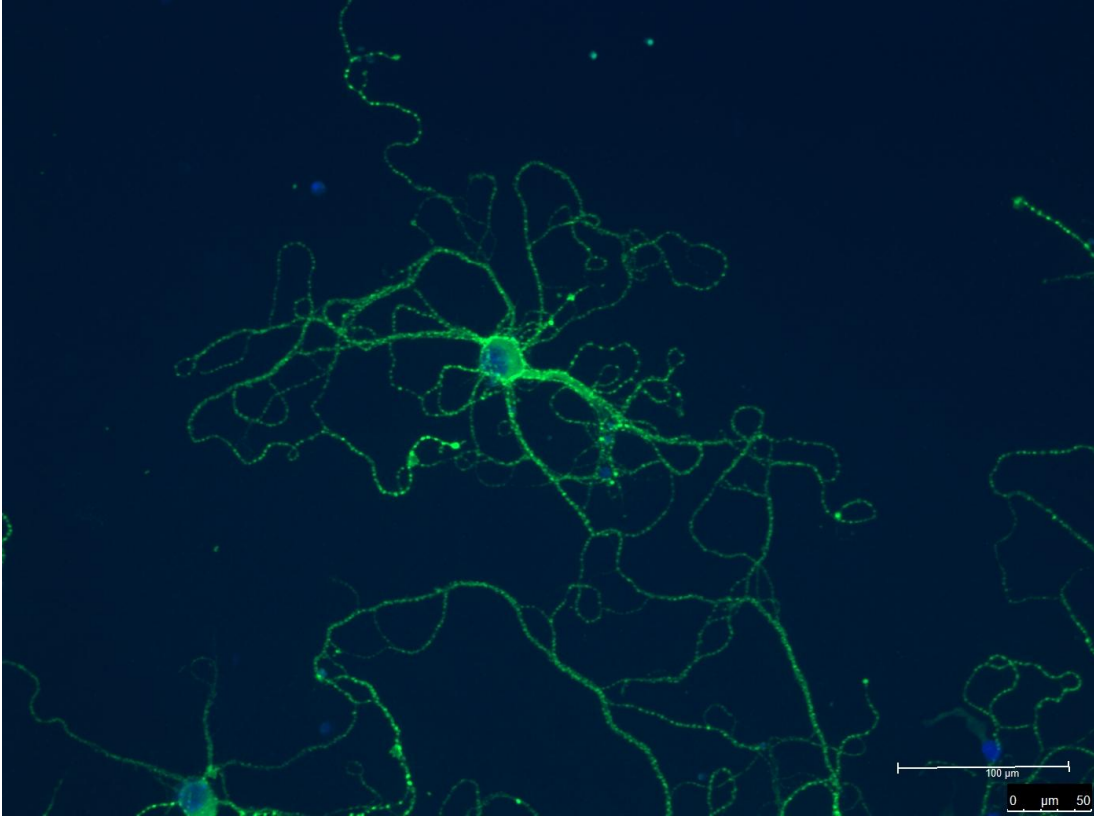
Figure 1: Continued

E. 0.5h 200ng cort
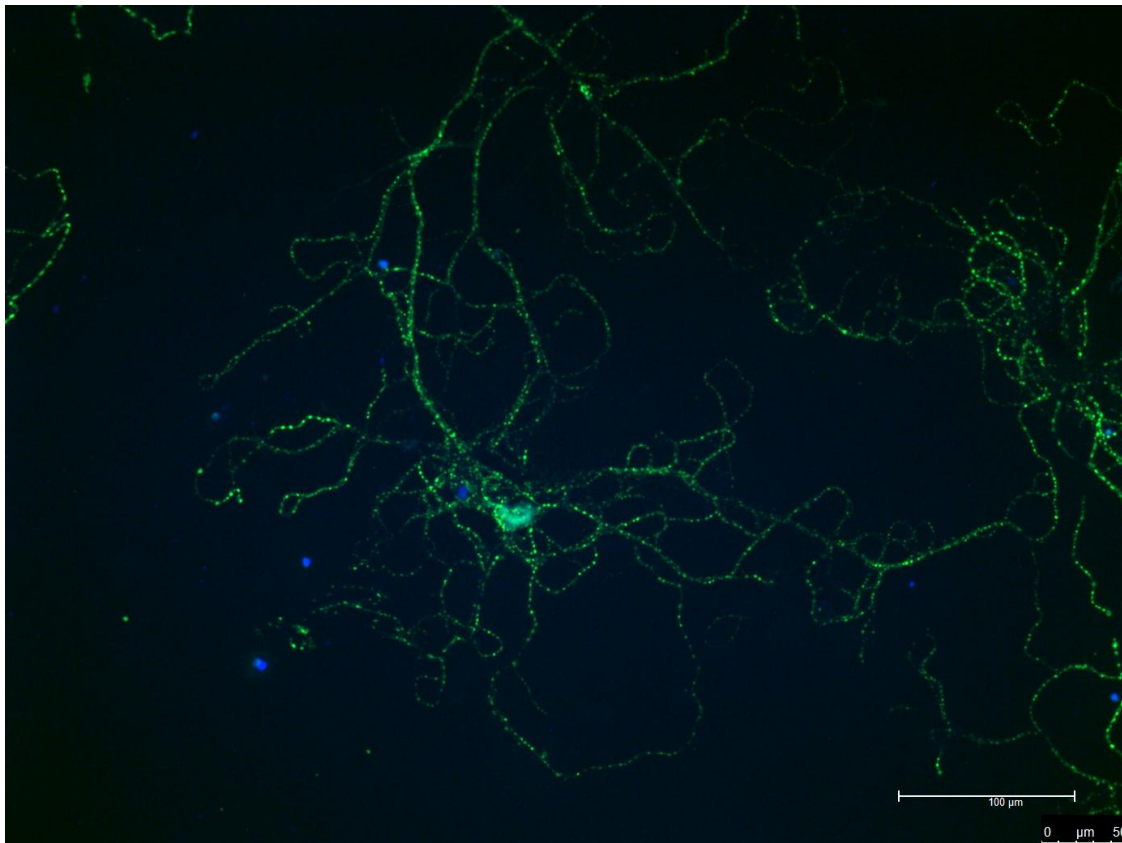
Figure 1: Continued

Figure 2: Upstream Mediators: Impact of Stress on GSK-3

Western blot analysis of (A) active GSK-3 (pY216), (C) inactive GSK-3 (pS9) and (E) total GSK-3 in both GSK-3α and GSK-3β isoforms of cultured mouse hippocampal cells exposed to low or high concentrations of stress hormones CRF(1uM or 10uM), or cort (200ng or 400ng) over a period of 0, 0.5, 2, 4, 8, or 24 hours. B-actin was used as loading control. Quantitative analysis of (B) active GSK-3 (pY216), (D) inactive GSK-3 (pS9) and (F) total GSK-3expression of CRF treated (top) and cort treated (bottom) cells. *Low treatments differs significantly from untreated control ($p < 0.01$); ** ($p < 0.001$); *** ($p < 0.0001$). † High treatments differ significantly from vehicle control ($p < 0.01$); ††($p < 0.001$); ††† ($p < 0.0001$).
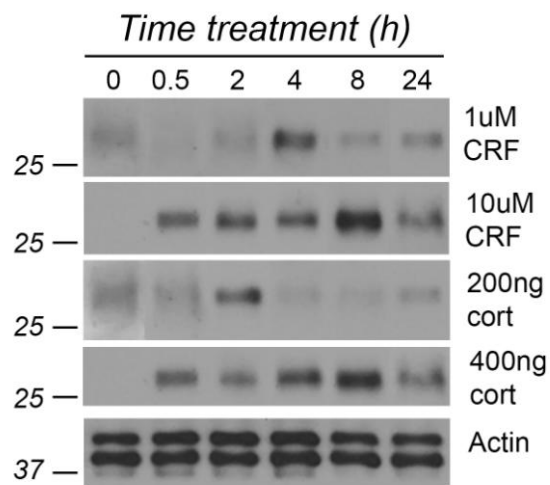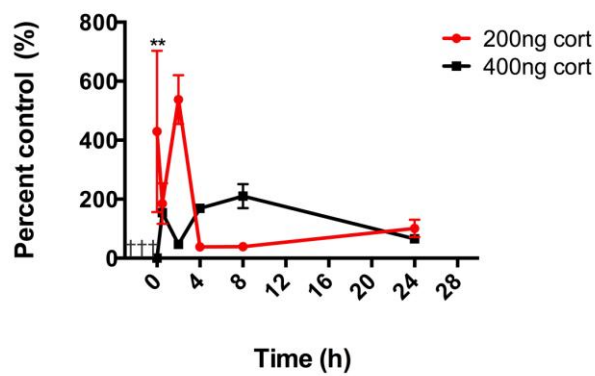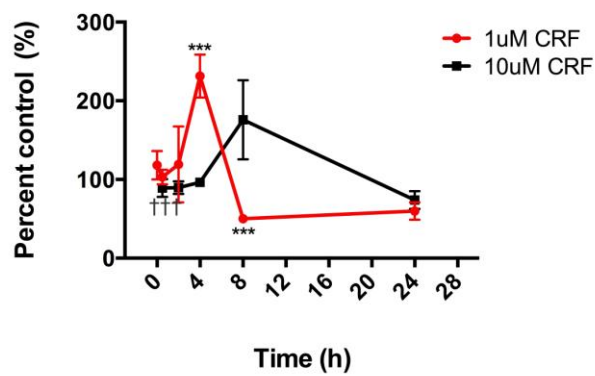
A. Active GSK-3 (pY216)
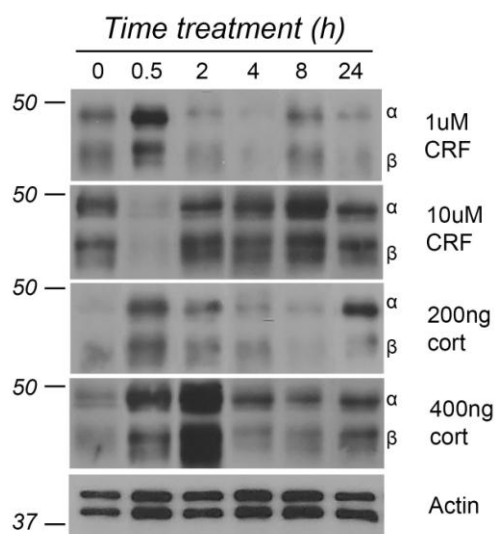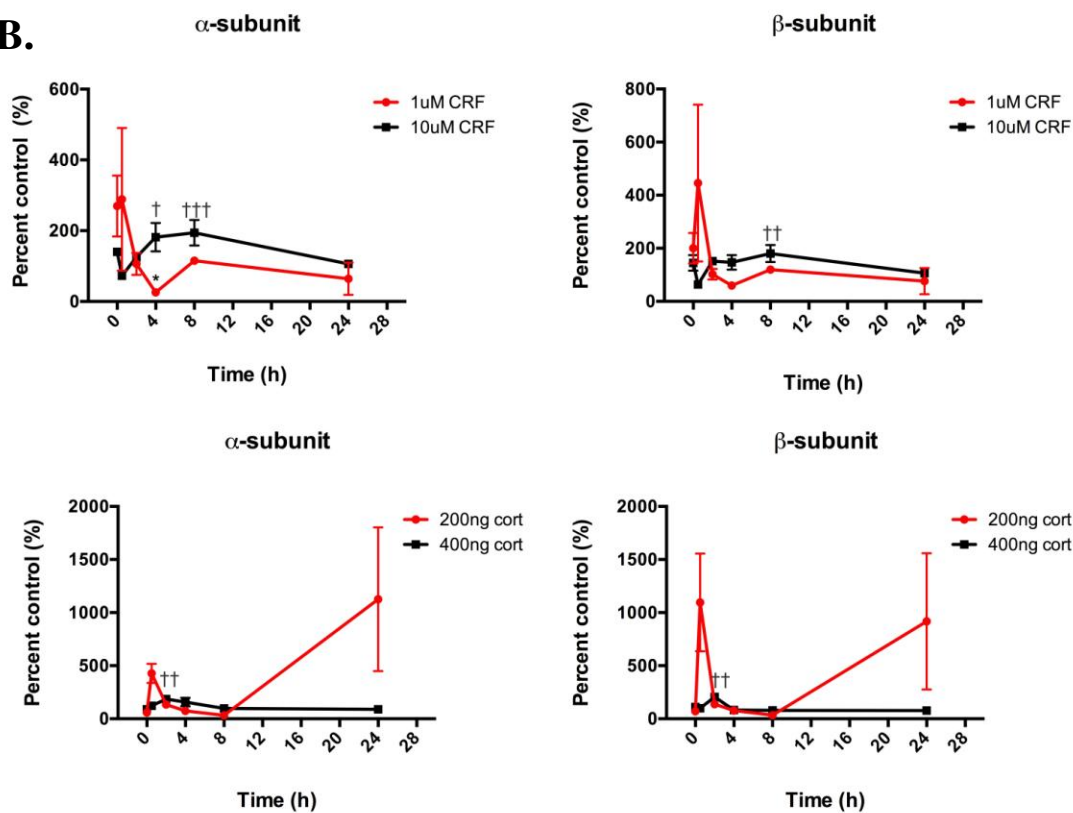
C. Inactive GSK-3 (pS9)
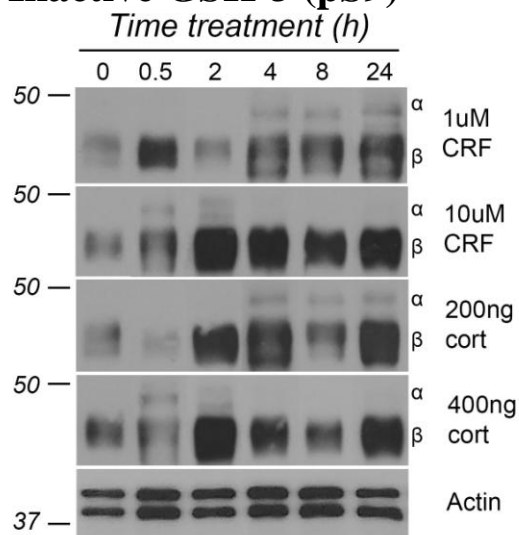
D.
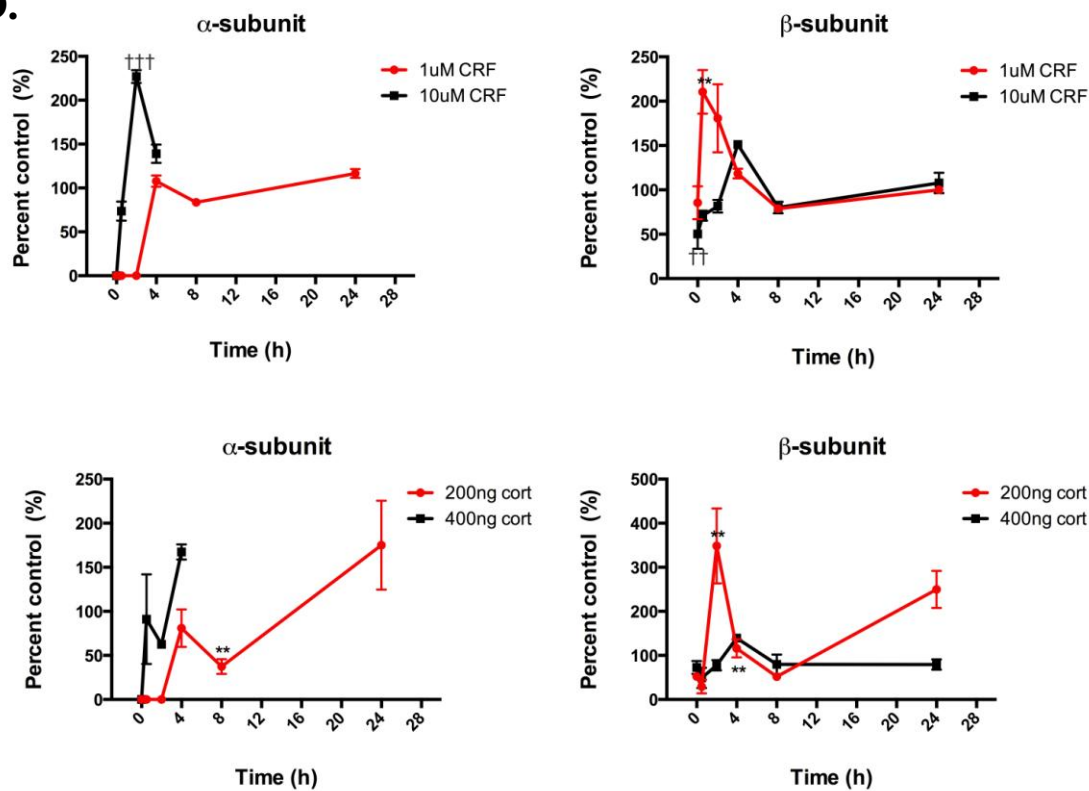
Figure 2: Continued

E. Total GSK-3
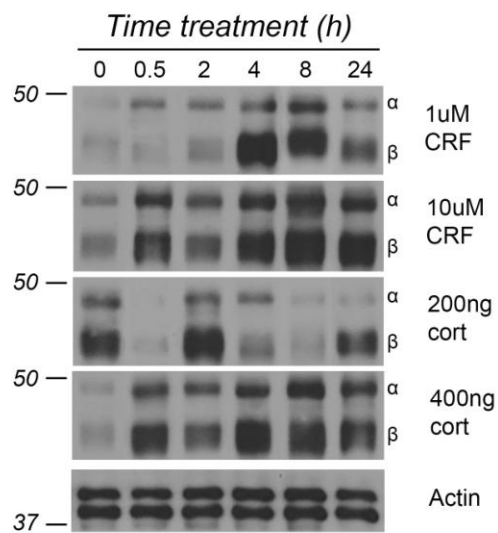
F.
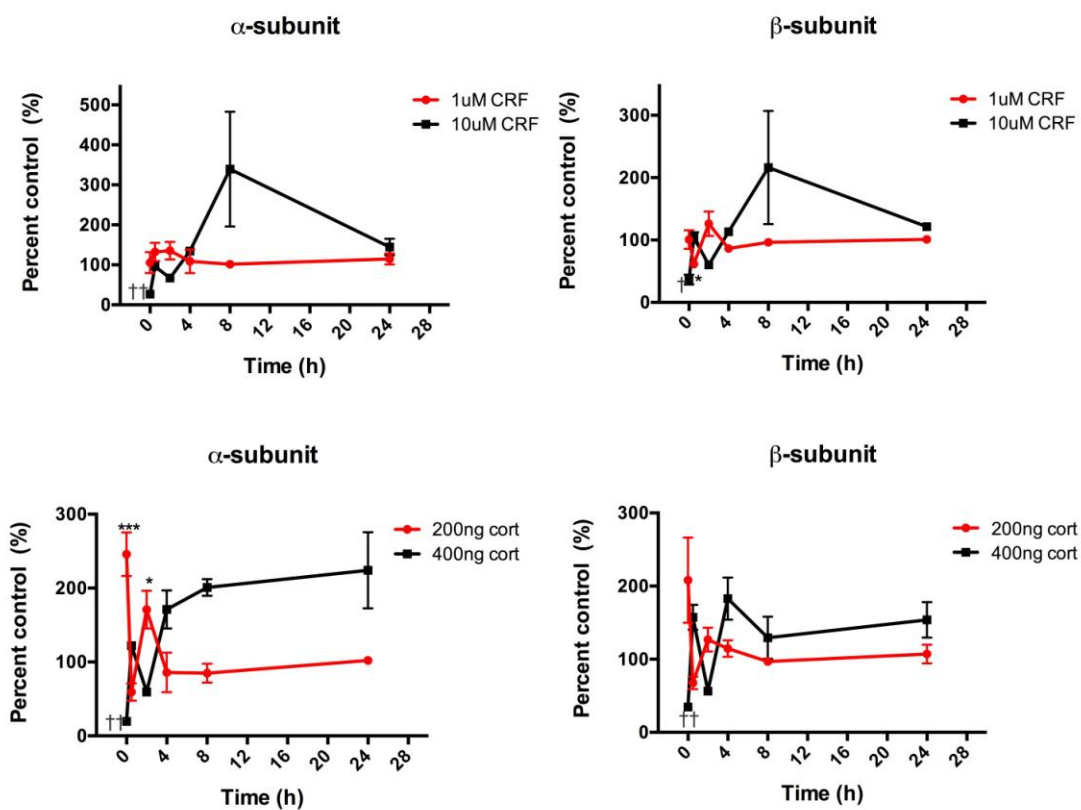
Figure 2: Continued

Figure 3: Upstream Mediators: Impact of Stress on JNK

(A) Western blot analysis of JNK of both pJNK54 and pJNK46 isoforms in cultured mouse hippocampal cells exposed to low or high concentrations of stress hormones CRF(1uM or 10uM), or cort (200ng or 400ng) over a period of 0, 0.5, 2, 4, 8, or 24 hours. B-actin was used as loading control. (B) Quantitative analysis of JNK expression of CRF treated (top) and cort treated (bottom) cells. *Low treatments differs significantly from untreated control (p<0.01); ** (p<0.001); *** (p<0.0001). † High treatments differ significantly from vehicle control (p<0.01); ††(p<0.001); ††† (p<0.0001).

A.
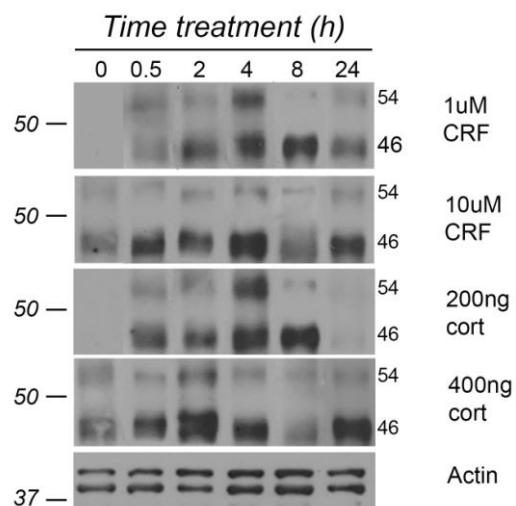
B.
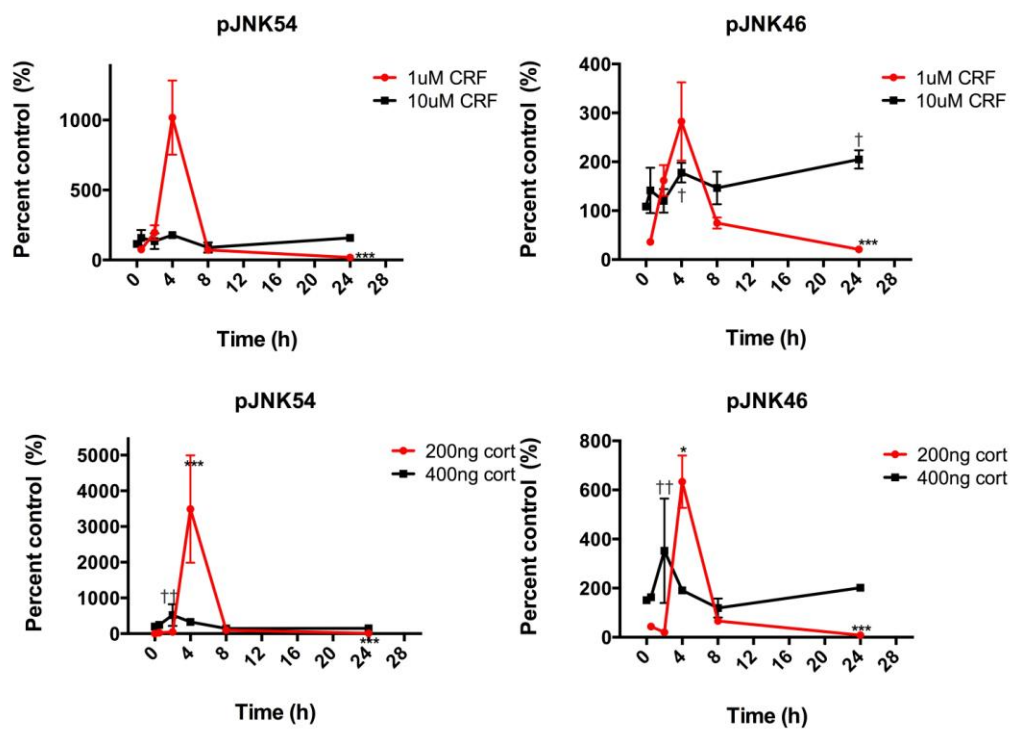
Figure 4: Upstream Mediators: Impact of Stress on ERK

(A) Western blot analysis of ERK of both isoforms in cultured mouse hippocampal cells exposed to low or high concentrations of stress hormones CRF(1uM or 10uM), or cort (200ng or 400ng) over a period of 0, 0.5, 2, 4, 8, or 24 hours. B-actin was used as loading control. (B) Quantitative analysis of ERK1/2 expression of CRF treated (top) and cort treated (bottom) cells. *Low treatments differs significantly from untreated control (p<0.01); ** (p<0.001); *** (p<0.0001). † High treatments differ significantly from vehicle control (p<0.01); ††(p<0.001); ††† (p<0.0001).

A.
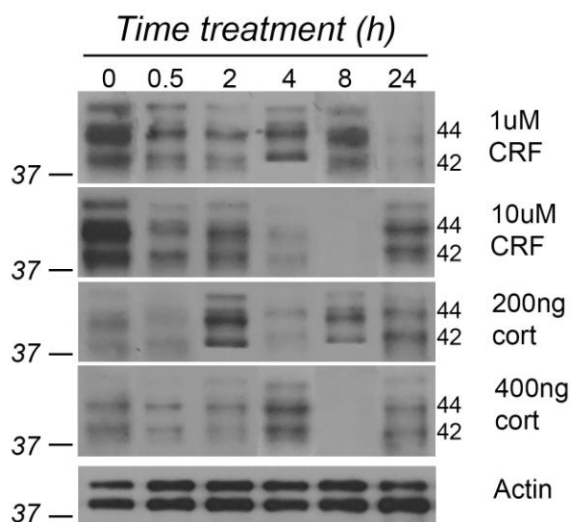
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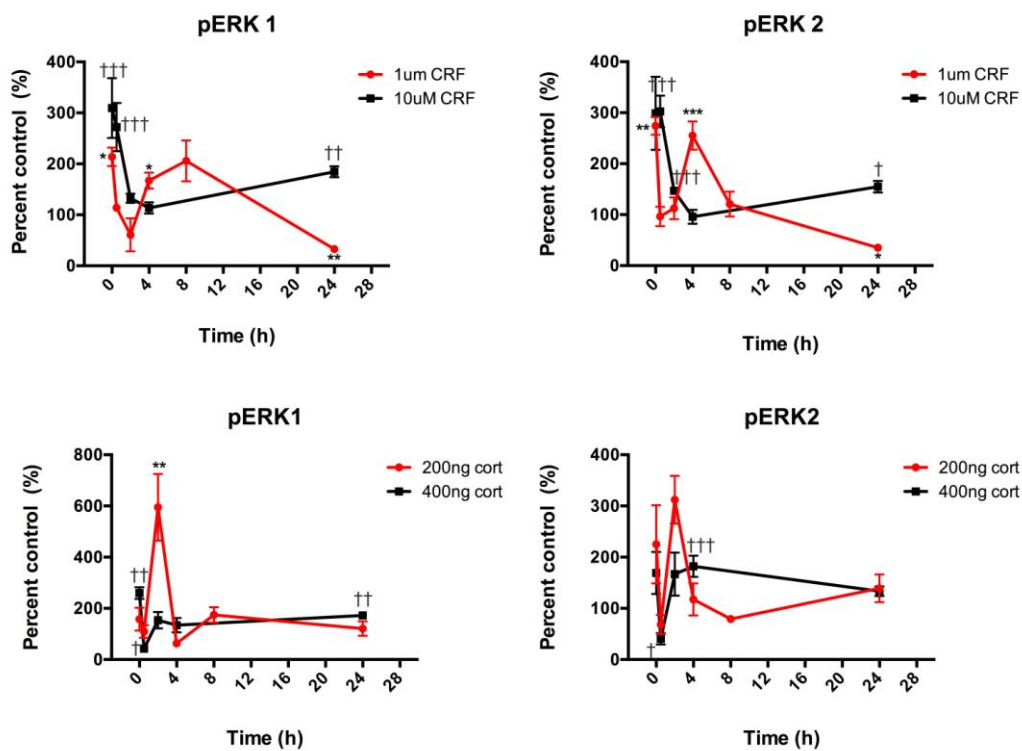
Figure 5: Upstream Mediators: Impact of Stress on cdk5

(A) Western blot analysis of CDK5 in cultured mouse hippocampal cells exposed to low or high concentrations of stress hormones CRF(1uM or 10uM), or cort (200ng or 400ng) over a period of 0, 0.5, 2, 4, 8, or 24 hours. B-actin was used as loading control. (B) Quantitative analysis of cdk5 expression of CRF treated (top) and cort treated (bottom) cells. *Low treatments differs significantly from untreated control ($p<0.01$); ** ($p<0.001$); *** ($p<0.0001$). † High treatments differ significantly from vehicle control ($p<0.01$); ††($p<0.001$); †††($p<0.0001$).

A.

Time treatment (h)

| 0 | 0.5 | 2 | 4 | 8 | 24 |

25 — 1uM CRF

25 — 10uM CRF

25 — 200ng cort

25 — 400ng cort

37 — Actin

B.

Figure 6: Upstream Mediators: Impact of Stress on P35

(A) Western blot analysis of P35 in cultured mouse hippocampal cells exposed to low or high concentrations of stress hormones CRF(1uM or 10uM), or cort (200ng or 400ng) over a period of 0, 0.5, 2, 4, 8, or 24 hours. B-actin was used as loading control. (B) Quantitative analysis of P35 expression of CRF treated (top) and cort treated (bottom) cells. *Low treatments differs significantly from untreated control (p<0.01); ** (p<0.001); *** (p<0.0001). † High treatments differ significantly from vehicle control (p<0.01); ††(p<0.001); ††† (p<0.0001).
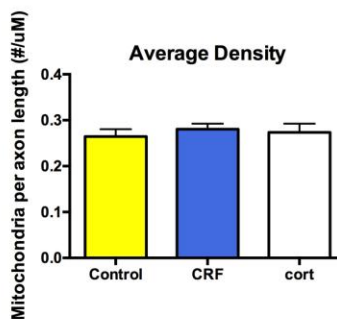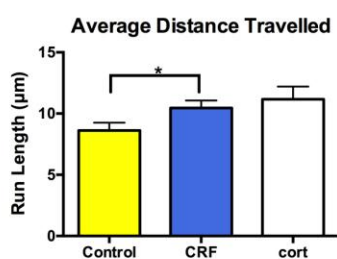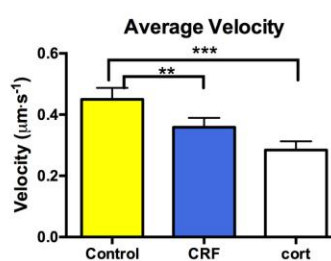
A.
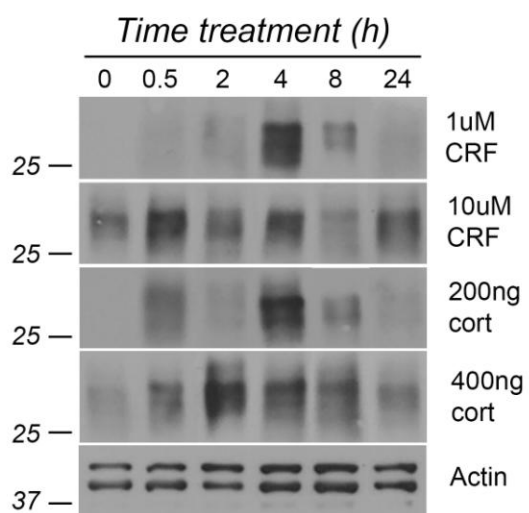
B.
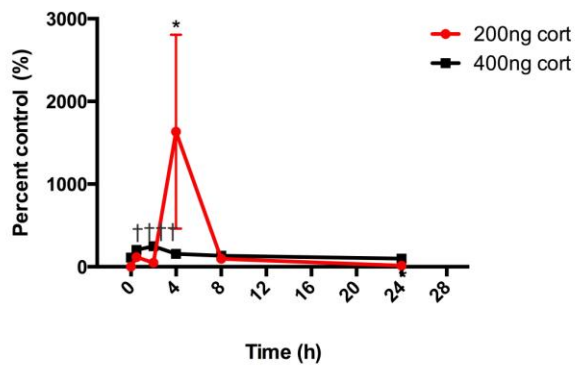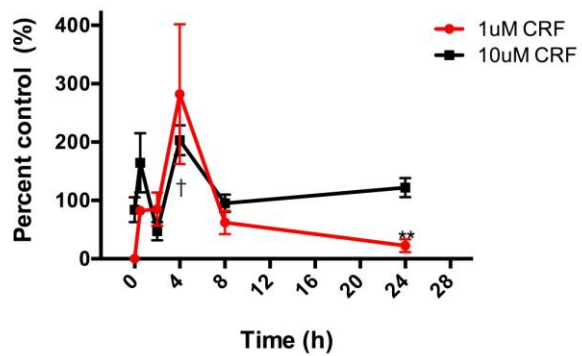
Figure 7: Impact of Stress-induced Tau Phosphorylation on Mitochondria Transport

Quantitative analysis of fluorescent live images acquired from axons of cultured mouse hippocampal cells. (A) Average velocity, (B) total distance travelled, and (C) percentage of moving mitochondria were examined in vehicle control, 0.5h, and 2h high treatments (10μM CRF, 400ng cort) in axons. *Treatments differs significantly from vehicle control ($p < 0.01$); ** ($p < 0.001$); *** ($p < 0.0001$).
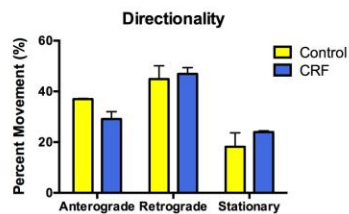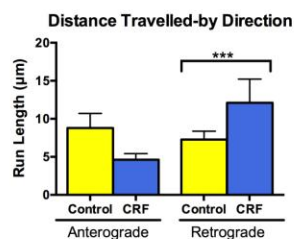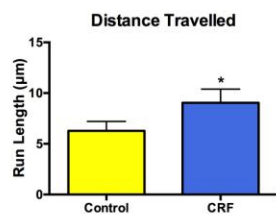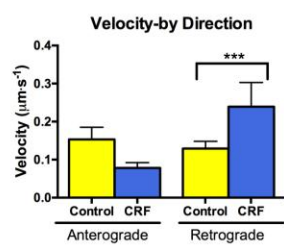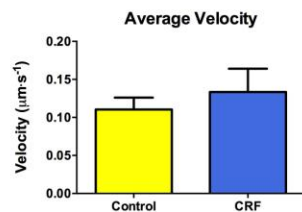
0.5h Treatment
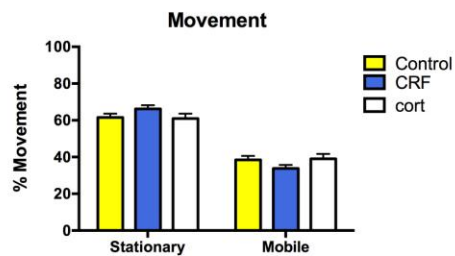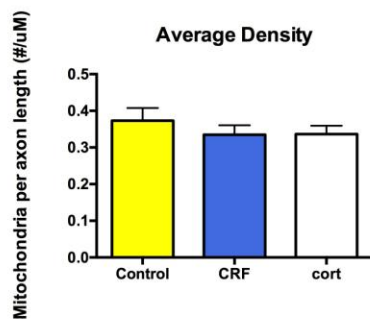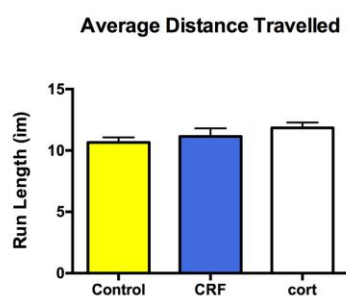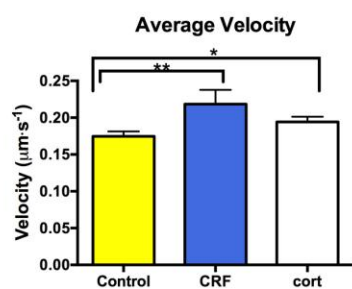
2h Treatment
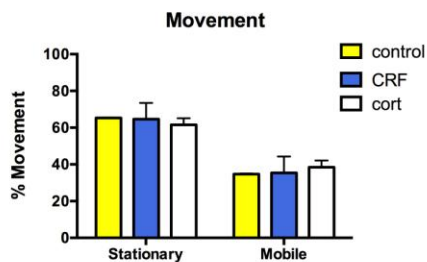
Figure 8: Impact of Stress-induced Tau Phosphorylation on BDNF Transport

Quantitative analysis of fluorescent live images acquired from axons of cultured mouse hippocampal cells. Average velocity, velocity-by direction, distance travelled, distance travelled-by direction, and direction of movement in axons were examined in vehicle control, 0.5h, and 2h high treatments of (A) 10uM CRF and (B) 400ng cort. *Treatments differs significantly from vehicle control ($p < 0.01$); ** ($p < 0.001$); *** ($p < 0.0001$); **** ($P < 0.00001$)
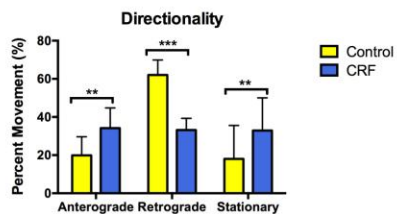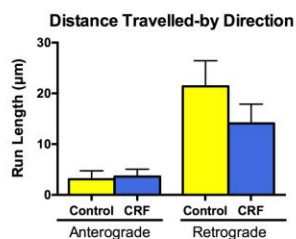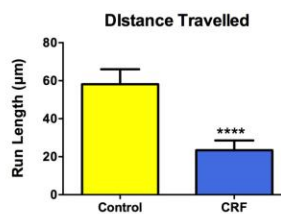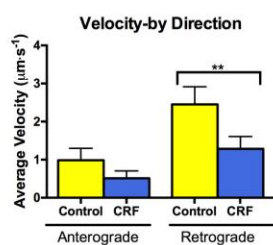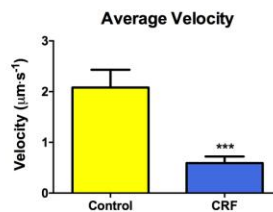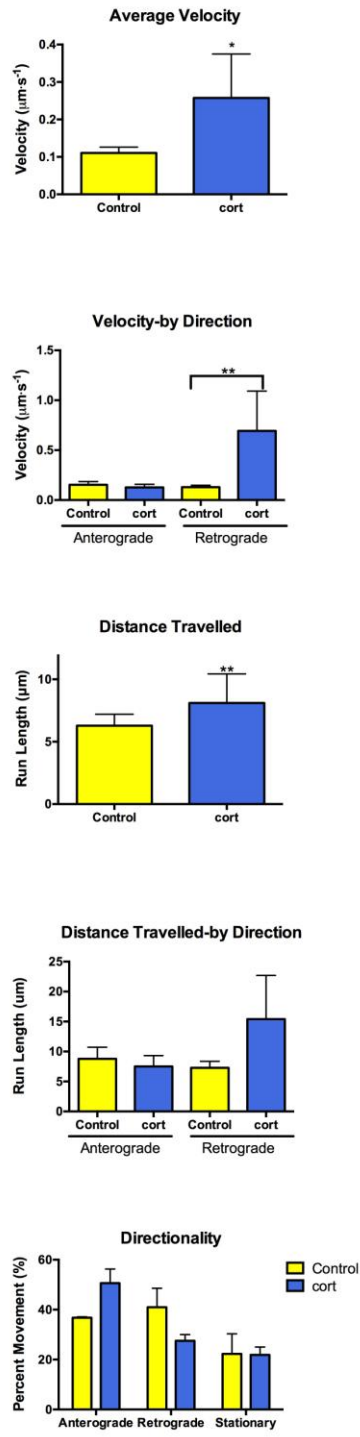
A. CRF

0.5h Treatment

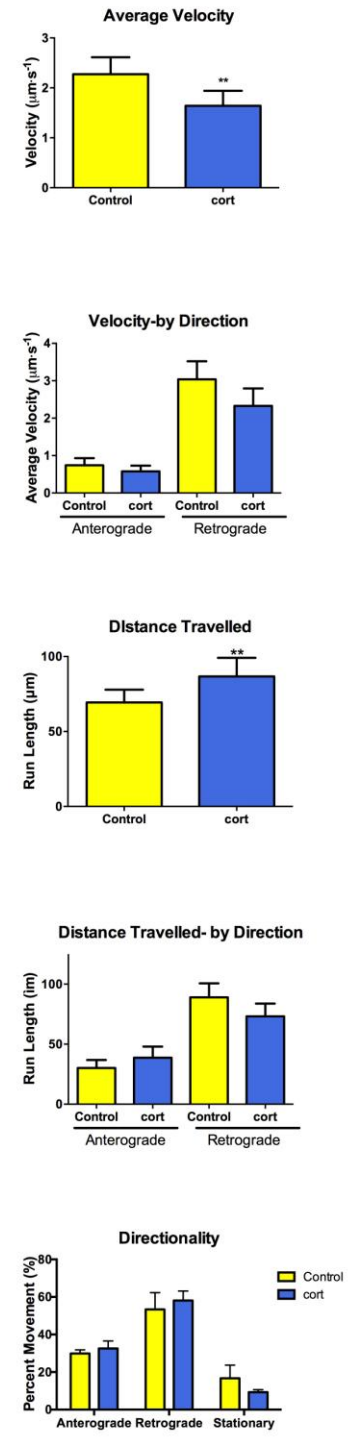## 2h Treatment

B. cort

0.5h Treatment

2h Treatment

Figure 8: Continued

REFERENCES

Aisen, P. S., Davis, K. L., Berg, J. D., Schafer, K., Campbell, K., Thomas, R. G., Weiner, M. F., Farlow, M. R., Sano, M., Grundman, M., & Thal, L. J. (2000). A randomized controlled trial of prednisone in Alzheimer's disease. Alzheimer's Disease Cooperative Study. *Neurology, 54*(3), 588-593.

Arriagada, P. V., Growdon, J. H., Hedley-Whyte, E. T., & Hyman, B. T. (1992). Neurofibrillary tangles but not senile plaques parallel duration and severity of Alzheimer's disease. *Neurology, 42*(3 Pt 1), 631-639.

Assoc, Alzheimers. (2012). 2012 Alzheimer's disease facts and figures. *Alzheimers & Dementia, 8*(2), 131-168.

Chang, C. P., Pearse, R. V., 2nd, O'Connell, S., & Rosenfeld, M. G. (1993). Identification of a seven transmembrane helix receptor for corticotropin-releasing factor and sauvagine in mammalian brain. *Neuron, 11*(6), 1187-1195.

Chen, R., Lewis, K. A., Perrin, M. H., & Vale, W. W. (1993). Expression cloning of a human corticotropin-releasing-factor receptor. *Proc Natl Acad Sci U S A, 90*(19), 8967-8971.

Davis, K. L., Mohs, R. C., Marin, D. B., Purohit, D. P., Perl, D. P., Lantz, M., Austin, G., & Haroutunian, V. (1999). Neuropeptide abnormalities in patients with early Alzheimer disease. *Arch Gen Psychiatry, 56*(11), 981-987.

Dugich-Djordjevic, M. M., Peterson, C., Isono, F., Ohsawa, F., Widmer, H. R., Denton, T. L., Bennett, G. L., & Hefti, F. (1995). Immunohistochemical visualization of brain-derived neurotrophic factor in the rat brain. *Eur J Neurosci, 7*(9), 1831-1839.

Ebneth, A., Godemann, R., Stamer, K., Illenberger, S., Trinczek, B., & Mandelkow, E. (1998). Overexpression of tau protein inhibits kinesin-dependent trafficking of vesicles, mitochondria, and endoplasmic reticulum: implications for Alzheimer's disease. *J Cell Biol, 143*(3), 777-794.

Frame, S., & Cohen, P. (2001). GSK3 takes centre stage more than 20 years after its discovery. *Biochem J, 359*(Pt 1), 1-16.

Gomez-Isla, T., Hollister, R., West, H., Mui, S., Growdon, J. H., Petersen, R. C., Parisi, J. E., & Hyman, B. T. (1997). Neuronal loss correlates with but exceeds neurofibrillary tangles in Alzheimer's disease. *Ann Neurol, 41*(1), 17-24.

Iqbal, K., Grundke-Iqbal, I., Zaidi, T., Merz, P. A., Wen, G. Y., Shaikh, S. S., Wisniewski, H. M., Alafuzoff, I., & Winblad, B. (1986). Defective brain microtubule assembly in Alzheimer's disease. *Lancet, 2*(8504), 421-426.

Jope, R. S., Yuskaitis, C. J., & Beurel, E. (2007). Glycogen synthase kinase-3 (GSK3): inflammation, diseases, and therapeutics. *Neurochem Res, 32*(4-5), 577-595.

Kenessey, A., & Yen, S. H. (1993). The extent of phosphorylation of fetal tau is comparable to that of PHF-tau from Alzheimer paired helical filaments. *Brain Res, 629*(1), 40-46.

Kopke, E., Tung, Y. C., Shaikh, S., Alonso, A. C., Iqbal, K., & Grundke-Iqbal, I. (1993). Microtubule-associated protein tau. Abnormal phosphorylation of a non-paired helical filament pool in Alzheimer disease. *J Biol Chem, 268*(32), 24374-24384.

Korneyev, A., Binder, L., & Bernardis, J. (1995). Rapid reversible phosphorylation of rat brain tau proteins in response to cold water stress. *Neurosci Lett, 191*(1-2), 19-22.

Landfield, P. W., Blalock, E. M., Chen, K. C., & Porter, N. M. (2007). A new glucocorticoid hypothesis of brain aging: implications for Alzheimer's disease. *Curr Alzheimer Res, 4*(2), 205-212.

Lucas, J. J., Hernandez, F., Gomez-Ramos, P., Moran, M. A., Hen, R., & Avila, J. (2001). Decreased nuclear beta-catenin, tau hyperphosphorylation and neurodegeneration in GSK-3beta conditional transgenic mice. *EMBO J, 20*(1-2), 27-39.

Mandelkow, E. M., Stamer, K., Vogel, R., Thies, E., & Mandelkow, E. (2003). Clogging of axons by tau, inhibition of axonal traffic and starvation of synapses. *Neurobiol Aging, 24*(8), 1079-1085.

Morfini, G. A., Burns, M., Binder, L. I., Kanaan, N. M., LaPointe, N., Bosco, D. A., Brown, R. H., Jr., Brown, H., Tiwari, A., Hayward, L., Edgar, J., Nave, K. A., Garberrn, J., Atagi, Y., Song, Y., Pigino, G., & Brady, S. T. (2009). Axonal transport defects in neurodegenerative diseases. *J Neurosci, 29*(41), 12776-12786.

Nagahara, A. H., Merrill, D. A., Coppola, G., Tsukada, S., Schroeder, B. E., Shaked, G. M., Wang, L., Blesch, A., Kim, A., Conner, J. M., Rockenstein, E., Chao, M. V., Koo, E. H., Geschwind, D., Masliah, E., Chiba, A. A., & Tuszynski, M. H. (2009). Neuroprotective effects of brain-derived neurotrophic factor in rodent and primate models of Alzheimer's disease. *Nat Med, 15*(3), 331-337.

Potter, E., Sutton, S., Donaldson, C., Chen, R., Perrin, M., Lewis, K., Sawchenko, P. E., & Vale, W. (1994). Distribution of corticotropin-releasing factor receptor mRNA expression in the rat brain and pituitary. *Proc Natl Acad Sci U S A, 91*(19), 8777-8781.

Prince, M., Bryce, R., Albanese, E., Wimo, A., Ribeiro, W., & Ferri, C. P. (2013). The global prevalence of dementia: a systematic review and metaanalysis. *Alzheimers Dement, 9*(1), 63-75 e62.

Rissman, R. A. (2009). Stress-induced tau phosphorylation: functional neuroplasticity or neuronal vulnerability? *J Alzheimers Dis, 18*(2), 453-457.

Rissman, R. A., Lee, K. F., Vale, W., & Sawchenko, P. E. (2007). Corticotropin-releasing factor receptors differentially regulate stress-induced tau phosphorylation. *J Neurosci, 27*(24), 6552-6562.

Rissman, R. A., Staup, M. A., Lee, A. R., Justice, N. J., Rice, K. C., Vale, W., & Sawchenko, P. E. (2012). Corticotropin-releasing factor receptor-dependent effects of repeated stress on tau phosphorylation, solubility, and aggregation. *Proc Natl Acad Sci U S A, 109*(16), 6277-6282.

Sapolsky, R. M., Krey, L. C., & McEwen, B. S. (1985). Prolonged glucocorticoid exposure reduces hippocampal neuron number: implications for aging. *J Neurosci, 5*(5), 1222-1227.

Sheng, Z. H., & Cai, Q. (2012). Mitochondrial transport in neurons: impact on synaptic homeostasis and neurodegeneration. *Nat Rev Neurosci, 13*(2), 77-93.

Smith, G. W., Aubry, J. M., Dellu, F., Contarino, A., Bilezikjian, L. M., Gold, L. H., Chen, R., Marchuk, Y., Hauser, C., Bentley, C. A., Sawchenko, P. E., Koob, G. F., Vale, W., & Lee, K. F. (1998). Corticotropin releasing factor receptor 1-deficient mice display decreased anxiety, impaired stress response, and aberrant neuroendocrine development. *Neuron, 20*(6), 1093-1102.

Smith, M. A., Makino, S., Kvetnansky, R., & Post, R. M. (1995). Stress and glucocorticoids affect the expression of brain-derived neurotrophic factor and neurotrophin-3 mRNAs in the hippocampus. *J Neurosci, 15*(3 Pt 1), 1768-1777.

Stamer, K., Vogel, R., Thies, E., Mandelkow, E., & Mandelkow, E. M. (2002). Tau blocks traffic of organelles, neurofilaments, and APP vesicles in neurons and enhances oxidative stress. *J Cell Biol, 156*(6), 1051-1063.

Stokin, G. B., & Goldstein, L. S. (2006). Axonal transport and Alzheimer's disease. *Annu Rev Biochem, 75*, 607-627.

Trinczek, B., Ebneth, A., Mandelkow, E. M., & Mandelkow, E. (1999). Tau regulates the attachment/detachment but not the speed of motors in microtubule-dependent transport of single vesicles and organelles. *J Cell Sci, 112 (Pt 14)*, 2355-2367.

Van Pett, K., Viau, V., Bittencourt, J. C., Chan, R. K., Li, H. Y., Arias, C., Prins, G. S., Perrin, M., Vale, W., & Sawchenko, P. E. (2000). Distribution of mRNAs encoding CRF receptors in brain and pituitary of rat and mouse. *J Comp Neurol, 428*(2), 191-212.

Vita, N., Laurent, P., Lefort, S., Chalon, P., Lelias, J. M., Kaghad, M., Le Fur, G., Caput, D., & Ferrara, P. (1993). Primary structure and functional expression of mouse

pituitary and human brain corticotrophin releasing factor receptors. *FEBS Lett, 335*(1), 1-5.

Wilson, R. S., Evans, D. A., Bienias, J. L., Mendes de Leon, C. F., Schneider, J. A., & Bennett, D. A. (2003). Proneness to psychological distress is associated with risk of Alzheimer's disease. *Neurology, 61*(11), 1479-1485.

Yoshida, H., & Ihara, Y. (1993). Tau in paired helical filaments is functionally distinct from fetal tau: assembly incompetence of paired helical filament-tau. *J Neurochem, 61*(3), 1183-1186.