Kinase-Based Regulation of Fast Axonal Transport in Health and Disease

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THESIS

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I dedicate this thesis to my mother, Sunja Oh, who sacrificed everything to support me in every possible way and without whom it would have never been accomplished.

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MK

Contribution of Authors

Parts of the work presented in **Chapter I** and **Chapter II** have been adapted from a previously published manuscript featuring the author of this thesis as its first author. The contributions of each author are described below.

Minsu Kang – Prepared the initial draft of the manuscript, prepared figures, and edited the manuscript to its final version.

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Scott T. Brady – Reviewed the draft of the manuscript and edited the manuscript to its final version.

Gerardo Morfini – Prepared figures, reviewed the draft of the manuscript, and edited the manuscript to its final version.

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LIST OF ABBREVIATIONS

AD	Alzheimer's disease
Akt	Protein kinase B
ALS	Amyotrophic lateral sclerosis
AMPA	α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid
ANOVA	Analysis of variance
aPKC	Atypical protein kinase C
ARPP-16	cAMP-regulated phosphoprotein 16
ARPP-21	cAMP-regulated phosphoprotein 21
ASO	Antisense oligonucleotide
ATC	Axon terminal compartment
ATP	Adenosine triphosphate
Ax	Axonal compartment
BCA	Bicinchoninic acid
BDNF	Brain-derived neurotrophic factor
BICD1/2	Bicaudal 1/2
$Ca2^{+}$	Calcium ion
CAMK	Ca^{2+}/calmodulin- dependent protein kinase
CCD	Charge-coupled device
CDyn	Cytoplasmic dynein
CI	Confidence interval
CIPN	Chemotherapy-induced peripheral neuropathy

CK2	Casein kinase 2
CNTF	Ciliary neurotrophic factor
CO_2	Carbon dioxide
cPKC	Conventional kinesin
CRIB	Cdc42- and Rac-interactive binding
CSK	C-terminal Src kinase
DAG	Diacylglycerol
DARPP-32	Dopamine- and cAMP-regulated phosphoprotein 32kDa
DHC	Dynein heavy chain
DIC	Dynein intermediate chain
DIV	Days in vitro
DLC	Dynein light chain
DLIC	Dynein light intermediate chain
DMEM	Dulbecco's modified eagle medium
Drp1	Dynamin-related protein 1
DTI	Diffusion tensor imaging
DTT	Dithiothreitol
EDTA	Etyhlendiaminetetraacetic acid
EGF	Epithelial growth factor
ERK	Extraceullar signal-regulated kinase
FANTOM5	Functional Annotation of the Mammalian Genome 5 project
FAT	Fast axonal transport

FBS	Fetal bovine serum
FGF	Fibroblast growth factor
Fis1	Mitochondrial fission 1
FRET	Förster resonance energy transfer
GABA	gamma-Aminobutyric acid
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GDNF	Glial cell-derived neurotrophic factor
GDP	Guanosine diphosphate
GEF	Guanine nucleotide exchange factor
GPe	External globus pallidus
GPi	Internal globus pallidus
Grb2	Growth factor receptor-bound protein 2
GSK3β	Glycogen synthase kinase 3 beta
GST	Glutathione S-transferase
GTEx	Genotype-Tissue Expression project
GTP	Guanosine triphosphate
HCl	Hydrochloric acid
HD	Huntington's disease
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HIP1	Huntingtin-interacting protein 1
HIV	Human immunodeficiency virus
HPA	Human Protein Atlas project

Htt	Huntingtin
IL-11	Interleukin 11
IL-6	Interleukin 6
IP3	Inositol triophosphate
JNK1/2/3	c-Jun N-terminal kinase 1/2/3
K•Aspartate	Potassium aspartate
KHC	Kinesin heavy chain
KLC	Kinesin light chain
L-Z	Leucine zipper
Lis1	Lissencephaly 1
M	Mean
MAPK	Mitogen-activated protein kinase
MAPKK	Mitogen-activated protein kinase kinase
MAPKKK	Mitogen-activated protein kinase kinase kinase
MBO	Membrane-bound organelle
MEK	Extracellular signal-regulated kinase kinase
MeOH	Methanol
MFC	Microfluidic chamber
Mfn1/2	Mitofusin 1/2
$MgCl_2$	Magnesium chloride
mHtt	Mutant huntingtin
MKK7	Mitogen-activated protein kinase kinase 7

MLK	Mixed-lineage kinase
MOPS	3-(N-morpholino)propanesulfonic acid
MPP+	1-methyl-4-phenylpyridinium
MSN	Medium spiny neuron
MT	Microtubule
n.a.	Numerical aperture
Na_3VO_4	Sodium orthovanadate
NaCl	Sodium chloride
NaF	Sodium floride
Ndel1	Nuclear distribution protein nudE-like 1
NGF	Nerve growth factor
NIR	Near-infrared imaging
NMDA	N-methyl-D-aspartate
nPKC	Novel protein kinase C
NT-3	Neurotrophin 3
NT-4/5	Neurotrophin 4/5
$p75^{NTR}$	Neurotrophin receptor p75
PAGE	Polyacrylamide gel electrophoresis
PD	Parkinson's disease
PDK1	Phosphoinositide-dependent kinase
Phos-Tag	Phosphate-binding tag
PI3K	Phosphoinositide 3 kinsae

PIS	Post-immunoprecipitation supernatant
PKC	Protein kinase C
PKD1	Protein kinase D1
PLCγ	Phospholipase C gamma
PMA	Phorbol 12-myistate 13-acetate
polyQ	Polyglutamine
PRD	Proline-rich domain
PSD-95	Postsynaptic density 95
PVDF	Polyvinylidene difluoride
QD	Quantum-dot
rpm	Rotation per minute
RTK	Receptor tyrosine kinase
SD	Standard deviation
SDC	Somatodendritic compartment
SDS	Sodium dodecyl sulfate
SFK	Src family kinase
SH2	Src homology 2
SH3	Src homology 3
SN	Substantia nigra
SOD1	Superoxide dismutase 1
Sos	Son of sevenless
TBS	Tris-buffered saline

TBS-T	Tris-buffered saline with tween-20
TEMED	Tetramethylethylenediamine
TGF-β	Transforming growth factor beta
TH	Tyrosine hydroxylase
TrkA/B/C	Tropomyosin A/B/C
UIC	University of Illinois at Chicago
VEC-DIC	Video-enhanced contrast differential interference contrast
VEGF	Vascular endothelial growth factor
YFP	Yellow fluorescent protein
Zn^{2+}	Zinc ion

SUMMARY

Neurons have unique morphologies with distinct intracellular compartments responsible for particular functions. The most essential function of neurons is to receive information from and deliver to other cells by electrochemical connections at the synapses. Synthesis and post-translational processing of proteins required for proper activity and maintenance of neuronal connections occur in the neuronal soma, and synthesized proteins must be transported to the site of utilization by microtubule-based molecular motor proteins conventional kinesin and cytoplasmic dynein, a process referred to as fast axonal transport. Defective fast axonal transport lead to neurodegeneration, and impairments fast axonal transport have been found in several neurodegenerative diseases such as Alzheimer's disease, Huntington's disease, and amyotrophic lateral sclerosis. Phosphorylation of motor proteins represents a major regulatory mechanism of fast axonal transport, and a body of evidence accumulated from decades of research in our lab demonstrated that aberrant kinase signaling in neurodegenerative diseases lead to abnormal phosphorylation of motor proteins and impairments in fast axonal transport. This work extends our understanding of kinase signaling-based regulation of axonal transport in both pathological and non-pathological conditions.

Chapter I provides an overview of axonal transport machineries, previous work describing the regulation of axonal transport, and examples of neurodegenerative disease featuring impaired axonal transport. **Chapter II** describes amelioration of Huntington's disease pathology by genetic deletion of c-Jun N-terminal kinase 3 (JNK3), a kinase our lab previously identified to be mediating the axonal transport defects in Huntington's disease, in R6/2 mouse model of Huntington's disease, which highlights therapeutic

potentials of maintaining intact fast axonal transport by correcting aberrant kinase signaling. Work presented in **Chapter III** demonstrates identification of a novel signaling cascade involving tropomyosin receptor kinase B (TrkB), Src family kinases (SFKs), and protein kinase D1 (PKD1) that activates retrograde axonal transport upon neurotrophin signaling by phosphorylation of intermediate chain subunit of cytoplasmic dynein. Finally, **Chapter IV** discusses the conclusions that can be drawn from this work and their implications for future research.

CHAPTER I

I. Introduction

Parts of this chapter were adapted from previously published work with the permission from the publisher. The citation for the published manuscript is as follows:

Minsu Kang, Lisa Baker, Yuyu Song, Scott T. Brady, Gerardo Morfini. Biochemical analysis of axon-specific phosphorylation events using isolated squid axoplasms. Methods in Cell Biology. 2016;131:199-216. doi: 10.1016/bs.mcb.2015.06.003 Epub 2015 Sep 2.

1.1 Background

1.1.1 Neuronal morphology

Neurons are highly polarized cells with distinct compartments. The neuronal soma contains the nucleus, endoplasmic reticulum and Golgi apparatus that carry out basic cell functions such as transcription and translation. Specialized cytoplasmic compartments projecting from the soma called dendrites and axons receive and propagate electrochemical information from and to other neurons, respectively, by forming synaptic connections. The lack of axonal protein synthesis and long distances between the cell body and discrete axonal and synaptic compartments, which can be thousands-fold times the length of their cell body, impose a unique challenge on neurons. Therefore, appropriate functions and survival of neurons rely on adequate delivery of membrane-bound organelles in both anterograde and retrograde direction by motor proteins,

conventional kinesin and cytoplasmic dynein respectively, in a process referred to as fast axonal transport (FAT).

1.1.2 Axonal transport of intracellular cargoes by molecular motors

Axons comprise the bulk of the total cell volume for most neurons, yet the vast majority of components needed for the generation and maintenance of this compartment, including lipids and membrane proteins, are synthesized in the neuronal cell body. Within the neuronal soma, these components are sorted and packaged into specific membrane-bound organelles (MBOs), which are later delivered to their final sites of utilization in axons by a cellular process collectively known as fast axonal transport (FAT) (Morfini et al. 2012). Anterograde FAT of MBOs from the cell body to axons and dendrites is largely executed by microtubule (MT)-based motor proteins of the kinesin superfamily, whereas MBOs moved in retrograde FAT are transported by cytoplasmic dynein (Hirokawa and Takemura 2005).

1.1.2.1 Conventional kinesin

Conventional kinesin represents the best characterized and most abundant member of the kinesin superfamily in mature neurons (Wagner et al. 1991) and is responsible for anterograde FAT of a wide variety of MBOs including mitochondria, plasmalemmal proteins, and synaptic vesicle precursors (Leopold et al. 1992). This multi-subunit motor protein is a heterotetramer consisting of two kinesin heavy chain (kinesin-1, KHC) and two kinesin light chain (KLC) homodimer subunits and is responsible for specific functional activities of the holoenzyme (DeBoer et al. 2008). Kinesin-1 activities

involve adenosine triphosphate (ATP) hydrolysis and MT-binding, which are essential for processive movement of conventional kinesin along axonal MTs (Wagner et al. 1991). KLCs contain tandem repeat domains that play a major role in the binding of conventional kinesin to MBOs (Stenoien and Brady 1997). Three kinesin-1 and two KLC genes are expressed in mammalian nerve tissue, and isoform-specific sequences located at the carboxy terminus of each subunit appear to mediate the sorting of biochemically heterogeneous forms of conventional kinesin to specific MBOs (DeBoer et al. 2008). The critical role of conventional kinesin and cytoplasmic dynein on neuronal function and the maintenance of axonal connectivity is illustrated by the identification of mutations in specific subunits of these motor proteins, which cause neurodegenerative diseases featuring axonal degeneration as a major pathological hallmark (Morfini et al. 2009; Roy et al. 2005).

1.1.2.2. Cytoplasmic dynein

Cytoplasmic dynein (CDyn) is a large protein complex comprising two heavy chains (DHCs) and multiple other subunits such as intermediate chains (DICs), light intermediate chains (DLICs), and a variety of light chains (DLCs) (Hirokawa et al. 2010). Several accessory proteins have been reported to interact with one or more of the dynein subunits such as lissencephaly1 (Lis1) (Maday et al. 2014), nuclear distribution protein nudE-like 1 (Ndel1) (Kardon and Vale 2009), Bicaudal 1/2 (BICD1/2) (McKenney et al. 2014), and dynactin complex (Kardon and Vale 2009). The compositions of CDyn complex and accessary proteins are thought to take part in various CDyn functions.

The roles of individual subunits and interacting proteins are not well known except for DHCs, which have ATPase activity and MT-binding domains (Carter 2013), and DICs, which appear to mediate the interaction between CDyn and its cargoes (Vaughan et al. 2001; Pfister 2015). Potential roles of other subunits have been studied but are not as well established as those of DHCs and DICs.

1.1.3 Regulation of axonal transport

The restricted distribution of different kinesin-1 MBO cargoes at specific axonal subcompartments (i.e., sodium channels at nodes of Ranvier, or synaptic vesicle precursors to presynaptic terminals) long implied the existence of regulatory mechanisms that allow for localized unloading of protein cargoes (Morfini et al. 2001). A hint on such mechanisms first came from studies showing that both kinesin-1 and KLC subunits are phosphorylated *in vivo* (Hollenbeck 1993; Elluru et al. 1995). More recently, a number of specific protein kinases were identified, which regulate functional activities of conventional kinesin by phosphorylating specific subunits. For example, both c-Jun amino-terminal kinase 3 (JNK3), p38α MAP kinase, and casein kinase 2 (CK2) phosphorylate kinesin-1 subunits at residues located in close proximity to MT-binding domains. Consistent with the enzymatic activities of kinesin-1, these phosphorylation events were found to inhibit processive movement of this subunit along axonal MTs (Morfini et al. 2013; Morfini et al. 2009). On the other hand, both glycogen synthase kinase 3 (GSK3β) and CK2 were shown to phosphorylate KLCs instead, and these events were associated with detachment of conventional kinesin from MBOs (Morfini 2002; Morfini et

al. 2001). Other proteins were found to regulate this motor protein indirectly, by modulating the activity of these kinases above (Morfini et al. 2004; Ratner et al. 1998).

1.1.4 Dying-back neuropathy

Dying-back neuropathy is a pattern of neurodegeneration where distal portion of neurons progressively degenerate towards the cell body *prior* to the neuronal cell death. This type of neurodegeneration has been shown in chemotherapy-induced peripheral neuropathy (CIPN) caused by anti-tumor chemical agents such as vincristine, paclitaxel, eribulin, and ixabepilone, which exert their anti-tumor properties by disrupting microtubule dynamics to inhibit mitosis (Legha 1986; Lobert et al. 1996; Jordan 2012; Boyette-Davis et al. 2015). Dying-back neuropathy is also well characterized in axonal injury where the onset of neuronal cell death is delayed for several days after the injury (Goshgarian et al. 1983; Berkelaar et al. 1994; Dale et al. 1995; Fishman and Parks 1998). In addition, neurons affected in several adult-onset neurodegenerative diseases such as Alzheimer's disease (Kowall and Kosik 1987; Ihara et al. 2010; Bell and Claudio Cuello 2006; Shankar and Walsh 2009; Kanaan et al. 2013), Parkinson's disease (Dauer and Przedborski 2003), amyotrophic lateral sclerosis (Fischer et al. 2004), and Huntington's disease (Morfini et al. 2009; Gatto et al. 2015) follow dying-back pattern of degeneration.

The etiologies of CIPN, axonal injury, and adult-onset neurodegenerative diseases vary significantly from one another, yet they somehow converge onto dying-back neuropathy. One common pathology found in all of them is disruption of microtubule-based fast axonal transport where there is a failure to deliver membrane-bound cargoes to appropriate locations within specific domains within a neuron such as nodes of Ranvier

and synapses. Axonal injuries such as axotomy evidently cause disruption of FAT (Delcroix et al. 1997; Goshgarian et al. 1983), and microtubule-targeting drugs that causes CIPN have been shown to inhibit axonal transport as well (LaPointe et al. 2013). Various adult-onset neurodegenerative diseases also feature axonal transport deficits (Morfini et al. 2006; Morfini et al. 2009; LaPointe et al. 2009; Pigino et al. 2009; Bosco et al. 2010; Kanaan et al. 2011). These findings indicate that inhibition of FAT can lead to dying-back neuropathy.

Studies of genetic mutations in microtubule-based molecular motor proteins conventional kinesin and cytoplasmic dynein (CDyn) further confirmed that FAT impairments suffice to cause dying-back neurodegeneration. For instance, loss-of-function mutations in kinesin heavy chain KIF5A cause axonal degeneration in hereditary spastic paraplegia type 10 and Charcot-Marie-Tooth disease type 2 (Reid et al. 2002; Crimella et al. 2012; Karle et al. 2012; López et al. 2015). Similarly, mutations in CDyn accessory protein p150glued lead to degeneration of motor neurons in several diseases such as spinal-bulbar muscular atrophy (Puls et al. 2005; Hafezparast 2003) and Perry syndrome (Farrer et al. 2009; Konno et al. 2017). Impaired FAT, as evidenced by neuropathological conditions described above, causes synaptic, and axonal dysfunctions eventually leading to dying-back neurodegeneration. The link between FAT defects and dying-back pattern of degeneration is further supported by the differential vulnerability of neurons to such pathology where neurons with long axons (i.e. projection neurons) and extensive axonal arborization are particularly susceptible to dying-back neuropathy compared to those with short axons (i.e. interneurons). However, many of the adult-onset neurodegenerative diseases that feature dying-back neuropathy are not caused by

mutations in motor proteins or physical injury to the axons while still involving FAT impairments. As described above, motor proteins exist as phosphoproteins and phosphorylation of motor proteins by different kinases regulate their functions. This suggests that abnormal activation of protein kinases involved in regulation of motor proteins may underlie FAT defects in neurodegenerative diseases that are not caused by mutations in motor proteins or axonal injury.

1.1.5 Disruption of fast axonal transport in neurodegenerative diseases via aberrant kinase signaling

Several neuropathogenic proteins in these diseases were shown to activate protein kinases involved in motor protein phosphorylation (Morfini et al. 2009). For example, pathogenic forms of huntingtin protein that cause Huntington's disease (HD) were found to inhibit kinesin-1-based FAT through a mechanism involving JNK3 activation (Morfini et al. 2009). Also, both mutant and misfolded forms of superoxide dismutase 1 (SOD1) associated with familial and sporadic forms of amyotrophic lateral sclerosis (ALS) similarly inhibited kinesin-1-based motility. Further study revealed that inhibitory effect of pathogenic forms of SOD1 on axonal transport was mediated by p38 kinase (Bosco et al. 2010; Morfini et al. 2013). Inhibitory effects of the Alzheimer's disease-related proteins tau and beta-amyloid on kinesin-1 function were also identified, which were mediated by GSK3 and CK2, respectively (LaPointe et al. 2009; Pigino et al. 2009; Kanaan et al. 2011). Many neurodegenerative diseases feature dying-back neurodegeneration, and *in vitro* and *in vivo* experimental models demonstrate that neuropathogenic proteins implicated in those diseases inhibit FAT through dysregulation of kinase signaling. Therefore, the

identification of specific kinases that alter phosphorylation of kinesin and CDyn within axonal compartment has important implications for the elucidation of specific pathogenic mechanisms, and the identification of therapeutic targets.

1.2 Specific aims and overall hypothesis

Over decades, our lab and others have identified dozens of kinases that regulate fast axonal transport through phosphorylation of motor proteins. Some kinases inhibit axonal transport of molecular cargoes while others activate it, indicating that phosphoregulation of axonal transport does not *only* represents pathogenic conditions unless there are disturbances in activities of kinases and phosphatases. The work described in the following chapters embodies the overall hypothesis that phosphorylation of motor proteins represents a major regulatory mechanism of FAT. Here, this hypothesis is examined in two different contexts: Huntington's disease in which an aberrant kinase signaling impairs axonal transport, and neurotrophin signaling in which activation of specific kinases promotes retrograde FAT of signaling endosomes.

1.2.1 To address role of c-Jun N-terminal kinase 3 in Huntington's disease pathology

Published work from our lab (Morfini et al., 2006; Morfini et al., 2009) has identified c-Jun N-terminal kinase 3 (JNK3) as the kinase mediating the toxic effect of mutant huntingtin on FAT via phosphorylation of kinesin-1. Impaired axonal transport leads to dying-back pattern of neurodegeneration, which has been shown in Huntington's disease (HD) patients as well as animal models (Gatto et al. 2015). To investigate the role of JNK3

in mutant huntingtin-mediated neurodegeneration *in vivo*, we combined a mouse model of HD (R6/2 mice, as reviewed in Ehrnhoefer et al. 2014) with genetic deletion of JNK3 (R6/2-JNK3$^{-/-}$). Based on our previous finding that JNK3 inhibits fast axonal transport, we hypothesized that HD neuropathology which is recapitulated by R6/2 mice will be ameliorated by genetic deletion of JNK3.

1.2.2 To identify protein kinases that regulate retrograde fast axonal transport of neurotrophin-containing signaling endosomes

Neurotrophins are essential for neuronal survival and functions (Kaplan and Miller 2000). They are released from postsynaptic cells and bind to neurotrophin receptors at the presynaptic terminal. Neurotrophin signaling triggers various local signaling cascades as well as modulating transcriptional activation of multiple genes that play critical roles in neuronal survival and synaptic plasticity (Kaplan and Miller 2000; Reichardt 2006; Park and Poo 2013). In order to produce their transcriptional activation effect, neurotrophins that bind to their receptors must be transported to the neuronal cell body by cytoplasmic dynein (CDyn) in a membrane-bound organelle called signaling endosome (Wu and Mobley 2010; Marlin and Li 2015). While multiple signaling cascades are activated upon ligand binding and subsequent activation of neurotrophin receptors, mechanisms underlying the activation of CDyn-based retrograde FAT of signaling endosomes that contain activated neurotrophin receptors remain elusive. Experiments under this aim were designed to identify protein kinase pathways that trigger retrograde FAT of signaling endosomes in the context of brain-derived neurotrophic factor (BDNF).

CHAPTER II.

II. ROLE OF JNK3 IN MUTANT HUNTINGTIN-MEDIATED AXONAL TRANSPORT DEFECTS

Parts of this chapter were adapted from previously published work with the permission from the publisher. The citation for the published manuscript is as follows:

Minsu Kang, Lisa Baker, Yuyu Song, Scott T. Brady, Gerardo Morfini. Biochemical analysis of axon-specific phosphorylation events using isolated squid axoplasms. Methods in Cell Biology. 2016;131:199-216. doi: 10.1016/bs.mcb.2015.06.003 Epub 2015 Sep 2.

2.1 Background

2.1.1 Huntington's disease

Huntington's disease (HD) is a fatal hereditary neurodegenerative disease featuring motor dysfunctions such as chorea (Reilmann et al. 2001) as well as cognitive (Paulsen 2011) and psychiatric impairments such as depression (Paulsen et al. 2005; Julien et al. 2007). The world-wide prevalence of HD is approximately 3 in 10,000 and it is more common among European and North American populations (Pringsheim et al. 2012). HD is a monogenic disease caused by polyglutamine (polyQ) expansion mutation in the huntingtin (htt) protein (CAG repeat expansion in htt gene) where healthy individuals have between 8 and 36 glutamine repeats while HD patients have 37 or more repeats. Interestingly, the typical age of onset is around 35-50 years (Harper 1996), but the length of the polyQ repeats correlate with earlier onset of the disease with. Patients

with 70 or more repeats often have a juvenile form of HD in which symptoms begin to develop in teenage years (Vonsattel and DiFiglia 1998).

A neuroanatomical hallmark of HD is the massive degeneration of medium spiny neurons (MSNs) in the striatum (Reiner et al. 1988; Deng et al. 2004) as well as the cerebral cortex (Mann et al. 1993). MSNs, which comprise up to 95% of total neurons in the striatum, are GABAergic projection neurons with characteristic medium-sized cell bodies, spiny dendrites and a long axon that projects to external (GPe) or internal (GPi) segments of the globus pallidus and substantia nigra (SN) depending on the subtypes: dopamine receptor D1 -expressing “direct” and D2-expressing “indirect” pathway neurons (Han et al. 2010; Smith et al. 1998; I. J. Mitchell et al. 1999). These MSNs with a long axon are particularly susceptible to degeneration in HD while striatal interneurons, which have short axons and are involved in local circuitry rather than connecting different areas of the brain, are largely spared in HD (Ferrante et al. 1985; Ferrante et al. 1986; Ferrante et al. 1987; Dawbarn et al. 1985; Massouh et al. 2008). Similar to the striatum, there is differential loss of neuronal populations in the cerebral cortex where a subset of pyramidal projection neurons in layers V, VI, and III are lost (Cudkowicz and Kowall 1990; Macdonald and Halliday 2002; Hedreen et al. 1991) while the cortical interneurons are spared in HD (Cudkowicz and Kowall 1990; Sotrel et al. 1991), indicating that longer axons contribute to neuronal vulnerability in HD.

2.1.2 Animal models of HD

Following the discovery of the huntingtin gene as the causative gene and the nature of the mutation that leads to HD in 1993, several animal models of HD have been

generated by introducing either full-length or a fragment of huntingtin gene with varying CAG repeat lengths (Mangiarini et al. 1996; Hemachandra Reddy et al. 1998, 1999; Hodgson et al. 1999; Schilling et al. 1999; Menalled and Chesselet 2002; Slow et al. 2003; Heng et al. 2008; Pouladi et al. 2013). One of the earliest and best-characterized mouse models of HD is the R6/2 mouse, which expresses the *exon* 1 fragment of the human huntingtin gene with around 150 CAG repeats (Mangiarini et al. 1996). The transgene expression in R6/2 mice is driven by the human huntingtin promoter at approximately 75% of endogenous huntingtin expression level. R6/2 mice recapitulate several symptoms of human HD, including motor dysfunctions (J. Y. Li et al. 2005; Garland et al. 2016) and neuropathological changes such as atrophy of the striatum and cortex, progressive loss of cortical projections (Gatto et al. 2015). Transgenic expression of a huntingtin fragment by the exogenous human huntingtin promotor in R6/2 mice seems to cause and earlier pathology and faster progressing pathology than HD in human patients, where clinical symptoms typically develop between 30-50 years old and progression occurs over 15-20 years. In contrast, R6/2 mice have progressive motor dysfunctions starting as early as 4-6 weeks of age and the lifespan of around 3-4 months (Li et al. 2005).

In addition to transgenic mice that express either full-length or a fragment of the human huntingtin gene by an exogenous promoter, there are knock-in mouse models of HD that were generated by replacing parts of the endogenous mouse huntingtin gene with the corresponding domains of the human huntingtin gene with varying CAG repeats lengths. An advantage of knock-in mice is the expression fidelity as mutant huntingtin gene expression is driven by endogenous mouse huntingtin promoter. Some of the disadvantages of using knock-in mice are mild phenotypes—sometimes no phenotypes

in heterozygous mice—and slower disease progression, which makes the research efforts more time-consuming and costly. There are several knock-in mouse models of HD such as Hdh111 (Wheeler et al. 1999; Lloret et al. 2006), CAG140 (Menalled et al. 2003; Rising et al. 2011), Hdh 50, Hdh 100, Hdh 150, Hdh250 (Sathasivam et al. 2013) and zQ175 (Menalled et al. 2012; Peng et al. 2016). One of the key differences among these models is whether or not the exon 1 region is humanized. Hdh150 and Hdh250 mice have completely murine huntingtin gene with increased CAG repeats while others such as Hdh111, CAG140 and zQ175 have an *exon* 1 that features varying degrees of humanization, resulting in a chimeric mouse-human huntingtin.

More recently, zQ175 has gained popularity among HD researchers. Germ line expansions of the CAG track in an older knock-in mouse model with 140 CAG repeats called CAG140 mice (Menalled et al. 2003; Rising et al. 2011) gave rise to a new subline zQ175 with approximately 190 CAG repeats. This zQ175 model was the first knock-in mouse model to exhibit clear disease phenotypes as heterozygotes (Menalled et al. 2012). Although the phenotypes are still not as severe as R6/2 transgenic mice and their lifespan is unaffected, zQ175 mice and its variations are gaining increasing attention within HD research community as most human HD patients are heterozygous for the mutant allele.

2.1.3 Dying-back neurodegeneration in HD

Historically, research efforts on HD therapeutics largely focused on preventing neuronal cell death (Vila and Przedborski 2003; Portera-Cailliau et al. 1995; Estrada Sánchez et al. 2008; Zeron et al. 2001; Hickey and Chesselet 2003; Jakel and Maragos

2000; Chen et al. 2000). However, a growing body of evidence suggest that axonal pathology and synaptic dysfunctions occur prior to neuronal cell death and HD symptoms begin to manifest before cell loss, including previously published data from our lab reporting no neuronal cell death in R6/2 mice even at P90, which is a late stage nearing death in these mice (Gatto et al. 2015). These synaptic dysfunctions include severe deficiencies in dopamine signaling characterized by loss of several components of dopamine signaling such as DARPP-32, ARPP-16, and ARPP-21 (Bibb et al. 2000), increased excitotoxicity due to changes in NMDA receptor functions (Cepeda et al. 2001; Laforet et al. 2001; Klapstein et al. 2001) as well as loss and mislocalization of synaptic proteins (Diprospero et al. 2004; Modregger et al. 2002). Along with synaptic dysfunctions, axonal pathology that long precedes neuronal cell death has been extensively described by several groups. Marked reduction in enkephalin immunoreactivity was reported in (Waters et al. 1988) and GPe (Reiner et al. 1988; Albin et al. 1990) without loss of MSNs, suggesting that degeneration of MSN axons projecting to SN and GP. More recent studies using magnetic resonance imaging (MRI) show white matter abnormalities in early stage HD patients that correlated with cognitive dysfunction (Beglinger et al. 2005). A second imaging modality, diffusion tensor imaging (DTI), revealed white matter disorganization and decreased white matter integrity before significant cell loss in early stage HD patients (Reading et al. 2005; Weaver et al. 2009; Rosas et al. 2010; Ross and Tabrizi 2011). Consistent with findings from early stage HD patients, published work from our lab also demonstrated the pre-symptomatic axonal pathology of transcallosal axons in the R6/2 mouse model of HD by correlating morphometric light microscopy of axons expressing a yellow fluorescent protein (YFP)

reporter with DTI imaging (Gatto et al. 2015). The evidence accumulated over two decades strongly suggests that loss of neuronal connectivity is an early pathogenic event in HD and neuronal death in HD follows the dying-back pattern of neurodegeneration where synaptic terminals and distal portion of the axons degenerate before neuronal apoptosis (Han et al. 2010).

2.1.4 Axonal transport defects in HD

Consistent with the evidence supporting the dying-back pattern of neurodegeneration in HD and the causal relationship between impaired FAT and such degeneration, defects in FAT have been reported in several experimental models of HD. Work from our lab demonstrated that perfusion of mutant huntingtin fragment in squid axoplasms results in inhibition of axonal transport in both anterograde and retrograde directions (Szebenyi et al. 2003; Morfini et al. 2009). Similar results were observed *in vivo* in drosophila where *exon 1* fragment of mutant huntingtin lead to reduced rate of axonal transport as well as accumulation of vesicles and mitochondria in the axon (Gunawardena et al. 2003; Lee et al. 2004; Sinadinos et al. 2009). In addition, studies of transgenic mice expressing full-length huntingtin with polyQ expansion reported impaired transport of vesicles and mitochondria both *in vitro* and *in vivo* using retrograde axonal tracer FluoroGold and fluorescent-labeling of mitochondrial proteins (Trushina et al. 2004; Orr et al. 2008). These reports from various experimental models of HD both *in vitro* and *in vivo* clearly demonstrate that mutant huntingtin causes FAT impairments, and that *exon 1* of huntingtin with expanded CAG repeats is sufficient to cause such impairments.

2.1.5 Alterations in mitochondrial transport

Studies of axonal transport defects in HD often examine the movement of mitochondria that are fluorescently labeled with lipophilic fluorescent dyes (MitoTracker) or expression of mitochondria-targeted proteins with a fluorescent tag. Several groups have reported defects in mitochondrial transport with mutant huntingtin (mHtt) expression (Trushina et al. 2004; Chang et al. 2006; Orr et al. 2008; Shirendeb et al. 2012; Tian et al. 2014). However, recent studies by two independent group reported that mitochondria in mature neurons are predominantly stationary (over 90%) both in primary neurons and *in vivo* (Lewis et al. 2016; Smit-Rigter et al. 2016). Much of the work on mitochondrial transport defects by mHtt is done with either short-term cultures of primary neurons or focus primarily on the transport of mobile mitochondria. Since only a small fraction of mitochondria is mobile in mature neurons, the true contribution of alterations in the mitochondrial transport to mHtt-mediated neuronal dysfunction and degeneration remains unclear. Therefore, studying impairments in axonal transport of highly-mobile vesicles seems more critical in elucidating the pathogenic mechanism of HD.

2.1.6 Proposed mechanisms underlying axonal transport defects in HD

2.1.6.1 Loss-of-function upon polyQ expansion of huntingtin

Huntingtin (Htt) is a relatively large protein (350ka) with no enzymatic activity. The functions of wildtype Htt remain elusive but global knockout of Htt in mice leads to early embryonic lethality (Nasir et al. 1995; Zeitlin et al. 1995), indicating that Htt plays a critical role during development. Htt is enriched in the brain but is expressed in all tissues beyond embryonic development (The Human Protein Atlas). Several potential roles of Htt have

been proposed with the hypothesis that polyQ expansion in mHtt results in loss of the proposed functions, leading to HD phenotypes.

Many of the proposed functions of Htt are related to axonal transport either by direct interaction between Htt and motor proteins or by interactions of Htt with adapter proteins such as huntingtin-associated protein 1 (HIP1) that thought to interact with motor proteins. (Caviston et al. 2007) suggested that Htt binds to dynein in a complex with HIP1 and facilitates dynein-mediated vesicle transport. Another group indicated that Htt and HAP1 regulates autophagosome transport and polyQ expansion or knockout of Htt disrupts autophagosome transport (Wong and Holzbaur 2014). While these interactions have been proposed and some have been accepted for decades, essentially all of data identifying protein complexes were generated by artificial methods such as overexpression of the potential interaction proteins followed by co-immunoprecipitation, two-yeast hybridization and co-localization in confocal microscopy (DiFiglia et al. 1995; Velier et al. 1998; Harjes and Wanker 2003; McGuire et al. 2006; Caviston et al. 2007; Caviston and Holzbaur 2009; Deng et al. 2014; White et al. 2015). Methods such as co-immnoprecipitation of overexpressed protein and two-yeast hybridization are prone to type 1 errors (false positives) as they rely on ectopic overexpression of target proteins, and co-localization using confocal microscopy cannot determine protein-protein interaction because of the resolution limit of ~200nm. While techniques such as Förster resonance energy transfer (FRET) increases the resolution of protein-protein interaction to <10nm, it has the same disadvantage of reliance on overexpression of proteins with fluorescent tags.

With identification of more and more protein-protein interactions, the proposed protein complexes have become larger and larger, and a critical review of these “mega-protein complexes” is needed to address the biological feasibility of such complexes. For instance, one of the widely cited motor protein complexes involving huntingtin is a complex comprising dynein, HAP1, Htt, dynactin, and kinesin associated with membrane-bound cargo. Dynein complex, even without other accessory proteins, includes two heavy chains (530kDa each), two intermediate chains (74kDa each), two light intermediate chains (55kDa each) and two light chains (8kDa each). Dynactin complex is another multi-subunit protein complex with more than 20 subunits ranging from 22 to 150kDa each and has molecular weight around 1MDa. Kinesin complex consists of two heavy chains (~100kDa each) and two light chains (64kDa each). Combining dynein complex (1.2MDa), dynactin complex (1MDa), kinesin complex (320kDa), with HAP1 (75kDa) and Htt (350kDa) results in a >3MDa protein complex, even without including other accessary proteins, that associates with membrane-bound cargo and moves at a speed of 1-4μm/sec. No such complexes have been detected on the surface of vesicles by electron microscopic methods. As mentioned, such “mega-complexes” were inferred from multiple experiments using overexpression of one or two parts of the complex and their existence as a whole complex has never been demonstrated *in vivo* with endogenous proteins. In fact, *lack* of interaction between Htt and motor proteins without overexpression has been shown *in vivo*. Morfini et al. (2009) showed that endogenous Htt does not interact with molecular motors by co-immunoprecipitation of endogenous Htt, kinesin, or dynein. Kinesin light chains co-immunoprecipitate with kinesin heavy chain but not with dynein subunits, and dynein heavy chains co-immunoprecipitate with dynein intermediate chain

but not with kinesin subunits as they are *true* and *independent* complexes. However, Htt does not co-immunoprecipitate with either kinesin or dynein and *vice versa*, indicating that either endogenous Htt does not form a protein complex with motor proteins or such complex exists only transiently.

Alternatively, Colin et al. (2008) proposed that Htt is a positive regulator of fast axonal transport and a molecular switch that can determine the direction of cargo transport, and this function is lost with the polyQ expansion in HD. The same group later proposed another role of Htt as a scaffold protein that brings the full set of glycolytic enzymes to complex with motor proteins for transporting vesicles (Zala et al. 2013), suggesting that glycolysis provides ATP for the transport of vesicles. The reasoning being that mHtt fails to bring the glycolytic enzymes to the vesicles thus inhibiting axonal transport of those vesicles. The brain is the most energy-dependent organ in the body which consumes 20% of the energy while being only 2% of the body weight. Not surprisingly, the brain critically depends on oxidative phosphorylation to generate the most amount of ATP rather than glycolysis (Magistretti and Allaman 2015), and glucose is almost fully oxidized in the brain (Allaman and Magistretti 2013). Further, the fact that local anoxia will block fast axonal transport has been known for over four decades (Ochs 1971; Leone and Ochs 1978), demonstrating the importance of oxidative metabolism for axonal transport. Therefore, it seems unlikely that the inhibition of axonal transport by mHtt is due to failure of localizing glycolytic enzymes to motor proteins. Furthermore, the relative abundance of Htt is likely to be far less than that of glycolytic enzymes such as glyceraldehyde 3-phosphate dehydrogenase (GAPDH). While the exact intracellular molar concentration of Htt in the brain is not known, comparisons of mRNA levels of Htt

and GAPDH within the same sample by three different projects (HPA, GTEx and FANTOM5) reporting in three different dataset (transcripts per million, reads per kilobase per million mapped reads and cap analysis of gene expression tags per million) revealed that Htt mRNA level in the brain is over 100-fold lower than GAPDH mRNA level (The Human Protein Atlas), which suggests that Htt protein is expressed at a far over 100-fold lower level than GAPDH. Combining the low ATP yield by glycolysis and the mismatched stoichiometry of proposed interaction between Htt and GAPDH, the contribution of diminished localization of glycolytic enzymes to vesicles in mHtt-mediated axonal transport defects and even the proposed role of Htt as a scaffold for glycolytic enzymes seem biologically unlikely.

While Htt knockouts are embryonic lethal (Nasir et al. 1995; Zeitlin et al. 1995), a recent and somewhat controversial study using conditional knockout of neuronal Htt in mice reported that the Htt knockout in adult mice did not lead to neurodegeneration (Wang et al. 2016). In addition, gene silencing of mutant Htt allele using antisense oligonucleotides (ASO) has recently passed a Phase 1/2a clinical trial and it demonstrated dose-dependent reduction of mHtt level as well as safety in human HD patients (IONIS-HTTRx; Kordasiewicz et al. 2012; Liu and Zeitlin 2017). These results challenge proposed roles of Htt that are essential for basic neuronal functions in adult neurons. Instead, the suggest that pathogenic mechanism of mHtt-mediated neurodegeneration is more likely to be via gain-of-function upon polyQ expansion rather than loss-of-function. The gain-of-function hypothesis of huntingtin mutation in HD pathogenesis is further supported by transgenic mouse models such as R6/2 mice where

transgenic expression of *exon 1* fragment of mutant huntingtin, without disrupting the expression of mouse huntingtin, is sufficient to cause HD pathology.

2.1.6.2 Kinase-dependent mechanism of fast axonal transport inhibition

In addition to mutations in motor proteins, abnormal kinase signaling has been shown to impair FAT. Molecular motors exist as phosphoproteins and phosphorylation of their subunits can regulate their activity. Works from our lab and others have demonstrated that pathogenic proteins implicated in several unrelated adult-onset neurodegenerative diseases cause aberrant activation of a specific kinase pathway, leading to FAT impairment by abnormal phosphorylation of motor proteins (Morfini et al. 2009; Brady and Morfini 2017).

In isolated axoplasms, perfusion of mHtt induced inhibition of both anterograde and retrograde fast axonal transport (Morfini et al. 2009). This inhibition was prevented by co-perfusion of mHtt with pharmacological inhibitors of c-Jun N-terminal kinases (JNKs) SP600125 or SB203580, indicating that the activation of JNKs by mHtt was necessary for the mHtt-mediated axonal transport inhibition. Further study with perfusions of active recombinant JNK1, 2 and 3 in the isolated axoplasms revealed that JNK3 mediates the inhibition of fast axonal transport by mHtt as perfusion of JNK3 mimics the effect of mHtt on fast axonal transport (Morfini et al. 2009). Active JNK3 can directly phosphorylate kinesin heavy chain subunit of the conventional kinesin at S176 residue, and this this phosphorylation causes detachment of kinesin from microtubules, leading to inhibition of kinesin-based anterograde axonal transport (Morfini et al. 2009). Mechanisms by which mHtt inhibit dynein-based retrograde axonal transport remain unknown, but it is

likely to involve JNK activation since co-perfusion of mHtt with pharmacological inhibitors of JNKs rescues both anterograde and retrograde fast axonal transport. In addition, perfusion of JNK3, but not JNK1 or JNK2, in isolated axoplasms leads to retrograde fast axonal transport, suggesting that JNK3 also mediates the inhibition of retrograde fast axonal transport by mHtt (Morfini et al. 2009).

2.1.7 Rationale and hypothesis

Based on the previous work that identified JNK3 as the mediator of the toxic effect of mHtt on fast axonal transport in squid axoplasms (Morfini et al. 2009), we have generated a mouse model of HD with genetic deletion of JNK3 by crossing R6/2 mice with JNK3$^{-/-}$ mice (R6/2-JNK3$^{-/-}$). R6/2 mice recapitulate many of the hallmark behavioral and neuropathological phenotypes of HD such as striatal and cortical degeneration. Recent work from our lab has documented pre-symptomatic axonal degeneration of transcallosal axons in R6/2 mice with a YFP reporter (Gatto et al. 2015). As impairments in axonal transport lead to a dying-back pattern of neuropathy and JNK3 is critical for mHtt-mediated axonal transport defects, we hypothesize that JNK is aberrantly activated by mHtt, and genetic deletion of JNK3 will ameliorate HD phenotypes observed in R6/2 mice.

2.2 Methods and materials

2.2.1 Recombinant huntingtin proteins

Recombinant huntingtin proteins used in this study have been provided by CHDI Foundation. GST-tagged N-terminal fragment of human huntingtin protein corresponding to amino acids 1-548 with either 16 (Htt-Q16) or 55 glutamines (Htt-Q55) were produced in E. Coli, purified, and reconstituted in 20mM HEPES by Dr. Andrea Caricasole (IRBM Science Park, Italy). The quality and relative amounts of each construct were assessed prior to use by immunoblotting 20ng of each construct with anti-Htt antibody clone 4C9 (1:10,000 dilution) **(Figure 1)**.

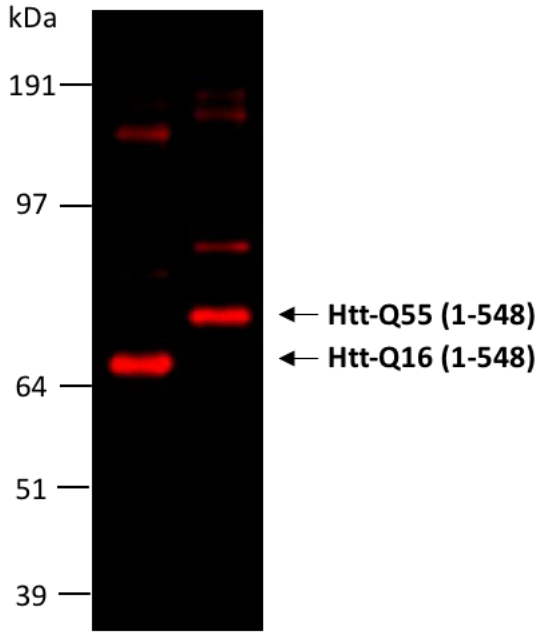

Figure 1. Recombinant huntingtin proteins used in this study. 20ng of each construct was loaded on 4-12% Bis-Tris gel and analyzed by immunoblotting with anti-Htt antibody clone 4C9. Western blot image shows comparable amount of proteins.

2.2.2 Mice

Female mice with ovary transplanted from heterozygous R6/2 mice expressing ~1kb of human huntingtin gene with 160 +/- 5 CAG repeats in CBA X C57BL/6J were purchased from The Jackson Laboratory (cat #002810). The ovarian transplanted female mice were paired with wild-type C57BL/6J mice (The Jackson Laboratory cat #000664) to generate wild-type and heterozygous R6/2 mice used in the study. Female JNK3 knockout ($JNK3^{-/-}$) mice in C57BL/6J background were purchased from The Jackson Laboratory (cat #004322) and paired with R6/2 males to generate R6/2-$JNK^{+/-}$ males, then paired with $JNK3^{-/-}$ females to generate R6/2-$JNK3^{-/-}$ mice. Both male and female mice were used in the study. All mice were housed in the barrier facility at University of Illinois at Chicago (UIC) and the protocols involving animal use have been approved by Institutional Animal Care and Use Committee at UIC.

2.2.3 Mouse brain dissection and preparation of mouse brain lysate

Upon euthanasia by CO2, mouse brain was obtained and sliced using ice-cold sagittal slicing matrix with 1.0mm intervals (Zivic Instruments cat #BSMAS001-2). From ML +/-1-3mm slices, cortex, striatum and cerebellum tissues were collected using biopsy punch with 2.0mm diameter (Miltex #33-31-P/25) in fresh lysis buffer (1% SDS in 50mM HEPES with 2mM NaF and 10mM Na_3VO_4). Collected tissues were quickly boiled at 90˚C for 2 minutes, homogenized using plastic pestle, then sonicated (20% amplification, 6 cycles; 1 cycle = 5s on, 2s off). Sonicated samples were centrifuged at 21130 x g in Eppendorf 5424 tabletop centrifuge (15000 rpm) at 4˚C for 15 minutes twice, transferring the supernatant to a new tube each time. The protein concentrations of the prepared

lysates were measured by BCA protein assay using Pierce BCA protein assay kit (ThermoFisher #23225). Following BCA assay, sample concentrations were adjusted to 1µg/µL by mixing appropriate amount of the lysate, lysis buffer and 6X Laemmli sample buffer.

2.2.4 Isolated squid axoplasm preparation and perfusion

Detailed descriptions of isolated squid axoplasm preparation and perfusion can be found in published work from our lab (Song et al., 2016; Kang et al., 2016) **(Figure 2)** and an example of the methods of validating antibodies for use in squid axoplasms and detecting changes in kinase activity in isolated squid axoplasms are provided in **Figure 3**. Briefly, pairs of "sister" axons were dissected from the Atlantic squid *Loligo pealeii* at Marine Biological Laboratory in Woods Hole. After removing connective tissues, the axoplasms were extruded on glass slides (Corning cat #2948-75X25) and a hydrophobic boundary was drawn along the contour of the axoplasm to keep perfusion solution on the slides. One of the sister axoplasm was perfused with 200nM recombinant Htt-Q16 (described above) in Buffer X/2 and the other sister axoplasm was perfused with 200nM recombinant Htt-Q55. After 50 minutes of incubation, the axoplasms were lysed with 1% SDS and the lysates were collected into 1.5mL Eppendorf tubes and 6X Laemmli sample buffer was added and stored in -20˚C until analysis by western blotting with antibodies validated to recognize squid variants of their target. A non-exhaustive list of antibodies validated in the squid axoplasm is provided in **Table 1**.

Antigen	Host	Clone	Source
Kinesin-1A, 1B, 1C	Mouse monoclonal	H2	Chemicon
Kinesin light chains	Mouse monoclonal	63-90	In-house
Kinesin light chains	Mouse monoclonal	KLC-All	In-house
α-Tubulin	Mouse monoclonal	DM1A	Sigma-Aldrich
Phosphorylated neurofilament	Mouse monoclonal	SMI-31	BioLegend
Phosphorylated neurofilament	Mouse monoclonal	RMO55	EMD Millipore
SNAP-25	Mouse monoclonal	71.1	Synaptic Systems
p-JNK (Thr183/Tyr185)	Mouse monoclonal	G9	Cell Signaling #9255
p-JNK (Thr183/Tyr185)	Rabbit polyclonal		Cell Signaling #9251
p-JNK (Thr183/Tyr185)	Rabbit monoclonal	81E11	Cell Signaling #4668
p-MKK3 (Ser189)/MKK6 (Ser207)	Rabbit monoclonal	22A8	Cell Signaling #9236
p-MKK7 (Ser271/Thr275)	Rabbit polyclonal		Cell Signaling #4171
p-p38 (Thr180/Tyr182)	Rabbit monoclonal	3D7	Cell Signaling #9215
p-GSK3β (Ser9)	Rabbit monoclonal	D85E12	Cell Signaling #5558
p-ERK (Tyr204)	Mouse monoclonal	E-4	Santa Cruz Biotech sc-7383

Table 1. Validated antibodies that cross-react with squid versions of their target proteins. (Table originally published in Kang et al. 2016)

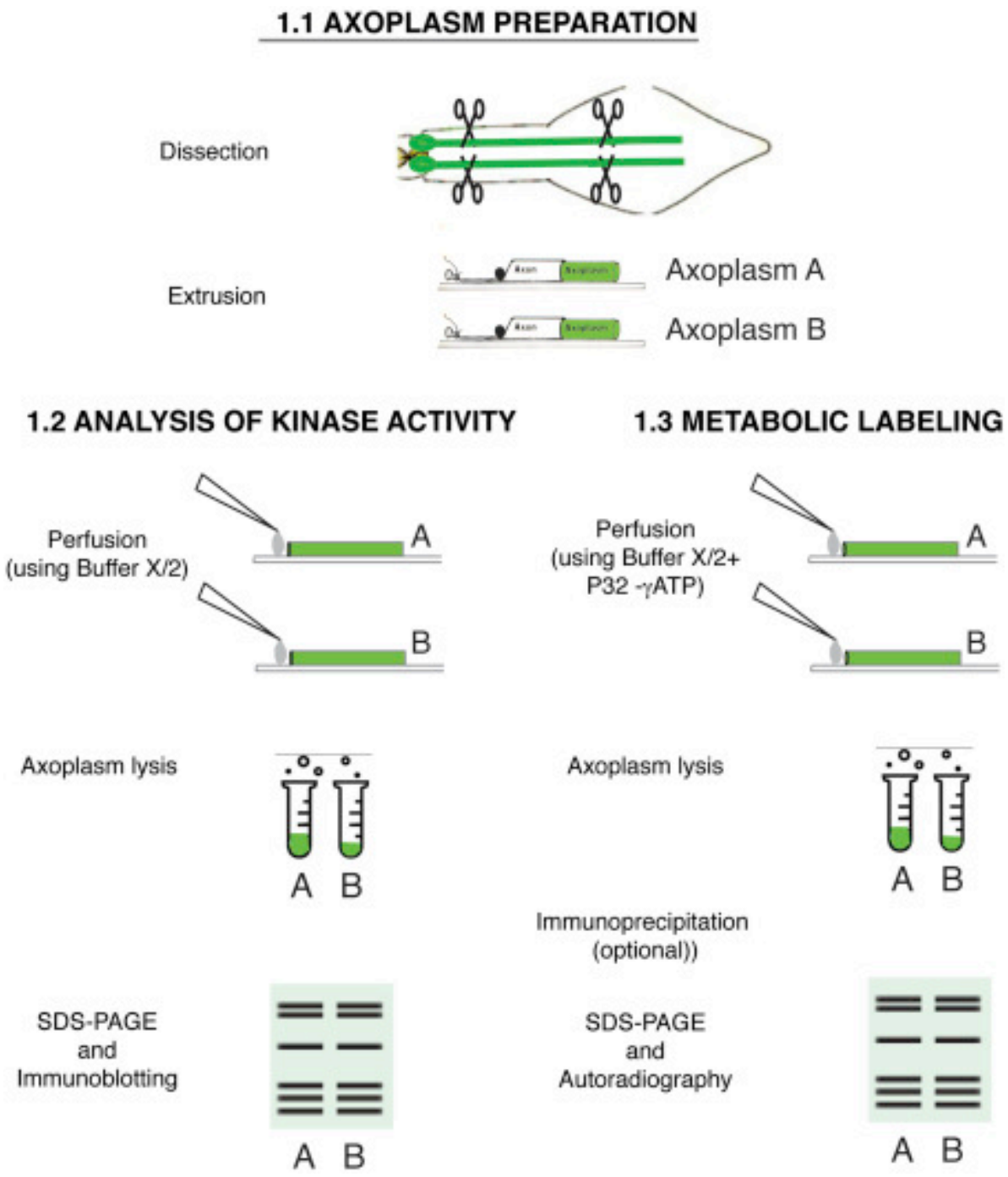

Figure 2. Outline of procedures in isolated squid axoplasms. Owing to intrinsic differences between different squid, two "sister" axons need to be dissected from the same squid, extruded, and placed on the glass coverslips. One axoplasms is perfused with control perfusion mix and its "sister' axoplasms with experimental perfusion mix. Metabolic labeling experiments require perfusion mixes containing P32-radiolabeled adenosine triphosphate. After 50-min incubation, axoplasms are lysed and processed for immunoblotting. For metabolic labeling procedures, radiolabeled axoplasmic proteins are immunoprecipitated and analyzed by autoradiography. (Figure originally published in Kang et al. 2016)

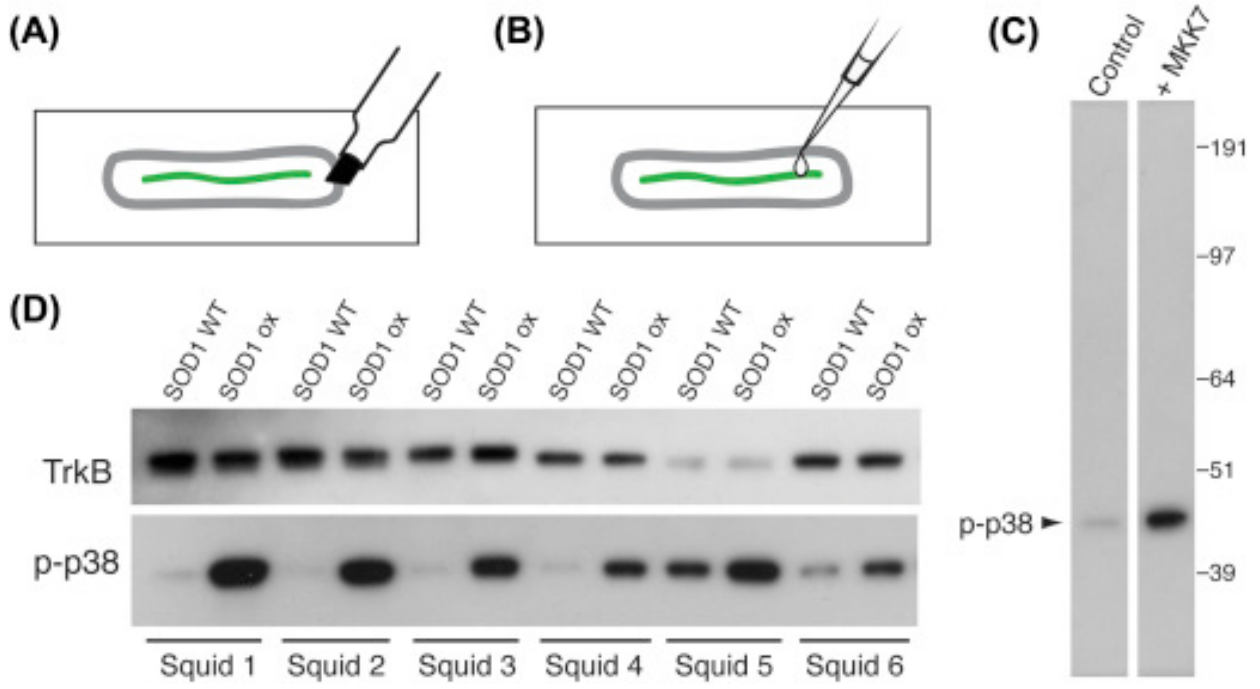

Figure 3. An example of biochemical analysis of axoplasms upon perfusion. Axon-autonomous effects of properly folded, wild type (SOD1-WT) and oxidized (SOD1-ox) forms of superoxide dismutase 1 on p38 mitogen-activate kinase (MAPK) activity. **(A)** A schematic of the use of the liquid blocker pen (i.e. Super Pap Pen). A liquid blocking line is drawn along the contour of the axoplasm on the glass slide approximately 3 mm away from the axoplasm. **(B)** Using a P100 or P200 pipette, perfusion mixes are placed within the boundary drawn with the Pap Pen. If needed, coverslips are tilted to ensure thorough perfusion of the axoplasm. **(C)** Validation of rabbit monoclonal antibody clone 3D7 (p-p38 antibody; Cell Signaling #9215) in squid axoplasm. Two axoplasms were homogenized in Buffer X/2 with ATP using a pipette. The homogenate was briefly centrifuged and similar aliquots of the supernatant were incubated with (+MKK7) or without (Control) recombinant mitogen-activated kinase kinase (MKK7) (purchased from EMD Millipore), a kinase that directly phosphorylates and activates p38 MAPK. After a 30-min incubation, samples were processed for immunoblotting with antibody 3D7. As expected, increased immunoreactivity was observed in the aliquot incubated with MKK7, compared to its control. **(D)** "Sister" axoplasms were dissected from six squid (Squid 1-6) and perfused with recombinant forms of superoxide dismutase 1 (SOD1, 5μM). One axoplasm was perfused with SOD1-WT and its "sister" axoplasm with SOD1-ox associated with sporadic forms of amyotrophic lateral sclerosis (Bosco et al., 2010). After a 50-min incubation, axoplasms were lysed, separated by SDS-PAGE and processed for immunoblotting using monoclonal antibody 3D7 (as in C). Note the variable levels of active (phosphorylated) p38 among different squid. Despite this variability, p-p38 immunoreactivity is consistently higher in axoplasm perfused with SOD1-ox than their "sister" counterparts perfused with SOD1-WT. A band recognized by a polyclonal antibody against TrkB (Santa Cruz, sc-11, Lot E0708) provided an internal control for protein loading. A protion of the p-p38 immunoblot (Squid 1-3) was shown in Boscoe et al, 2010. (Figure originally published in Kang et al. 2016)

2.2.5 Post-mortem tissues

Human post-mortem cortex tissues from non-HD and HD patients were provided by Dr. Jeffery Kordower at Rush University (originally obtained from New York Brain Bank at Colombia University). Subjects included both male and female with age ranging from 28 to 75. HD patients were diagnosed as HD stage 3/4 and CAG repeat lengths ranged from 42 to 47. Detailed information for each subject is listed in **Table 2**.

Case ID	Age	Sex	Cold PMI (HH:MM)	Frozen PMI (HH:MM)	Diagnosis	PolyQ Length
4915	62	M	04:00	04:30	Encephalopathy of hypoxic-ischemic type, moderate	N/A
4523	62	F	04:33	22:38	Encephalopathy of hypoxic-ischemic type, acute	N/A
320	28	F	02:09	05:57	Control	N/A
4518	56	M	N/A	08:50	Control	N/A
2476	60	M	00:00	22:55	HD 3/4	43
4225	72	F	00:15	48:35	HD 3/4	42
4340	75	M	02:45	08:20	HD 3/4	43
4921	47	M	N/A	34:45	HD 3/4	46
4988	56	M	00:05	15:25	HD 3/4	47
5109	64	M	00:06	38:01	HD 3/4	43
5124	57	M	00:48	18:38	HD 3/4	43

Table 2. Demographic information of human samples used in this study. PMI: Post-mortem interval, which indicates the interval between the time of death and the time cadavers were placed in cold or frozen. Huntington's disease clinical progression follows 5 diagnostic stages based on symptoms: HD 1 – Preclinical/Prodromal Stage, HD 2 – Early Stage, HD 3 – Middle Stage, HD 4 – Late Stage, HD 5 – End of Life.

2.2.6 Western blotting

For western blotting, 10-20µg of brain lysate samples prepared as described above were loaded on 4-12% Bis-Tris gels (Invitrogen cat #NP0336) and ran in MOPS running buffer at 120V for 2 hours. Following electrophoresis, the proteins were transferred onto a 0.45µm Immobilon-P PVDF membrane (Millipore cat #IPVH00005) or Immobilon-FL (Millipore cat #IPFL00005) in Towbin buffer (25mM Tris, 192 mM glycine and 10% MeOH) at 400mA for 2 hours with cooling system maintained at 4˚C. After transfer, the membranes were incubated in blocking buffer (1% non-fat milk in tris-buffered saline with 0.1% Tween-20; TBS-T) for 1hr in room temperature. After blocking, membranes were washed three times with TBS-T for 5 minutes each wash. Then the membranes were incubated overnight with primary antibodies diluted in 1% BSA in tris-buffered saline (TBS) with following dilutions: anti-phospho-JNK (Cell Signaling #9251, 1:500), anti-GAPDH (Sigma #G8795, 1:300,000), anti-JNK1 (BD Biosciences #554286, 1:1000), anti-JNK2 (Cell Signaling #4672, 1:500), anti-JNK3 (Cell Signaling #2305, 1:6000) for human samples; anti-DARPP32 (Cell Signaling #2306, 1: 2,000,000), anti-GAPDH (Sigma #G8795 ,1:200,000), anti-PSD95 (BD Biosciences #610495, 1:4000), anti-TH (Pel-Freez #P40101-0, 1:200,000), anti-JNK1 (BD Biosciences #554286, 1:500), anti-JNK2 (Cell Signaling #4672, 1:500), anti-JNK3 (Cell Signaling #2305, 1:10,000), anti-phospho-JNK (Cell Signaling #4668, 1:30,000) for mouse samples; anti-KHC (H2, In-house, 1:10,000), anti-phospho-JNK (Cell Signaling #4668, 1:10,000), anti-phospho-MKK7 (Cell Signaling #4171, Lot 3, 1:500) and anti-Htt (4C9, CHDI Foundation, 1:100,000) for isolated squid axoplasm samples. Characterization of antibodies used in this study is shown in **Figure 4**. The next day, the membranes were washed three times with TBS-T for 5 minutes each

wash and incubated with secondary antibodies diluted in 1% milk in TBS for 1 hour in room temperature with following dilutions: goat anti-mouse IgG-HRP conjugate (Jackson Laboratories #115-035-146, 1:30,000), goat anti-rabbit IgG-HRP conjugate (Jackson Laboratories #111-035-144, 1:30,000). After incubation with secondary antibodies, membranes were washed three times with TBS-T for 5 minutes each wash and the signals were detected via chemiluminescence using WesternSure PREMIUM chemiluminescent substrate (LI-COR) and HyBlot CL autoradiography film (Denville Scientific). Some immunoblots were analyzed using LI-COR Odyssey CLx/Fc near-infrared (NIR) imaging systems. Electrophoresis and transfer procedures for LI-COR NIR imaging was identical as chemiluminescence. Following transfer, the PVDF membranes were incubated with blocking buffer without tween-20 (1% non-fat milk in TBS). Primary antibody incubation procedure for LI-COR NIR imaging was identical as chemiluminescence. Secondary antibodies were diluted in 1% milk in TBS-T with 0.01% SDS and incubated for 45 minutes to 1 hour in room temperature with following dilutions: goat anti-mouse IgG IRDye 680RD (LI-COR #925-68070, 1:10,000) and goat anti-rabbit IgG IR Dye 800CW (LI-COR #925-32211, 1:10,000).

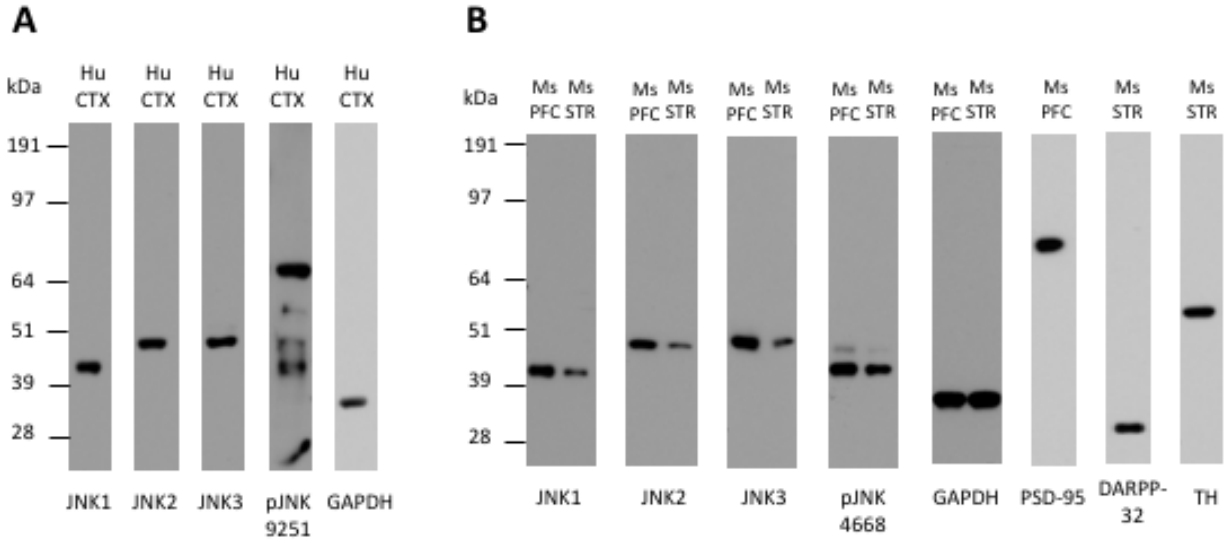

Figure 4. Characterization of antibodies used in this study. (A) Characterization of antibodies used in post-mortem human cortex samples. **(B)** Characterization of antibodies used in mouse prefrontal cortex (Ms PFC) and mouse striatum (Ms STR) samples. JNK1 (mouse monoclonal, BD Biosciences #554286), JNK2 (rabbit polyclonal, Cell Signaling #4672), JNK3 (Rabbit monoclonal, Cell Signaling #2305), pJNK 9251 (rabbit monoclonal, Cell Signaling #9251), pJNK 4668 (rabbit monoclonal, Cell Signaling #4668), GAPDH (mouse monoclonal, Sigma #G8795), PSD-95 (mouse monoclonal, Cell Signaling #36223), DARPP-32 (rabbit monoclonal, Cell Signaling #2306), TH (rabbit polyclonal, Pel-Freeez #40101-0)

2.2.7 Statistical analysis

Western blot images from chemiluminescence were scanned via negative film scanning and analyzed by densitometry tool in ImageJ. Western blot images from LI-COR near-infrared imaging system were analyzed by LI-COR ImageStudio software. Statistical analyses were performed using Prism 7 software for all experiments. For the comparisons of non-HD and HD human post-mortem tissues, two-tailed unpaired t-tests were performed to assess statistical significance between groups. For the analysis of isolated squid axoplasms, a two-tailed paired t-test was performed to compare two experimental conditions within sister axoplasm pairs due to variability among squid. For the comparisons of different genotypes in mice, two-tailed unpaired t-tests were performed for comparisons between two groups, and one-away analysis of variance (ANOVA) was performed for comparisons among three groups. Following statistically significant one-way ANOVA results, Tukey's post-hoc analysis was performed in order to reveal the statistically significant comparisons.

2.3 Results

2.3.1 Activation of JNK in HD

Previous work from our lab demonstrated activation of JNK in the cell lysates of NSC34 cells transfected with Htt-Q56 compared to those transfected with Htt-Q18 (Morfini et al. 2009). Here, we report activation of JNK in post-mortem cortex of HD patients as well as experimental models of HD: isolated squid axoplasms used in previous study (Morfini et al. 2009) and R6/2 mice used in current study.

2.3.1.1 Increased activation of JNK in post-mortem cortex of HD patients

Western blot analysis of post-mortem cortex samples from non-HD controls (n=4) and HD patients (n=7) showed increased level of pJNK normalized to that of GAPDH in HD patient samples compared to control **(Figure 5A, 5B)**. Non-parametric unpaired two-tailed t-test revealed that this difference between non-HD controls (M[1] =0.07425, SD[2]=0.04648, 95% CI[3] [<0.001, 0.1482]) and HD patients (M=0.9389, SD=0.5975, 95% CI [0.3863, 1.491]) was statistically significant (p=0.0061, 95% CI [0.149, 1.524]). Non-parametric unpaired two-tailed t-test analysis found no significant difference in the level of total JNK3 normalized to that of GAPDH between non-HD controls (M=2.49, SD=0.3368, 95% CI [1.954, 3.025]) and HD patients (M=2.292, SD=0.6394, 95% CI [1.701, 2.883]) (p=0.4121, 95% CI [-0.825, 0.596]) **(Figure 5A, 5C)**. The demographic information regarding the human samples used in this study is listed in **Table 2**.

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[1] M: mean
[2] SD: standard deviation
[3] CI: confidence interval

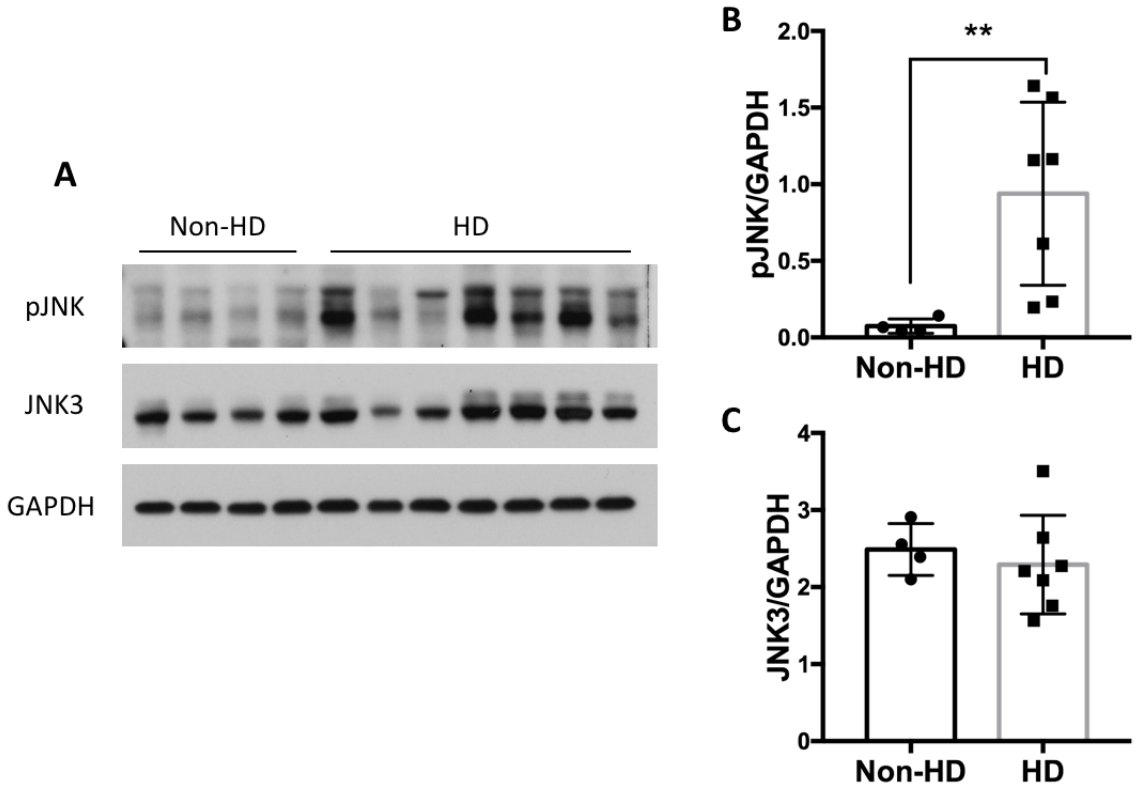

Figure 5. Increased activation of JNK in the cortex of HD patients. (A) Western blot analysis of the levels of pJNK and JNK3 in the cortices of non-HD controls and HD patients. GAPDH is provided as a loading control. **(B)** Two-tailed t-test revealed a statistically significant increase in the level of pJNK normalized to that of GAPDH in HD patients compared to non-HD controls, **p=0.0.0061. **(C)** Two-tailed t-test revealed no significant difference between the level of JNK3 normalized to that of GAPDH in non-HD controls compared to HD, p=0.4121. Error bars represent SD.

2.3.1.2 Activation of JNK by mHtt in isolated squid axoplasms

Activation of JNK by mHtt was analyzed in two different experimental models of HD used in this study: isolated squid axoplasms and R6/2 mice. Axoplasms were isolated from squid giant axons as described in **2.2**. A total of 7 pairs of isolated squid axoplasms were used, with one axon of each pair perfused with recombinant Htt-Q16 (Htt) and the other with Htt-Q55 (mHtt) proteins described in **2.2** at a concentration of 200nM and analyzed by western blotting and imaged on a LI-COR Odyssey Fc near-infrared imaging system. Following image acquisition, the signals for KHC and pJNK were obtained using LI-COR Image Studio software. Non-parametric paired two-tailed t-test using Prism 7 software revealed a statistically significant increase in the level of pJNK that were normalized to that of KHC in axoplasms perfused with Htt-Q55 (M=0.0239, SD=0.007671, 95% CI [0.01629, 0.03048]) compared to their Htt-Q16 perfused sister axoplasms (M=0.02066, SD=0.008452, 95% CI [0.01284, 0.02847]), p=0.0156 **(Figure 6)**. Due to the inherent differences among wild-caught squid **(see Figure 3)**, the differences between sister axoplasms are represented as % change in Htt-Q55 from Htt-Q16 instead of raw normalized values **(Figure 6B)**.

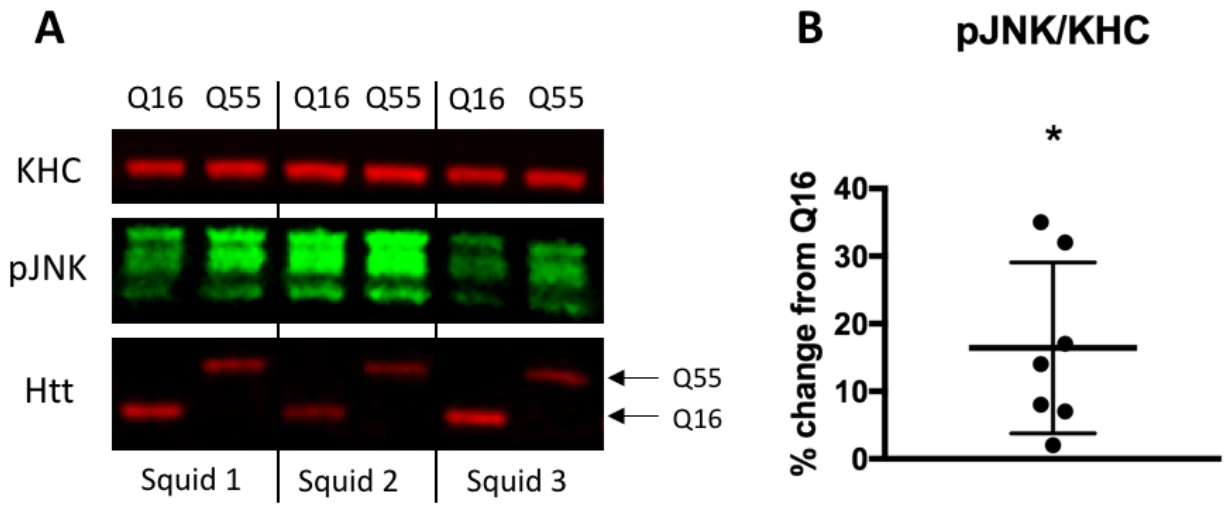

Figure 6. Increased activation of JNK in isolated squid axoplasms perfused with mHtt. (A) A representative immunoblot from 3 pairs of isolated squid axoplasms perfused with either recombinant Htt-Q16 or Htt-Q55 (200nM). Axoplasms perfused with Htt-Q55 shows increased level of pJNK compared to their sister axoplasms perfused with Htt-Q16. KHC is provided as a loading control and Htt demonstrates comparable level of perfused recombinant Htt-Q16 and Q55. **(B)** Two-tailed paired t-test of 7 pairs of axoplasms revealed a statistically significant increase in the level of pJNK normalized to that of KHC in axoplasms perfused with Htt-Q55 compared to their sister axoplasms perfused with Htt-Q16, *p=0.0156. The difference between sister axoplasms are represented as % change in Htt-Q55 from Htt-Q16. Error bars represent SD.

2.3.1.3 Activation of JNK in R6/2 mouse model of HD

To evaluate the activation of JNK by mHtt *in vivo*, cortices and striata from postnatal day 50 (P50) wildtype (n=5) and R6/2 (n=5) mice, which is a symptomatic age for R6/2 mice, were collected and analyzed by western blot as described in **2.2**. Unpaired two-tailed t-test with Welch's correction revealed a statistically significant increase in the level of pJNK normalized to that of GAPDH loading control in the prefrontal cortex (PFC) of R6/2 mice (M=1.556, SD=0.2809, 95% CI [1.207, 1.905]) compared to that of wildtype mice (M=1.098, SD=0.2697, 95% CI [0.7634, 1.433]), t(7.987)=2.63, p=0.0302 **(Figure 7A, 7C)**, as well as in the striatum (STR) of R6/2 mice (M=0.8432, SD=0.2357, 95% CI [0.5505, 1.136]) compared to that of wildtype mice (M=0.453, SD=0.1501, 95% CI [0.2667, 0.6393]), t(6.785)=3.122, p=0.0175 **(Figure 7B, 7D)**.

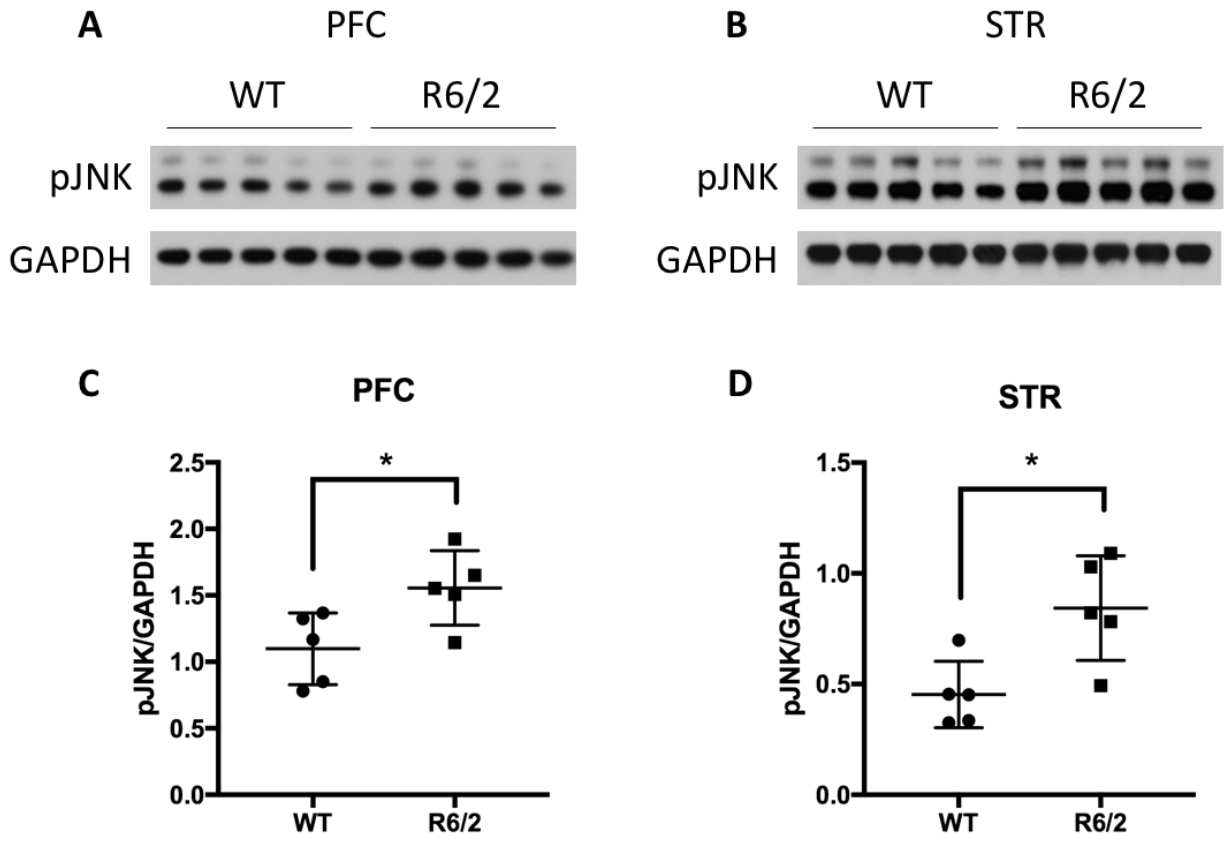

Figure 7. Increased activation of JNK in R6/2 mouse model of HD. R6/2 mice (P50) shows increased level of pJNK compared to age-matched wildtype mice in both prefrontal cortex (PFC) **(A)** and striatum (STR) **(B)**. Unpaired two-tailed t-test with Welch's correction revealed a statistically significant increase in pJNK in PFC, *p=0.0302 **(C)**, and STR, *p=0.0175 **(D)**. Error bars represent SD.

2.3.2 Characterization of R6/2 mice

R6/2 mice begin to show defects in motor functions around P45 and typically die around P100. Before investigating the effect of JNK3 knockout in these mice, we characterized the biochemical pathology of R6/2 mice at P45-P50, which have been previously reported by others. R6/2 mice at this age do not have significant neuronal cell loss (Gatto et al. 2015), but several reports demonstrate reduced levels of synaptic markers in the striatum, indicating synaptic dysfunction and loss prior to cell death.

2.3.2.1 Biochemical analysis of loss of synaptic connectivity in the striatum of R6/2 mice

Mice were euthanized and cortex and striatum samples were collected as described in **2.2**. Total levels of two synaptic markers, postsynaptic density protein (PSD-95) and dopamine- and cyclic-AMP-regulated phosphoprotein of molecular weight 32,000 (DARPP-32), were determined in the striata of wildtype (n=5) and R6/2 (n=7) mice at P45. Unpaired two-tailed t-test with Welch's correction revealed a statistically significant reduction in the level of PSD-95 normalized to that of GAPDH in R6/2 mice (M=0.1754, SD=0.03074, 95% CI [0.1469, 0.2038]) compared to wildtype mice (M=0.6852, SD=0.2848, 95% CI [0.3316, 1.039]), t(4.067)=3.986, p=0.0158 **(Figure 8A, 8B)** as well as the level of DARPP-32 normalized to that of GAPDH in R6/2 mice (M=0.223, SD=0.1031, 95% CI [0.1277, 0.3184]) compared to wildtype mice (M=0.7239, SD=0.3197, 95% CI [0.3269, 1.121]), t(4.599)=3.38, p=0.0224 **(Figure 8A, 8C)**.

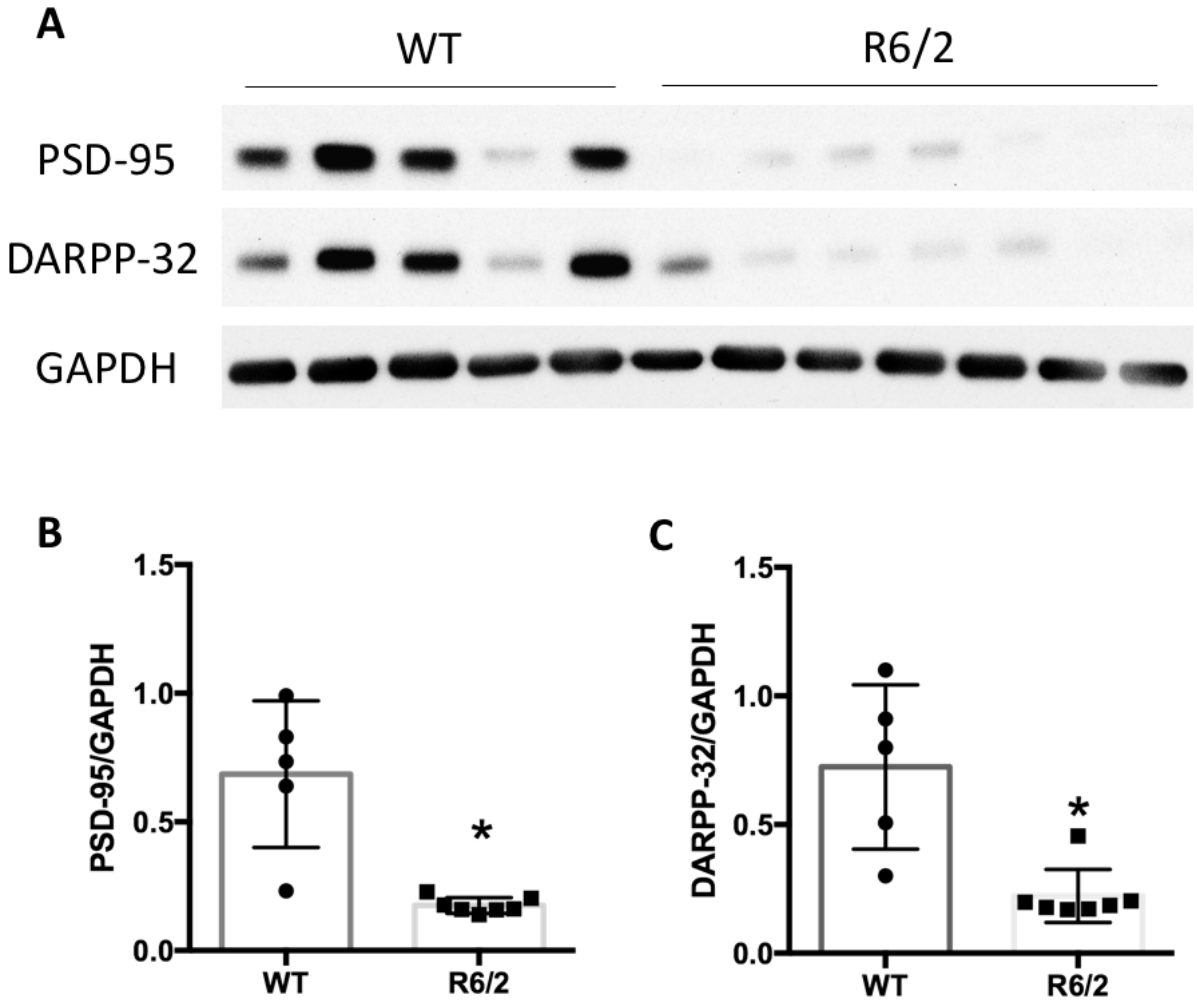

Figure 8. Loss of synaptic markers in the striatum of R6/2 mice. (A) Western blot image of synaptic markers PSD-95, DARPP-32 in the striatum of R6/2 mice at P45. GAPDH is provided as a loading control. Unpaired two-tailed t-test with Welch's correction revealed a statistically significant reduction in the level of PSD-95, *p=0.0158, **(B)** and DARPP-32, *p=0.0224, **(C)** in R6/2 mice. Error bars represent SD.

2.3.3 Characterization of JNK3$^{-/-}$ mice

Genetic deletion of one isoform of can lead to alterations in the expression levels of other isoforms in order to compensate for the deletion. To test whether or not the deletion of JNK3 in mice affect the expression levels of JNK isoforms as well as the expression levels of biochemical markers of interest, we compared striatum of wildtype and JNK3$^{-/-}$ mice at P50.

2.3.3.1 JNK isoform expression in JNK3$^{-/-}$ mice

Mice were euthanized and striatum samples from wildtype (n=3) and JNK3$^{-/-}$ mice (n=3) were collected and analyzed by western blot with antibodies for JNK1, JNK2 and JNK3. As expected, no JNK3 was detected in JNK3$^{-/-}$ mice, confirming the genetic deletion in these mice **(Figure 9A, 9D)**. No statistical analysis could be done for the comparison of JNK3 levels between wildtype and JNK3$^{-/-}$ mice as their values in JNK3$^{-/-}$ mice were zero. Unpaired two-tailed t-test with Welch's correction revealed a statistically significant increase in the level of JNK1 normalized to that of GAPDH in the striata of JNK3$^{-/-}$ mice (M=0.878, SD=0.02536, 95% CI [0.815, 0.941]) compared to wildtype mice (M=0.369, S=0.09642, 95% CI [0.1295, 0.6085]), t(2.275)=8.843, p=0.0083 **(Figure 9A, 9B)**, but there was no significant difference between the level of JNK2 between wildtype (M=0.4967, SD=1.064, 95% CI [0.2325, 0.7609]) and JNK3$^{-/-}$ mice (M=0.474, SD=0.2671, 95% CI [-0.1895, 1.138]), t(2.619)=0.1366, p=0.9012 **(Figure 9A, 9C)**.

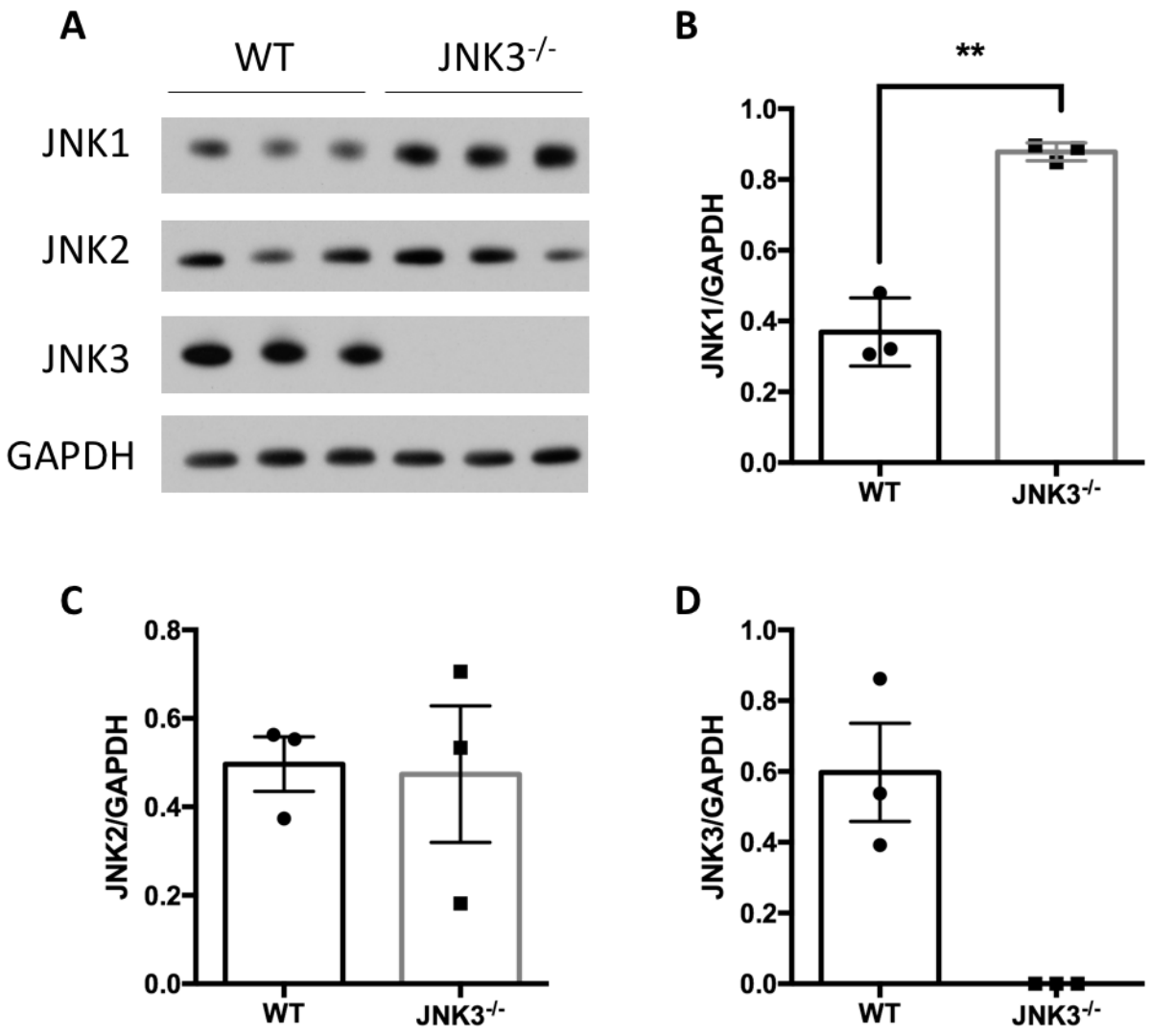

Figure 9. Expression of JNK isoforms in the striatum of JNK3$^{-/-}$ mice. (A) Western blot image of JNK1, JNK2 and JNK3 expression in the striatum of P50 wildtype and JNK3$^{-/-}$ mice. GAPDH is provided as a loading control. Unpaired two-tailed t-test with Welch's correction revealed a statistically significant increase in the amount of JNK1 **(B)**, **p=0.0083, but not JNK2 **(C)** in JNK3$^{-/-}$ mice compared to wildtype mice. No JNK3 expression was detected in JNK3$^{-/-}$ mice **(D)**. Error bars indicate SD.

2.3.3.2 Comparison of the expression of synaptic and neuronal connectivity markers in the striatum of JNK3$^{-/-}$ mice

Mice were euthanized and striata from wildtype (n=3) and JNK3$^{-/-}$ mice (n=3) were collected and analyzed by western blot with antibodies for PSD-95, DARPP-32 and tyrosine hydroxylase (TH). TH is a rate-limiting enzyme for the production of dopamine and is used as a marker for dopaminergic neurons. As resident neurons in the striatum are GABAergic and do not express TH, the amount of TH detected in the striatum is an indirect measure of processes stemming from the dopaminergic neurons in substantia nigra. Unpaired two-tailed t-test with Welch's correction showed no significant difference in the levels of PSD-95 ($M_{wildtype}$=0.9433, $SD_{wildtype}$=0.368, 95% $CI_{wildtype}$ [0.02917, 1.858]; $M_{knockout}$=1.06, $SD_{knockout}$=0.1078, 95% $CI_{knockout}$ [0.7921, 1.328], t(2.341)=0.5269, p=0.6440), DARPP-32 ($M_{wildtype}$=0.6147, $SD_{wildtype}$=0.1844, 95% $CI_{wildtype}$ [0.1566, 1.073]; $M_{knockout}$=0.8077, $SD_{knockout}$=0.1521, 95% $CI_{knockout}$ [0.4297, 1.186], t(3.861)=1.398, p=0.2370) and TH ($M_{wildtype}$=0.4623, $SD_{wildtype}$=0.1791, 95% $CI_{wildtype}$ [0.0174, 0.9073]; $M_{knockout}$=0.5123, $SD_{knockout}$=0.0.3506, 95% $CI_{knockout}$ [-0.3586, 1.383], t(2.977)=0.22, p=0.8401) normalized to that of GAPDH in the striata of wildtype and JNK3$^{-/-}$ mice **(Figure 10)**.

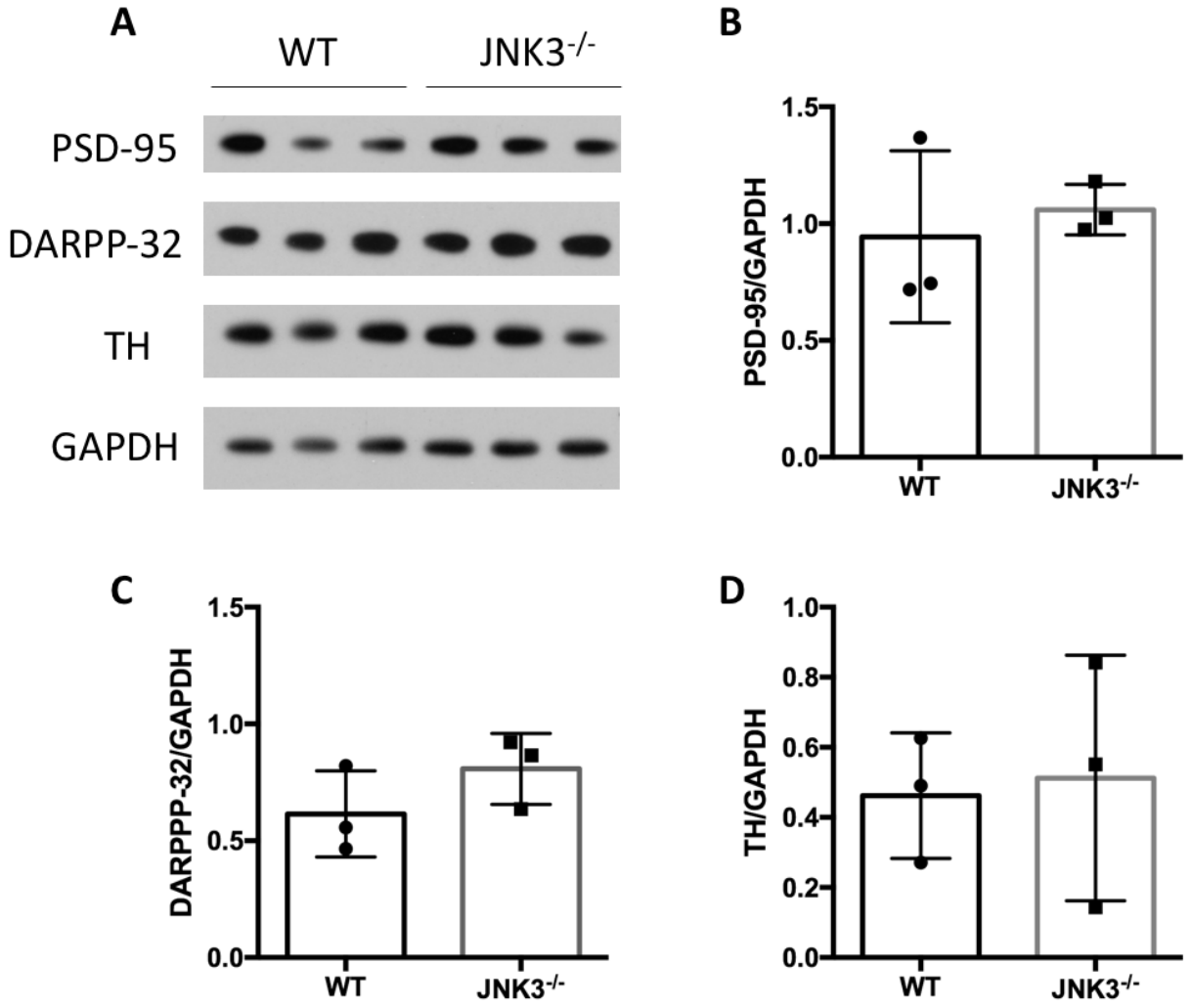

Figure 10. Levels of synaptic and neuronal connectivity markers in the striatum of JNK3$^{-/-}$ mice. (A) Western blot image of PSD-95, DARPP-32 and TH in the striatum of P50 wildtype and JNK3$^{-/-}$ mice. **(B)** Unpaired two-tailed t-test with Welch's correction revealed no statistically significant differences in the levels of PSD-95, DARPP-32 and TH between wildtype and JNK3$^{-/-}$ mice. Error bars represent SD.

2.3.4 Amelioration of HD phenotypes in R6/2-JNK3$^{-/-}$ mice

2.3.4.1 Recovery of biochemical markers of synaptic connectivity

With the establishment of baseline by comparing potential differences between wildtype and R6/2 mice as well as between wildtype and JNK3$^{-/-}$, we next examined the effects of JNK3 knockout in the R6/2 mice to investigate the contribution of JNK3 in HD phenotypes observed in R6/2 mice. Striata from P50 wildtype (n=4), R6/2 (n=4), R6/2-JNK3$^{-/-}$ (n=4) mice were collected as described in **2.2**. One-away ANOVA revealed statistically significant differences in the level of PSD-95 normalized to that of GAPDH among three groups ($F(2, 9)=18.38$, $p=0.0007$). Tukey's post-hoc analysis confirmed statistically significant differences in the normalized PSD-95 level between wildtype and R6/2 ($p<0.001$) and between wildtype and R6/2-JNK3$^{-/-}$ ($p<0.01$), but not between R6/2 and R6/2-JNK3$^{-/-}$ **(Figure 11A, 11B)**. One-way ANOVA revealed statistically significant differences in the level of DARPP-32 normalized to that of GAPDH among three groups ($F(2, 9)=10.53$, $p=0.0044$). Tukey's post-hoc analysis confirmed statistically significant differences in normalized DARPP-32 level between wildtype and R6/2 ($p<0.01$), but not between wildtype and R6/2-JNK3$^{-/-}$ and between R6/2 and R6/2-JNK3$^{-/-}$ **(Figure 11A, 11C)**. One-way ANOVA revealed statistically significant differences in the level of TH normalized to that of GAPDH among three groups ($F(2, 9)=7.143$, $p=0.0139$). Tukey's post-hoc analysis confirmed statistically significant differences in normalized TH level between wildtype and R6/2 ($p<0.05$), but not between wildtype and R6/2-JNK3$^{-/-}$ and between R6/2 and R6/2-JNK3$^{-/-}$ **(Figure 11A, 11D)**.

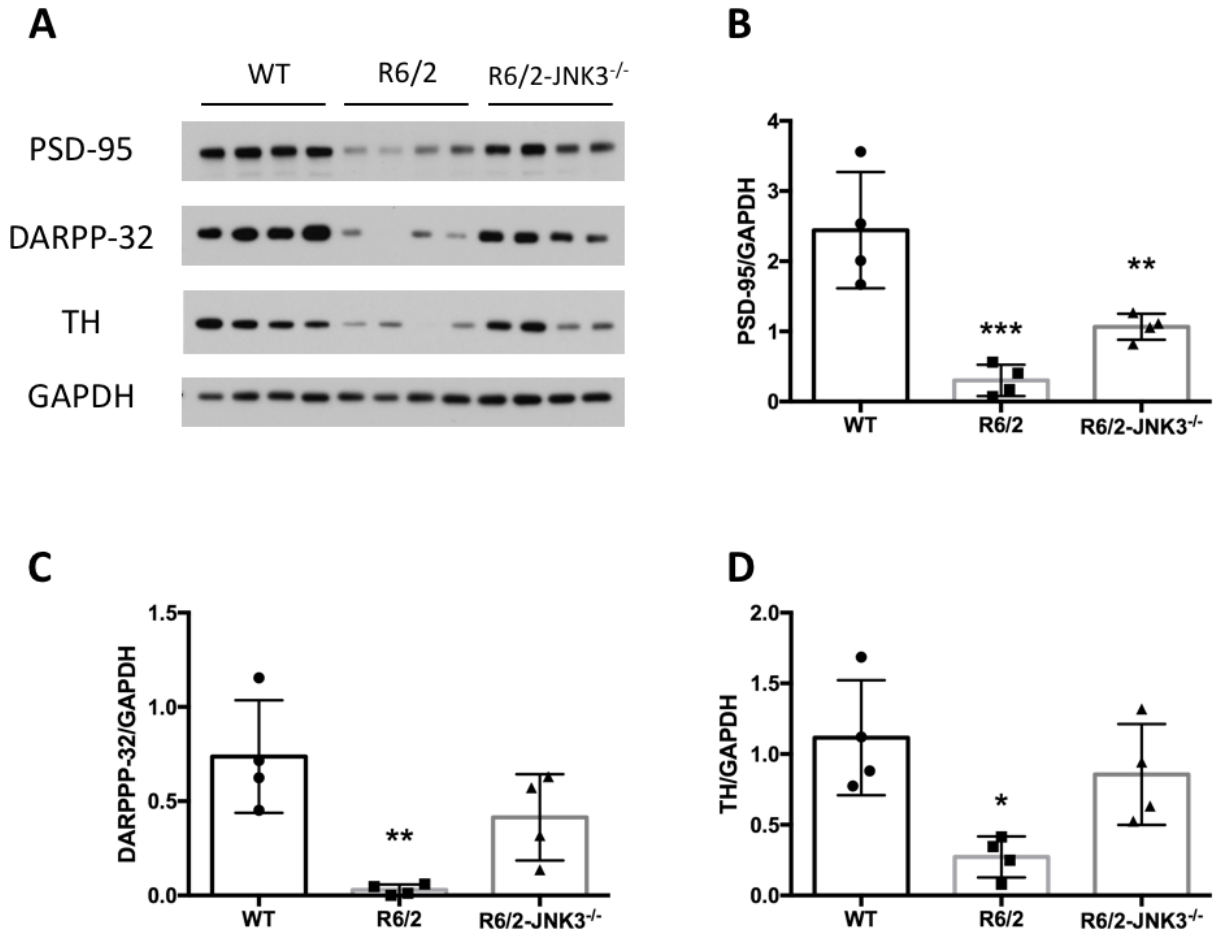

Figure 11. Amelioration of hallmark biochemical phenotypes of HD in R6/2-JNK3$^{-/-}$ mice. (A) Western blot image shows the loss of synaptic markers PSD-95 and DARPP-32 as well as TH, marker of dopaminergic innervation into the striatum in P50 R6/2 mice. This loss is partially and fully restored to normal levels in R6/2-JNK3$^{-/-}$. One-way ANOVA followed by Tukey's post-hoc analysis found **(B)** a statistically significant reduction in PSD-95 level in R6/2 (***$p<0.001$, compared to wildtype) and a partial rescue in R6/2-JNK3-/- (**$p=0.01$, compared to wildtype), **(C)** a statistically significant reduction in DARPP-32 level in R6/2 (**$p<0.01$, compared to wildtype), which is restored to normal level—non-significant difference from wildtype—in R6/2-JNK3$^{-/-}$ mice, and **(D)** a statistically significant reduction in TH level in R6/2 (*$p<0.05$, compared to wildtype), which is restored to normal level in R6/2-JNK3$^{-/-}$ mice. Error bars represent SD.

2.3.4.2 Expression of JNK isoforms in R6/2-JNK3$^{-/-}$ mice

The expression levels of different JNK isoforms were analyzed in R6/2-JNK3$^{-/-}$ mice to examine potential compensatory increase in other isoforms and interaction between Htt transgene expression and JNK3 knockout. The same set of samples used in **2.3.4.1** were analyzed by immunoblotting with antibodies for each of the JNK isoforms. One-way ANOVA revealed statistically significant differences in the level of JNK1 normalized to GAPDH among three groups ($F(2, 9)=7.393$, $p=0.0126$). Tukey's post-hoc analysis identified a significant difference in JNK1 level between wildtype and R6/2-JNK3-/- mice ($p<0.05$) as well as between R6/2 and R6/2-JNK3-/- ($p<0.05$) but no difference between wildtype and R6/2 mice **(Figure 12A, 12B)**, indicating that increase in level JNK1 is most likely from genetic deletion of JNK3, which was also observed in JNK3-/- mice **(Figure 9)**. One-way ANOVA showed no significant differences in the level of JNK2 normalized to GAPDH among three groups ($F(2, 9)=3.048$, $p=0.0976$) although there was a trend for increased level of JNK2 in R6/2-JNK3$^{-/-}$ **(Figure 12A, 12C)**. One-way ANOVA revealed significant differences in the level of JNK3 normalized to GAPDH among three groups ($F(2, 9)=8.254$, $p=0.0092$). As expected from genetic knockout of JNK3, Tukey's post-hoc analysis identified a significant reduction in the level of JNK3 in R6/2-JNK3$^{-/-}$ mice compared to wildtype ($p<0.01$) but only a trend compared to R6/2 mice ($p=0.0614$), and there was no significant difference between wildtype and R6/2 mice ($p=0.4150$) although there may be a trend for reduced level of JNK3 in R6/2 mice **(Figure 12A, 12D)**.

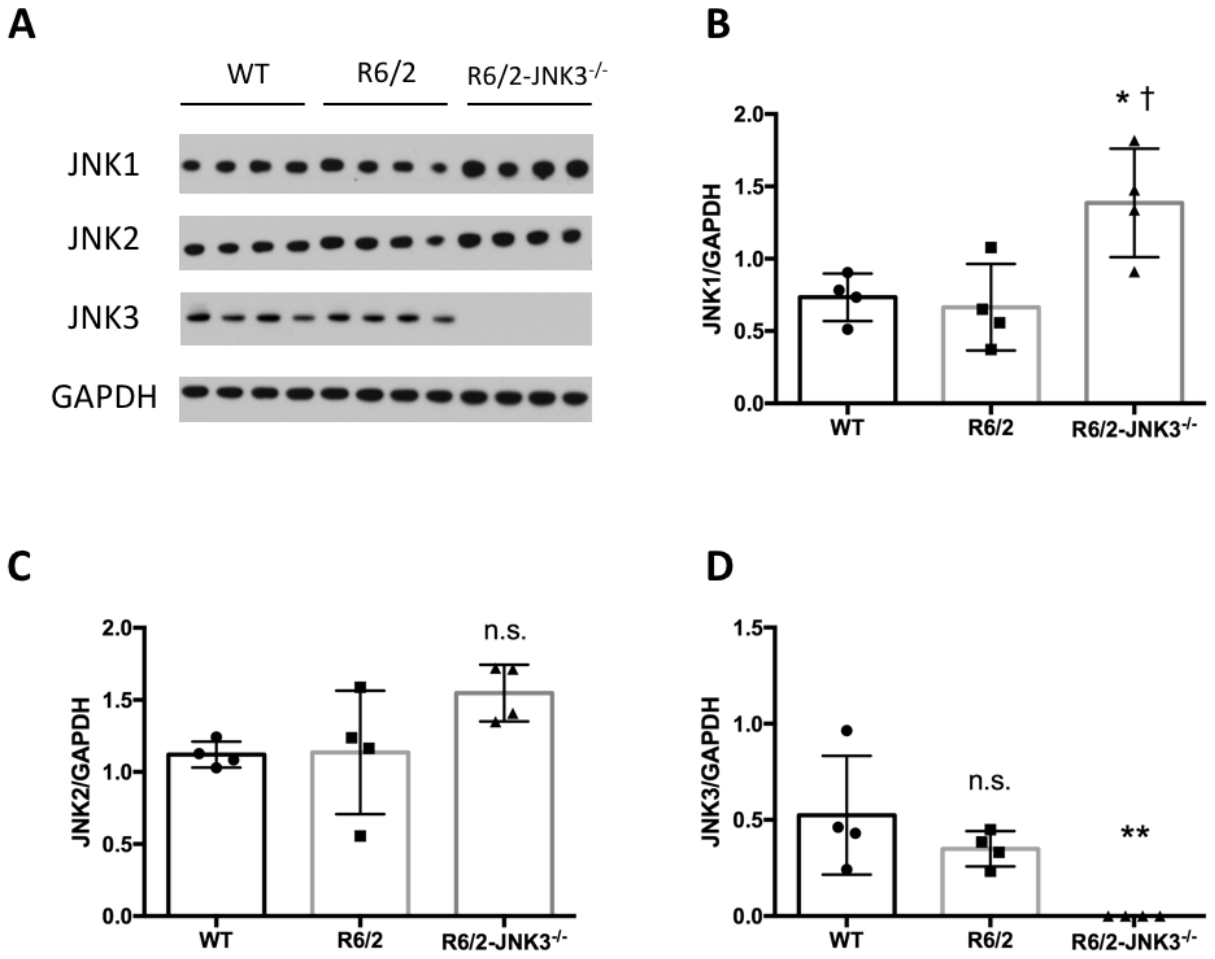

Figure 12. Expression of JNK isoforms in R6/2 and R6/2-JNK3$^{-/-}$ mice. (A) Western blot image shows levels of JNK1, JNK2 and JNK3 expression in the striatum of P50 wildtype, R6/2 and R6/2-JNK3$^{-/-}$ mice. GAPDH is provided as a loading control. **(B)** R6/2-JNK3$^{-/-}$ mice have a significant increase in the level of JNK1 compared to wildtype (*$p<0.05$) and R6/2 (†$p<0.05$). **(C)** There is no significant difference in the level of JNK2 although there is a trend for increased level of JNK2 in R6/2-JNK3$^{-/-}$ mice. **(D)** R6/2-JNK3$^{-/-}$ mice, as expected, do not express JNK3 (**$p<0.01$, compared to wildtype), and there is no significant difference in the level of JNK3 between wildtype and R6/2. Error bars represent SD.

2.3.5 Activation of JNK upstream kinase by mHtt in isolated squid axoplasms

JNK is a member of the mitogen-activated protein kinase (MAPK) family. Phosphorylation of the activation loop of MAPK by upstream kinases, mitogen-activated protein kinase kinases (MAPKKs), activate MAPK. MAPKKs themselves are also activated by phosphorylation of the activation loop by further upstream kinases, mitogen-activated protein kinase kinase kinases (MAPKKKs). To determine whether mHtt activates JNK via upstream kinases we examined activation of MAPKKs by mHtt.

Mitogen-activated protein kinase kinase 7 (MKK7) is one of the MAPKKs known to phosphorylate and activate JNK. Using the recombinant Htt-Q16 and Htt-Q55 proteins used in **Figure 6**, mHtt-mediated activation of MKK7 in isolated squid axoplasms was analyzed by western blot with an antibody that recognizes the active form of MKK7 (phospho-MKK7). The level of phospho-MKK7 in 6 pairs of isolated squid axoplasms were analyzed by non-parametric two-tailed paired t-test, and the analysis revealed a statistically significant increase in the level of phospho-MKK7, normalized to that of KHC loading control, in the axoplasms perfused with Htt-Q55 (M=0.01574, SD=0.007287, 95% CI [0.008091, 0.02338]) compared to their sister axoplasms perfused with Htt-Q16 (M=0.01258, SD=0.007089, 95% CI [0.005144, 0.02002]), p=0.0312 **(Figure 13)**.

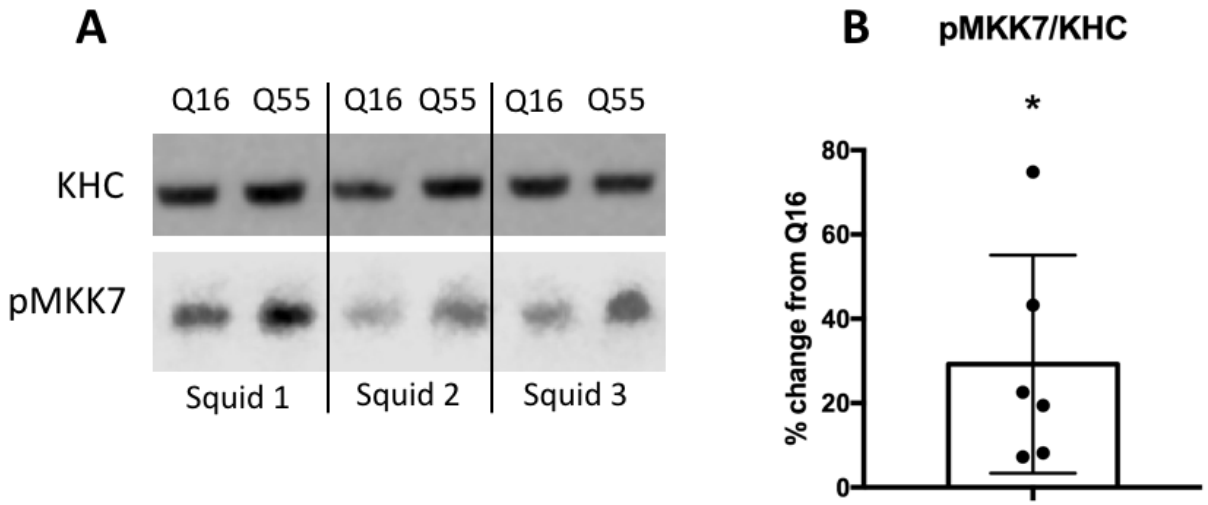

Figure 13. mHtt-mediated activation of MKK7 in isolated squid axoplasms. (A) A representative immunoblot image demonstrating the activation of MKK7 in isolated squid axoplasms perfused with mHtt (Htt-Q55). KHC is provided as a loading control. **(B)** Non-parametric two-tailed paired t-test of 6 pairs of axoplasms found a statistically significant increase in the level of pMKK7 in the axoplasms perfused with Htt-Q55 compared to Htt-Q16, *p=0.0312. The differences between sister axoplasms are represented as % change from Q16. Error bars indicate SD.

2.4 DISCUSSION

Experiments presented in this chapter were designed based on the previously published work from our lab demonstrating the activation of JNK3 by mHtt underlies mHtt-mediated axonal transport defects (Morfini et al. 2009; Morfini et al. 2006). To address the extent of the contribution that JNK3 activation plays in HD pathology, R6/2 mice, a transgenic mouse model of HD, were crossed with JNK3$^{-/-}$ mice. Based on our knowledge that defects in axonal transport lead to neurodegeneration (Reid et al. 2002; Karle et al. 2012; Crimella et al. 2012; López et al. 2015; Puls et al. 2005; Hafezparast 2003; Farrer et al. 2009; Konno et al. 2017), we hypothesized that correcting mHtt-mediated axonal transport deficits by genetic deletion of JNK3 would be beneficial in preserving dying-back neuropathy and loss of axonal connectivity as shown in our recent work (Gatto et al. 2015) and that it would alleviate mHtt-mediated neurodegeneration and HD symptoms recapitulated in R6/2 mice.

2.4.1 <u>Increased activation of JNK in HD patients and various experimental models of HD</u>

Western blot analysis of post-mortem cortex tissues collected from non-HD donors and HD patients showed a significant increase in the level of phosphorylated (active) JNK in cortices of HD patients **(Figure 5)**. The demographic of the donors varied widely in the age of death as well as post-mortem intervals **(Table 2)**, which contributed to the variability within the experimental groups, especially for the HD group. Nonetheless, non-parametric two-tailed t-test determined that the difference was statistically significant (**$p<0.01$). While activation of JNK has been shown in many animal and cellular models

of HD (Morfini et al. 2009; Morfini et al. 2006; Perrin et al. 2009; Taylor et al. 2013; Apostol et al. 2006; Liu 1998), this is the first demonstration of elevated level of active JNK in HD patients to our knowledge. This is an important finding because it suggests that increased activation of JNK is relevant to human HD and that any amelioration of HD phenotypes observed by inhibiting JNK in animal and cellular models may also apply to HD patients.

We also found increased activation of JNK in experimental models of HD used in this study: isolated squid axoplasms perfused with recombinant mHtt **(Figure 6)** and R6/2 mice **(Figure 7)**. The antibodies used in the analysis of pJNK in mouse and isolated squid axoplasms (Cell Signaling, #4668) and human tissue (Cell Signaling, #9251) were raised against the two phosphorylation sites within the activation loop (Thr183/Tyr185), which are conserved among all three isoforms of JNK (JNK1/2/3). Therefore, they detect all three isoforms of JNK and it is not clear whether or not they have same affinity for all isoforms. While some efforts can be made to discern isoform-specific activation of JNK based on their molecular weight (Figure 4) between JNK1 and JNK2/3, it is not yet possible to selectively detect activation of JNK3 with immunoblotting due to the lack of antibody that selectively recognizes active form of JNK3 but not other JNK isoforms, and it is unlikely that such antibody will be available in the future due to the conserved activation sites among JNK isoforms. Potential approaches to measure activation of JNK3 in an isoform-selective manner are an enzyme-linked immunosorbent assay (ELISA) using a JNK3 antibody to capture JNK3 in the lysate followed by detection of active JNK using a pJNK antibody (or *vice versa*), and immunoprecipitation of JNK3 followed by immunoblotting with a pJNK antibody (or *vice versa*). However, both approaches require JNK3 antibody and pJNK antibody to be generated from different hosts. We have

characterized many commercial JNK3 and pJNK antibodies. Unfortunately, JNK3 and pJNK antibodies validated in our lab are all from rabbit. If JNK3 and/or pJNK antibodies from hosts other than rabbit become available in the future, the proposed techniques could be utilized to examine isoform-specific activation of JNK3 in HD.

2.4.2 Changes in expression of JNK isoforms in JNK3$^{-/-}$, R6/2 and R6/2-JNK3$^{-/-}$ mice

When expression of one or more genes are disturbed by genetic manipulations such as knockouts and knockdowns, upregulation of other isoforms of the same gene or genes that encode for proteins with similar cellular functions is often observed in a compensatory response. Indeed, we observed upregulation of JNK1 in JNK3-/- **(Figure 9)** as well as R6/2-JNK3-/- mice **(Figure 12)**. JNK1 is ubiquitously expressed in all tissues, and JNK2 is expressed in many tissues and enriched in muscles. JNK3 has the most tissue-selective expression among the three JNK isoforms, mainly expressed in the brain and to a lesser extent in the heart, the pancreas and the testis (Yarza et al. 2016; Coffey 2014). A compensatory increase in JNK1 expression upon genetic deletion of JNK3 suggests some functional redundancy between JNK1 and JNK3 although tissue-specific expression of JNK3 indicates JNK3 has cellular functions distinct from JNK 1 and JNK2. In addition, JNK2/3 have been shown to be activated in response to stimuli such as stress while JNK1 is constitutively active in neurons (Coffey et al. 2002), indicating that the loss of JNK3 may not be well compensated by upregulation of JNK1.

There seemed to be a trend for a reduction in the expression of JNK3 in R6/2 mice, which could be a compensatory mechanism for increased activation of JNK3 with the transgenic expression of mHtt. Although the reduction in JNK3 expression in R6/2 mice

did not reach statistical significance in this study, results from another study involving a larger number of animals indicate a decreased expression of JNK3 in R6/2 mice (Kang et al. *in preparation*).

2.4.3 Alleviation of HD pathology by genetic deletion of JNK3 in R6/2 mice

Based on our previous results identifying JNK3 as the critical mediator of axonal transport defects by mHtt (Morfini et al. 2009; Morfini et al. 2006) and preliminary data demonstrating the rescue of transcallosal connectivity in R6/2-JNK3$^{-/-}$ mice (Kang et al. *in preparation*), we hypothesized that deletion of JNK3 would alleviate many of HD phenotypes observed in R6/2 mice. Indeed, R6/2-JNK3$^{-/-}$ mice showed normal or near normal levels of synaptic proteins PSD-95 and DARPP-32 as well as TH, an indirect marker of nigrostriatal dopaminergic projections to the striatum, at a symptomatic age (P50) **(Figure 11)**, indicating that neuronal connectivity has been spared in these mice. Behavioral phenotypes that are unmistakably observed in R6/2 mice such as deficits in rotarod performance, clasping test and nesting behavior also improved in R6/2-JNK3$^{-/-}$ mice, demonstrating functional recovery along with spared neuronal connections (Kang et al. *in preparation*). However, R6/2-JNK3$^{-/-}$ mice eventually succumb to the disease phenotypes in the late stage (P75 and beyond), and their lifespan is approximately P125, which is about 25% longer than that of R6/2 mice in our colony (Kang et al. *in preparation*), and 25% increase in the survival of R6/2 mice one of the most efficacious results with any treatments in these mice (Li et al. 2005). As an exaggerated model of HD, R6/2 mice have a very early disease onset and severe symptoms, and the global expression of the mHtt transgene confounds potential causes of death in these mice due to non-neuronal

complications such as metabolic syndromes and cardiac dysfunctions. We are currently investigating the effect of JNK3 deletion in a different mouse model of HD, Q175, which has much slower disease progression.

JNKs are traditionally described as stress-activated kinases and indeed they are activated in response to various stress stimuli such as ultraviolet irradiation, hypoxia, and cytokines. While the amelioration of HD pathology in R6/2-JNK3$^{-/-}$ mice may be in part due to potentially diminished stress responses from the genetic deletion of JNK3, our hypothesis and previously published data provide a specific mechanism of *one* of the roles JNK3 plays in the axonal transport impairment and subsequent neurodegeneration in HD.

In sum, the data presented in this chapter demonstrate that loss of neuronal connections was prevented and significant improvements in motor functions and survival were observed in R6/2-JNK3$^{-/-}$ mice, indicating that activation of JNK3 by mHtt is a critical pathogenic event in HD, and inhibiting JNK3 could be a promising therapeutic strategy for HD.

2.4.4 Potential effects of mHtt-mediated aberrant JNK activation in synaptic transmission

As shown in this chapter, synaptic connections vulnerable in HD were spared in R6/2-JNK3$^{-/-}$ mice, presumably from preventing dying-back neuropathy by rescuing axonal transport. While we have demonstrated that the *levels* of synaptic proteins are restored, whether or not the synaptic transmission is rescued in R6/2-JNK3$^{-/-}$ mice was not directly addressed in this study. A collaboration through CHDI Foundation is currently

underway to examine recovery of synaptic transmission in these mice. Furthermore, unpublished preliminary work in squid giant synapses demonstrated that microinjection of mHtt into squid giant synapses leads to dysfunctions in synaptic transmission, and the effect of mHtt was prevented by co-injection of a pharmacological inhibitor of JNK, SP600125, along with mHtt (Ivannikov et al, *unpublished*). This finding suggests that JNK3-mediated toxic effects of mHtt on neuronal connections may involve direct effects of JNK3 on synaptic function as well as on axonal transport.

2.4.5 Potential mechanism of activation of JNK by mHtt via upstream kinases

JNKs, as members of mitogen-activated protein kinase (MAPK) family, are activated by upstream kinases mitogen-activated protein kinase kinases (MKKs) such as MKK4/MKK7, which are activated by further upstream kinases mitogen-activated protein kinase kinase kinases (MAPKKKs). Data from this chapter shows activation of MKK7, an upstream kinase of JNKs, by mutant huntingtin (mHtt) in isolated squid axoplasms **(Figure 13)**, suggesting that mHtt may activate JNK via upstream kinases. Furthermore, studies using co-expression of mHtt and mixed-linage kinase 2 (MLK2), a member of MAPKKKs that is upstream of MKK7, in non-neuronal cell lines demonstrated a direct interaction between Htt and MLK2 (Liu et al. 2000). Preliminary result from our lab using mouse brain lysates show a direct interaction of Htt with MLK by co-immunoprecipitation, indicating that the Htt-MLK interaction occurs in the brain at endogenous levels. These results suggest that Htt may act as a scaffold for MAPK signaling components.

2.4.6 Future directions

As we further our understanding of the pathogenic mechanism of HD and regulation of axonal transport, there are many outstanding questions that warrant future studies. One of those questions is the mechanism of the inhibition of cytoplasmic dynein (CDyn)-based retrograde axonal transport by mHtt. It is clear that mHtt inhibits both anterograde and retrograde axonal transport of membrane-bound cargoes. Previous work from our lab has identified phosphorylation of kinesin heavy chain by JNK3 is the mechanism of anterograde axonal transport inhibition by mHtt. While the mechanism underlying the inhibition of retrograde axonal transport remains unknown, our data from isolated squid axoplasms strongly suggests that JNK3 may also mediate the toxic effect of mHtt on retrograde axonal transport as perfusion of recombinant JNK3 inhibits retrograde axonal transport and co-perfusion of mHtt with JNK inhibitor SP600125 rescues both anterograde and retrograde axonal transport (Morfini et al. 2009; Morfini et al. 2006).

Our ongoing research effort is focused on determining the interaction of Htt and MLKs, and the effects polyQ expansion on such interaction, as well as addressing the effect of JNK3 in another mouse model of HD, Q175 mice, which better represent human HD progression. Based on our findings described in this chapter, if a compound that can isoform-selectively inhibit JNK3 becomes available in the future, it could be a very promising therapeutic compound for HD patients.

CHAPTER III.

III. REGULATION OF RETROGRADE AXONAL TRANSPORT BY NEUROTROPHIN SIGNALING

3.1 Background

3.1.1 Discovery of neurotrophins

Neurotrophins are a family of secreted proteins that are critical for neuronal development, function, and survival (Hefti 1997; Binder and Scharfman 2004). Classic studies in 1930s using chick embryos have demonstrated that target cells innervated by neurons produce trophic signals that promote the survival of innervating neurons (Hamburger 1934), introducing the neurotrophic factor hypothesis. A couple of decades following this finding, the first neurotrophin was identified and named nerve growth factor (NGF) (Levi-Montalcini and Hamburger 1951; Levi-Montalcini 1966). Decades later, another neurotrophin was identified based on its effects on promoting survival of dorsal root ganglion neurons. This protein was eventually purified from pig brain and was named brain-derived neurotrophic factor (BDNF) (Barde et al. 1982). Following the discovery of NGF and BDNF, other neurotrophins such as neurotrophin-3 (NT-3) (Maisonpierre et al. 1990) and neurotrophin-4/5 (NT-4/5) (Hallböök et al. 1991; Ip et al. 1992; Davies et al. 1993) were also identified as a member of a neurotrophin family.

3.1.2 Classification of neurotrophic factors

Since the discovery of neurotrophins, other soluble proteins have been identified to exert trophic effects on neurons and have been classified into a larger family of proteins called *neurotrophic factors*. Neurotrophic factors are commonly classified into three main families based on structural and functional similarities: neurotrophins, ciliary neurotrophic factor (CNTF) family (also known as neuropoietic/neurotrophic cytokine family), glial cell line-derived neurotrophic factor (GDNF) family (also known as transforming growth factor beta (TGF-β) superfamily) (Sato 2017; Kalinowska-Lyszczarz and Losy 2012). In addition to the three main families, the list of proteins that have been identified to have neurotrophic effects continues to grow, which includes epidermal growth factor (EGF), neuregulins, vascular endothelial growth factor (VEGF), and fibroblast growth factor (FGF) (Malenka et al. 2009). A non-exhaustive list of neurotrophic factor families and their members are provided in **Table 3**.

Neurotrophic factors		
Neurotrophins	**GDNF Family (TGF-β superfamily)**	**CNTF family (Neurotrophic cytokines)**
• NGF • BDNF • NT-3 • NT-4/5	• GDNF family • TGF-β family • BMP family • Neuturin • Artemin • Persephin …	• CNTF • Leukemia inhibitory factor • IL-6 • IL-11 • EGF family • FGF • VEGF • Neuropoetin …

Table 3. Classification of neurotrophic factors. NGF: nerve growth factor, BDNF: brain-derived neurotrophic factor, NT-3: neurotrophin-3, NT-4/5: neurotrophin-4/5, GDNF: glial-derived neurotrophic factor, TGF-β: transforming growth factor, BMP: bone morphogenic protein, CNTF: ciliary neurotrophic factor, IL-6: interleukin 6, IL-11: interleukin 11, EGF: epidermal growth factor, FGF: fibroblast growth factor, VEGF: vascular endothelial growth factor.

3.1.3 Synthesis and release of neurotrophins

Neurotrophins are typically released from post-synaptic cells that receive inputs from neurons, neurons in case of neuron-neuron interaction, and from muscle cells, in case of neuron-muscle interaction at the neuromuscular junction. They bind to neurotrophin receptors on the innervating, pre-synaptic neurons. Neurotrophins are synthesized as a precursor form of pre-pro-neurortrophins (i.e. preproBDNF, preproNGF, …), which are cleaved into pro-neurotorphins (i.e. proBDNF, proNGF, …), which are then cleaved to produce mature neurotrophins (ie.g. BDNF, NGF, …). Mature neurotrophins exist as homodimers (Pirvola and Ylikoski 2003; Al-Qudah and Al-Dwairi 2016) although a BDNF/NT-3 heterodimer with 10-fold less affinity to their receptors has been reported in an artificially overexpressed setting (Jungbluth et al. 1994).

3.1.4 Neurotrophin receptors

Despite the fact that neurotrophins share more than 50% sequence homology, secreted neurotrophins isoform-specifically bind to their receptors called either tropomyosin receptor kinase or tyrosine receptor kinase A/B/C (TrkA/B/C). NGF binds to TrkA, BDNF and NT-4 binds to TrkB, and NT-3 binds to TrkC but it also binds to TrkA and TrkB with low affinity. All neurotrophins can also bind to another receptor called p75 neurotrophin receptor ($p75^{NTR}$) albeit with low affinity (Chao 2003; Skaper 2008) **(Figure 14)**. Trk receptors are members of receptor tyrosine kinase (RTK) family with a single transmembrane domain and a cytoplasmic tyrosine kinase domain surrounded by multiple tyrosine residues. $p75^{NTR}$ is thought to form a complex with Trk receptors and modulate their specificity and kinetics of ligand binding (Chao 2003; Skaper 2008).

Interestingly, $p75^{NTR}$ binds to pro-neurotrophins with much higher affinity than mature neurotrophins (R. Lee 2001). In addition, binding of pro-neurotrophins to $p75^{NTR}$ leads to apoptotic signaling via its cytosolic death domain rather than trophic signaling (R. Lee 2001; Beattie et al. 2002), indicating that neurotrophin signaling can be modulated by regulation of pro-neurotrophin cleavage and the balance of pro-neurotrophin and mature neurotrophin can affect the biological outcome of neurotrophin signaling. Researchers have reported differential expressions of Trk receptors and neurotrophins depending on neuronal subtypes as well as developmental stages (Ylikoski et al. 1993; McMahon et al. 1994; Fariñas et al. 1998; Huang and Reichardt 2001; Pirvola and Ylikoski 2003; Kemi et al. 2006), suggesting that each neurotrophin has unique functions as well as overlapping functions in different cell types and developmental stages.

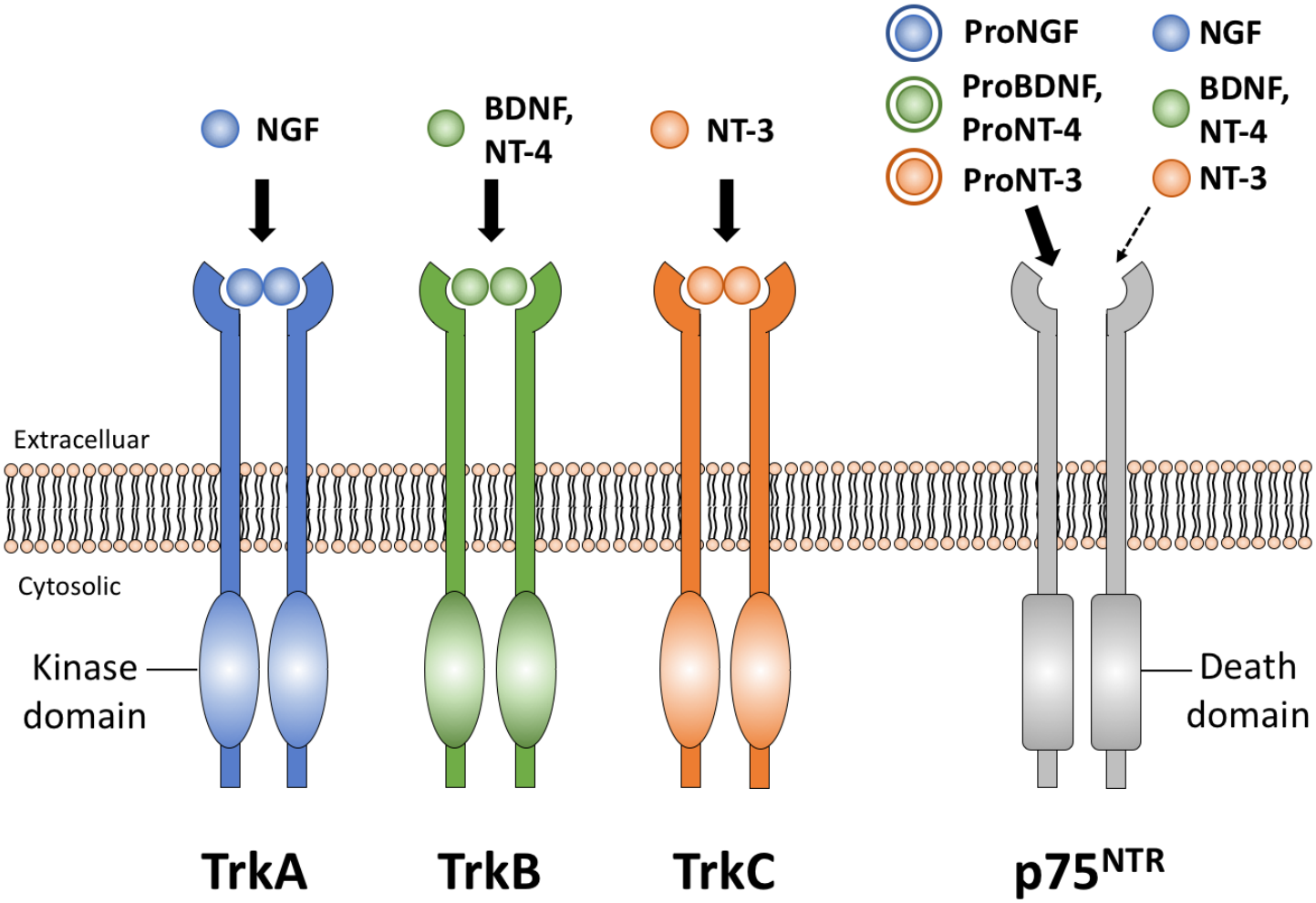

Figure 14. Receptor-specificity of neurotrophins. NGF binds to TrkA, BDN and NT-4 binds to TrkB, and NT-3 binds to TrkC. Receptors form a homodimer and trans-phosphorylate multiple tyrosine residues within and around the kinase domain of the other subunit upon ligand binding. Pro-neurotrophins (ProNGF, ProBDNF, …) bind to $p75^{NTR}$ receptor. All four mature neurotrophins (NGF, BDNF, …) can also bind to $p75^{NTR}$ but at lower affinity compared to pro-neurotrophins. Unlike Trk receptors, ligand binding to $p75^{NTR}$ leads to activation of pro-apoptotic signaling cascades via its death domain.

3.1.5 Neurotrophin signaling cascade

Upon binding of neurotrophins to their receptors, Trk receptors trans-phosphorylate several tyrosine residues within (Tyr705, Tyr706, and Tyr707 in case of TrkB) and around the kinase domain (Tyr515 and Tyr816) of the other subunit, then become activated. Active Trk receptors have similar downstream signaling cascades although there are differences in the extent of activation of specific signaling pathways depending on neuronal cell types and the dominant neurotrophin type. Phosphorylation of different tyrosine residues on Trk receptors leads to activation of different pathways. For instance, phosphorylation of Tyr515 on TrkB, which is located just outside of the kinase domain on the N-terminal side, recruits Shc (Src homology 2 domain-containing transforming protein) which acts as an adapter protein for Grb2[4]-Sos[5] protein complex. Sos is a guanine nucleotide exchange factor (GEF) which promotes the exchange of guanosine diphosphate (GDP) to guanosine triphosphate (GTP) and activates a small GTPase protein Ras, leading to activation of Ras-Raf-MEK[6]-ERK[7] pathway as well as PI3K[8]-PDK1[9]-Akt[10] pathway. Phosphorylation of Tyr816 on TrkB, which is located adjacent to the kinase domain on the C-terminal side, recruits phospholipase C gamma (PLCγ), leading to subsequent activation of PLCγ-IP3[11]-CAMK[12] and PLCγ-DAG[13]-PKC[14]

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[4] Grb2: growth factor receptor-bound protein 2
[5] Sos: Son of Sevenless
[6] MEK: mitogen-activated protein kinase kinase
[7] ERK: extracellular signal-regulated protein kinase
[8] PI3K: phosphatidylinositide 3-kinase
[9] PDK1: phosphoinositide-dependent kinase 1
[10] Akt: protein kinase B
[11] IP3: inositol triphosphate
[12] CAMK: Ca2+/calmodulin-dependent protein kinase
[13] DAG: diacylglycerol
[14] PKC: protein kinase C

pathways (as reviewed in Duman and Voleti 2012). Src family kinases (SFKs), which are non-receptor tyrosine kinases, are also found to be activated by TrkB upon neurotrophin binding. Interestingly, active SFKs can also directly phosphorylate and activate TrkB receptors, suggesting mutual regulation of SFKs and TrkB (Huang and McNamara 2010; Boltaev et al. 2017) **(Figure 15)**.

Activation of several signaling cascades, including ones described above, upon neurotrophin signaling promotes survival and growth of neurons as well as synaptic plasticity. Some of these effects are locally mediated by direct and indirect interaction between Trk receptors and ion channels at the synapse (Takasu et al. 2002; Li et al. 1999; Balkowiec et al. 2000; Nagano et al. 2003). For instance, autocrine neurotrophin signaling at the synapse can activate PKC and CaMK, which can directly modulate local synaptic transmission by phosphorylation of AMPA[15] and NMDA[16] receptors (I. Song and Huganir 2002; Nagy 2008). On the other hand, other effects are mediated by transcriptional activation of pro-survival genes in the nucleus via activation of transcription factors such as cAMP response element-binding protein (CREB) (Duman and Voleti 2012; Ito and Enomoto 2016; Cunha 2010) upon target-derived neurotrophin signaling **(Figure 15)**.

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[15] AMPA: α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid
[16] NMDA: N-methyl-D-aspartate

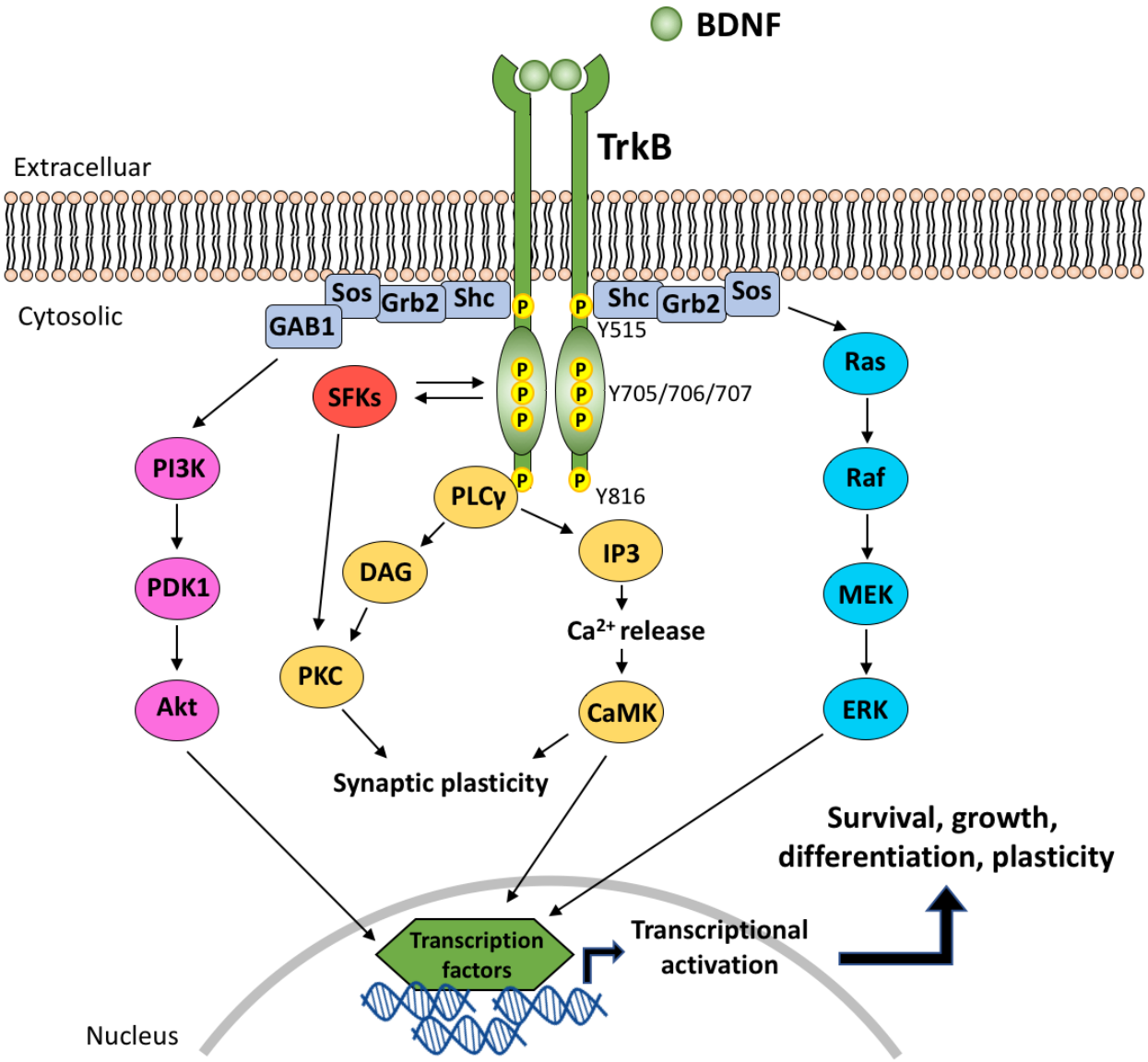

Figure 15. BDNF signaling cascade. Upon BDNF binding, TrkB receptors auto-phosphorylate at Tyr705/706/707 residues within its kinase domain as well as Tyr515 and Tyr816. Phosphorylation of these residues lead to activation of several kinase signaling cascades which may include SFKs (red), Ras-Raf-MEK-ERK (blue), PI3K-PKD1-Akt (pink), PLC-IP3/DAG-PKC/CaMK (yellow). PKC and CaMK can phosphorylate AMPA and NMDA receptors to modulate their functions. Other pathways can activate transcription of pro-survival genes by phosphorylation of transcription factors such as CREB.

3.1.6 Retrograde transport of neurotrophins

Some aspects of trophic effects by neurotrophins, such as transcriptional activation at the nucleus, require the active Trk receptors to be transported along the axon, sometimes over an extreme distance, to reach the neuronal soma (Heerssen et al. 2004; Ye et al. 2003). Upon activation, neurotrophin-bound Trk receptors are internalized by both clathrin-dependent endocytosis (Zheng et al. 2008) and clathrin-independent pinocytosis (Shao et al. 2002; Valdez et al. 2005). The endocytosed active neurotrophin-Trk receptor complex is transported to the cell body by cytoplasmic dynein (CDyn) in a membrane-bound organelle called signaling endosome with its neurotrophin-bound N-terminus contained in the lumen of the signaling endosome and the C-terminal kinase domain exposed to the cytoplasm during transport (Zweifel et al. 2005).

3.1.7 Alterations in BDNF expression and transport in neurodegenerative diseases

Brain-derived neurotrophic factor (BDNF) is a neurotrophin that promotes development, survival, and functions of neurons (Zuccato and Cattaneo 2009). Impaired neurotrophin signaling has been reported in several neurodegenerative diseases such as Alzheimer's disease (AD) (Phillips et al. 1991), Parkinson's disease (PD) (Murer et al. 2001), and Huntington's disease (HD) (Ferrer et al. 2000). Multiple groups have reported reductions in BDNF levels in HD (Ferrer et al. 2000; Zuccato et al. 2001, 2005; Gharami et al. 2008) as well as mHtt-mediated defects in transport of BDNF (Gauthier et al. 2004; del Toro et al. 2006). Several groups have proposed to alleviate HD pathology by enhancing BDNF signaling in various mouse models of HD. Systemic injection of recombinant BDNF in R6/2 resulted in increased level of BDNF expression and reduced

striatal atrophy (Giampá et al. 2013), and a similar result has been shown in N171-82Q HD mice treated with small molecule TrkB agonists (Jiang et al. 2013), but it is not clear whether the reduction in BDNF associated with HD is a direct or indirect effect of the pathology.

3.1.8 Rationale and hypothesis

The pro-survival signaling cascades activated by neurotrophins and the evidence for defective neurotrophin signaling in neurodegeneration prompted many scientists to use them as potential therapeutics for several neurodegenerative diseases such as AD, PD, HD, and amyotrophic lateral sclerosis. Unfortunately, these attempts were met with lack of efficacy and substantial side effects in dozens of clinical trials (Thoenen and Sendtner 2002). Even after decades of clinical trials with various neurotrophins including NGF and BDNF, there is currently no FDA-approved clinical use of neurotrophins in treating neurodegeneration, indicating that there is a need for deeper understanding of neurotrophin signaling in both health and diseases. One of the gaps in our knowledge of neurotrophin signaling is the regulatory mechanism underlying transport of signaling endosomes, which must contain active neurotrophin receptors for transcriptional activation by neurotrophin signaling. While numerous downstream signaling cascades of neurotrophin signaling have been identified, mechanisms underlying the activation of signaling endosome transport upon neurotrophin signaling remain elusive.

Based on evidence that regulation of axonal transport could involve phosphorylation of motor proteins, we hypothesized that activation of signaling endosome transport is regulated by phosphorylation of CDyn. Among several subunits of CDyn,

dynein intermediate chain (DIC) is particularly of interest because it has been associated with cargo-binding function of CDyn and shown to be phosphorylated *in vivo* (Dillman and Pfister 1994). Preliminary data from our lab using radiometric labeling of rat primary cortical neurons with ^{32}P followed by immunoprecipitation of CDyn and autoradiography indicates selective increase in the phosphorylation of DIC upon BDNF stimulation of rat primary cortical neurons (Morfini et al. *unpublished*). Based on this preliminary result and previously identified mechanisms of axonal transport regulation by phosphorylation of motor proteins, we hypothesized that neurotrophin signaling, in the context of BDNF, leads to phosphorylation of DIC, and that this phosphorylation of DIC activates retrograde transport of signaling endosomes upon neurotrophin signaling.

3.2 Methods and materials

3.2.1 Rat primary cortical neuronal culture

All procedures involving animals have been approved by the Institutional Animal Care and Use Committee (IACUC) at University of Illinois at Chicago (UIC). Timed-pregnant female Sprague-Dawley rats were ordered from Charles River Laboratories, and embryos were collected on embryonic day 18 (E18). Cortices from the embryos were dissected using fine forceps and collected in cold 1X Hank's balanced salt solution (HBSS, Gibco cat. 14185-052). Cortex tissues were trypsinized for 15 minutes in 37˚C water bath with manual swirling every 5 minutes using trypsin (Gibco cat. 1509-046) at 0.5% final concentration. Trypsin was washed from the tissues by 3 washes with 1X HBSS then deactivated by adding Dulbecco's modified eagle medium (Gibco cat. 11995-065) supplemented with 10% fetal bovine serum (FBS, Gibco cat. 16000-044) and 1X penicillin/streptomycin (Pen/Strep, Gibco cat. 15140-122) (DMEM++). Trypsinized tissues were manually dissociated with a glass Pasteur pipet then triturated into single-cell suspension with a flame-polished Pasteur pipet with 50% smaller diameter. The number of cells were measured by hemocytometer, and the cell stocks were diluted to desired concentration for plating in DMEM++. Cells were plated onto plasma-treated tissue culture dishes that were coated with 0.5mg/mL poly-L-lysine (Sigma cat. P2636) in borate buffer overnight prior to plating and washed for 30 minutes 3 times with sterile water. 2 x 10^6 cells ere plated on P60 dishes and 5 x 10^6 cells were plated on P100 dishes. After plating, cells were incubated in a humidified CO_2 incubator with 5% CO_2 at 37˚C for 3 hours to make sure they were attached to the dish, and the media was

completely exchanged with Neurobasal medium (Gibco cat. 10888-022) supplemented with 1X B27 (Gibco cat. 17504-044), 0.5mM GlutaMax (Gibco cat. 35050-061) (NB+++).

3.2.2 BDNF stimulation and inhibitor treatment

Recombinant human brain-derived neurotrophic factor (BDNF) was purchased from Alomone Labs (cat. B-250). For BDNF stimulation of rat primary cortical neurons, 25ng/mL of BDNF was added in the media and incubated in 37˚C CO_2 incubator for 10-20 minutes after 3 days *in vitro* (3DIV). For experiments involving kinase inhibitors, cells were pre-treated with inhibitors for 20 minutes prior to BDNF stimulation at following concentrations in NB+++: 200nM k252a (Millipore cat. 420297), 50μM SU6656 (Millipore cat. 572636), 30μM A419529 (Tocris cat. 3914), 30μM CRT 0066101 (Tocris cat. 4975), 10μM HIV TAT_PKD1 substrate peptide (synthesized at UIC Research Resources Center; Sequence: GRKKRRQRRRPPAALVRQMSVAFFFK), 10μM HIV TAT_PKCδ substrate peptide (synthesized at UIC Research Resources Center; Sequence: GRKKRRQRRRPPAKRKRKGSFFYGG). After inhibitor treatment and BDNF stimulation, the media was completely aspirated, and cells were scraped in 1% SDS. Cell lysates were sonicated (20% amplification, 6 cycles; 1 cycle = 5s on, 2s off), and centrifuged at 21130 x g in Eppendorf 5424 tabletop centrifuge (15000 rpm) at 4˚C for 15 minutes. Following centrifugation, clarified supernatant was transferred to a new Eppendorf tube and the same volume of 6X sample buffer was added for final concentration of 3X for western blot and Phos-tag SDS-PAGE.

3.2.3 Metabolic labeling of rat primary cortical neurons

For metabolic labeling of rat primary cortical neurons with ^{32}P, neurons were plated and cultured in as described above. On 2DIV, the 3/4 of media was exchanged with DMEM without phosphate (Gibco cat. 11971-025) to promote ^{32}P incorporation. On 3DIV, ^{32}P (PerkinElmer cat. NEX053H001MC) was added to the media, and cells were stimulated with BDNF for 20 minutes as described above.

3.2.4 Immunoprecipitation of dynein from rat primary cortical neurons

Endogenous dynein complex was immunoprecipitated from rat primary cortical neurons using a monoclonal antibody against dynein intermediate chain (DIC). This antibody, clone 74.1, immunoprecipitates DIC as well as co-immunoprecipitating other subunits of dynein complex including dynein heavy chain and light intermediate chains. For immunoprecipitation, rat primary cortical neurons were scraped in a non-denaturing lysis buffer ROLB (50mM HEPES pH=7.4, 100mM NaCl, 0.5% Triton X-11, 0.1% sodium deoxycholate, 50mM NaF, 80mM β-glycerophosphate, 1mM EDTA) supplemented with phosphatase inhibitor cocktail II (Calbiochem cat. 524625, 1:200 dilution), protease inhibitor cocktail (Sigma cat. P8340, 1:200 dilution), okadaic acid (Calbiochem cat. 459620, 100nM), microcystin (Calbiochem cat. 475816), and Na_3VO_4 (Sigma cat. S6508). Cell lysates were manually homogenized with a plastic pestle in 1.5mL Eppendorf tubes, then centrifuged at 21130 x g in Eppendorf 5424 tabletop centrifuge (15000 rpm) at 4˚C for 15 minutes twice, transferring the supernatant to new Eppendorf tubes each time. The clarified supernatant was pre-cleared by incubation with pre-clearing mix containing protein G agarose beads (ThermoScientific cat. 20399), mouse IgG agarose beads

(Jackson Immunoresearch cat. 015-000-052), and 0.1% bovine serum albumin (Jackson Immunoresearch cat. 001-000-162) for 1 hour in 4˚C. After pre-clearing, samples were centrifuged at 1,000RPM for 1 minute in Eppendorf 5424 tabletop centrifuge to pellet the agarose beads, and the supernatant was transferred to 1.5mL Eppendorf tubes containing protein G agarose beads and 74.1 antibody, and incubated for 3 hours with rotation in 4˚C. After immunoprecipitation, samples were centrifuged at 1,000RPM for 1 minute in Eppendorf 5424 tabletop centrifuge to pellet the agarose beads, and washed with wash buffer (10mM HEPES pH=7.4, 200mM NaCl, 0.5% Triton X-100) 3 times, and a final wash with 20mM HEPES pH=7.4 to remove Triton X-100. After final wash, samples were centrifuged at 1,000RPM for 1 minute in Eppendorf 5424 tabletop centrifuge, and the agarose beads were dried with a 27G needle attached to a vacuum trap. Appropriate volume of 3X sample buffer was added to the beads for SDS-PAGE.

3.2.5 ^{32}P autoradiography

Metabolically labeled dynein complex was immunoprecipitated from the rat primary cortical neurons treated with BDNF in the presence of ^{32}P as described, and separated by gel electrophoresis on 4-12% Bis-Tris gels (Invitrogen cat. NP0336) in MOPS running buffer at 120V for 2 hours. Following electrophoresis, the gels were stained with Coomassie brilliant blue R-250 solution (Bio-Rad cat. 1610436), detained with destaining solution (10% acetic acid, 25% MeOH in water), and dried using a gel dryer. Dried gels were scanned on Typhoon 9410 phosphoimager to measure the incorporation of ^{32}P on the phosphorylated subunits of dynein complex.

3.2.6 PKD1 *in vitro* kinase assay

Active recombinant PKD1 (Sigma-Aldrich cat. K4268 Lot# SLBR2717V) with an N-terminal GST tag was used to phosphorylate recombinant full-length rat DIC 1A (produced by UIC Research Resources Center in 2007 then reconstituted to 30μM in 20mM HEPES; stored in -80˚C). 100nM PKD1 was incubated with 3μM DIC 1A in kinase assay buffer (50mM K•Aspartate, 10mM $MgCl_2$, 1mM DTT, 20mM HEPES pH=7.4) with 0.5mM ATP and 0.1mCi radiolabeled ATP [γ-^{32}P] (PerkinElmer cat. NEG035C010MC) for 1 hour. The reaction was stopped by adding 6X sample buffer to final concentration of 3X sample buffer. The samples were separated by gel electrophoresis on a 4-12% Bis-Tris gel (Invitrogen cat. NP0336) in MOPS running buffer at 120V for 2 hours. Following electrophoresis, the gel was stained with Coomassie brilliant blue R-250 solution (Bio-Rad cat. 1610436), detained with destaining solution (10% acetic acid, 25% MeOH in water), and dried using a gel dryer. The dried gel was exposed to HyBlot CL autoradiography film (Denville Scientific cat. NC9550782) for 3 hours, and the film was developed using a film developer.

3.2.7 Western blotting

For western blotting, rat primary cortical neuronal lysates prepared as described above were loaded on 4-12% Bis-Tris gels (Invitrogen cat #NP0336) and ran in MOPS running buffer at 120V for 2 hours. Following electrophoresis, the proteins were transferred onto a 0.45μm Immobilon-P PVDF membrane (Millipore cat #IPVH00005) or Immobilon-FL (Millipore cat #IPFL00005) in Towbin buffer (25mM Tris, 192 mM glycine, 10% MeOH) at 400mA for 2 hours with cooling system maintained at 4˚C. After transfer,

the membranes were incubated in blocking buffer (1% non-fat milk in TBS-T) for 1hr in room temperature. After blocking, membranes were washed three times with TBS-T for 5 minutes each wash. Then the membranes were incubated overnight with primary antibodies diluted in 1% BSA in TBS with following dilutions: anti-phospho-TrkA/TrkB (Tyr706/Tyr707) (Cell Signaling #4621, 1:500), anti-TrkB (Cell Signaling #4603, 1:200), anti-phospho-PKD1 (S916) (Cell Signaling #2051, 1:500), anti-PKD1/PKCμ (Clone C-20, Santa Cruz #sc-639, 1:200), anti-phospho-(Ser/Thr) PKD substrate (Cell Signaling #4381, 1:500), anti-phopshotyrosine (clone PY99, Santa Cruz #sc-7020, 1:200), anti-DIC (clone 74.1, Santa Cruz #sc-13524, 1:500), and anti-GAPDH (Sigma cat. G8795, 1:2000). The next day, the membranes were washed three times with TBS-T for 5 minutes each wash and incubated with secondary antibodies diluted in 1% milk in TBS-T with 0.01% SDS, and incubated for 45 minutes to 1 hour in room temperature with following dilutions: goat anti-mouse IgG IRDye 680RD (LI-COR #925-68070, 1:10,000) and goat anti-rabbit IgG IR Dye 800CW (LI-COR #925-32211, 1:10,000). After incubation with secondary antibodies, membranes were washed three times with TBS-T for 5 minutes each wash , and one final wash with TBS to remove Tween-20, then analyzed using LI-COR Odyssey CLx/Fc near-infrared (NIR) imaging systems.

3.2.8 Phos-tag SDS-PAGE

For analysis of dynein intermediate chain phosphorylation using gel motility shift on Phos-tag SDS-PAGE, 50μM of Phos-tag acrylamide (Wako Chemicals cat. AAL-107) and 10mM MnCl2 were added to the resolving gel solution (7% w/v acrylamide, 0.1% w/v SDS, 375mM Tris-HCl pH=8.8, 0.001% v/v TEMED, 0.0004% v/v ammonium persulfate).

Stacking gel solution did not differ from typical recipe (4.5% w/v acrylamide, 125mM Tris-HCl pH=6.8, 0.1% w/v SDS, 0.001% v/v TEMED, 0.0004% v/v ammonium persulfate). To separate phosphorylated dynein intermediate chain, samples were loaded on the Phos-tag gels and electrophoresed at 25mA for 2 hours and 15 minutes. After SDS-PAGE, gels were incubated in towbin buffer (25mM Tris, 192mM glycine, 10% MeOH) supplemented with 10mM Na•EDTA with gentle agitation on a rocker for 1 hour (fresh buffer every 20 minutes), followed by 20 minute wash with Towbin buffer without 10mM Na•EDTA. After EDTA washes, gels were transferred and proceeded with western blot protocol for LI-COR Odyssey CLx near-infrared imaging system as described above.

3.2.9 Vesicle motility assay in isolated squid axoplasms

Isolated squid axoplasms were prepared and perfused as described in **2.2**. For vesicle motility assays, a video-enhanced contrast differential interference contrast (VEC-DIC) microscopy system equipped with was Zeiss Axiomat with a 100x, 1.3 n.a. objective, Hamamatsu Argus 20 and Model 2400 CCD camera, and Hamamatsu Photonics Microscopy C2117 video manipulator was used to visualize the movement of vesicular cargoes in isolated squid axoplasms. Detailed method for vesicle motility assay is described in a published protocol from our lab (Y. Song et al. 2016).

3.2.10 Primary neuronal culture in microfluidic chambers

Our microfluidic chamber (MFC) design consists of three individual somatodendritic compartments (SDC) that are each a 7x7 mm square and one axon terminal compartment (ATC) that is 20 mm in length at its maximum. A polydimethyl-

siloxane (PDMS) wall that is 1 mm in width separates the three SDC from one another. Our microfluidic chamber system has an open-top design with a 6 mm hole punched into each of the SDC squares and three 4 mm holes punched into the ATC. There are 106 microchannels (Ax) that connect each SDC to the shared ATC, and a single microchannel is 500 μm in length, 8 μm in width, and 2.5 μm in height **(Figure 16)**.

Prior to plating neurons into our custom microfluidic chambers (MFCs), the chambers were sterilized with 70% ethanol for 15 minutes, placed into sterile 100 mm culture dishes, and each of the compartments were washed three times with autoclaved diH_2O for 1 hr each in a sterile laminar flow hood. The microfluidic chamber compartments were coated with 1 mg/mL poly-L-lysine (Sigma cat. P2636) in 0.1 M borate buffer (pH 8.5) over-night at room temperature, and washed three times with autoclaved diH_2O for 2 hr each. The compartments were then filled with DMEM(++), and placed inside an incubator at 37°C and 5% CO_2. Fluidic isolation is maintained within our custom MFC for over 96 hours, which can be sustained over longer periods of time with media changes occurring every 3 days to keep the fluidic height between the compartments stable. After preparation of MFCs, rat primary cortical neurons were prepared as described in **3.3.1**, and 70,000 neurons were plated in each SDC and cultured for 12 days with fluidic isolation.

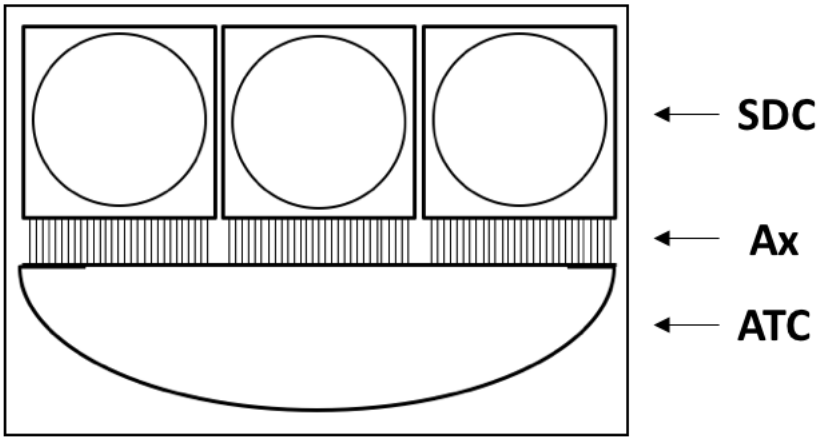

Figure 16. Schematic of microfluidic chambers. Chambers made with PDMS feature somatodendritic (SDC), axonal (Ax), and axon terminal (ATC) compartments. Neurons are plated in SDC, only neurites can enter Ax due to the narrow width (8μm) of Ax, allowing the separation of axons from neuronal cell bodies. Fluidic isolation of ATC from SDC and Ax can be achieved by maintaining higher fluidic height in SDC compared to ATC.

3.2.11 QD-BDNF treatment

To prepare the quantum-dot-BDNF conjugate (QD-BDNF), streptavidin-conjugated Quantum-Dot-655 (1μM stock; ThermoFisher cat. Q10123MP) was incubated with biotin-conjugated BDNF (1μM stock; Alomone Labs cat. B-250-B) at a 1:1 volume ratio on ice for 1 hr prior to starting the cellular treatments. On 12 days *in vitro* (12DIV) cortical neuron axon terminals were treated with QD-BDNF with or without inhibitors within the axon terminal compartment (ATC) of MFC. First, the axon terminals were incubated with either 200nM K252a or 30μM CRT 0066101 for 20 min at 37°C. The QD-BDNF (final concentration 1nM) was mixed with the ATC media with or without inhibitors and incubated for an additional 20 min at 37°C. Following QD-BDNF incubation, the ATC media was completely replaced with new NB(+++) to remove free QD-BDNF in the media, and cells were incubated for an additional 30 min. The samples then underwent fixation for immunocytochemistry.

3.2.12 Immunocytochemistry

Cortical neurons were fixed within the intact microfluidic chamber in preparation for immunocytochemistry. The media was rinsed out of each main compartment with 37°C 1X phosphate buffered saline (PBS). The neurons underwent microtubule extraction and stabilization with a BRB80 (80mM PIPES, 1mM $MgCl_2$, 1mM EGTA) and glutaraldehyde containing extraction buffer (1X BRB80, 0.3% Triton-X, 0.25% glutaraldehyde) for 5 min, followed by fixation with 4% paraformaldehyde in PBS for 1 hr, and washed twice with PBS for 30 min each. Fresh PBS was then added to each of the compartments, and the MFC were stored at 4°C overnight. Autofluorescence was quenched for 30 min with 50

mM NH_4Cl, and the MFC compartments were washed twice with PBS for 30 min each. The neurons were permeabilized with 0.1% Triton-X for 1 hr, prior to blocking and incubation with the anti-βIII tubulin antibody (Sigma cat. T8660, 1:500 dilution) overnight at 4°C. On the following day, the compartments were washed with PBS twice for 1 hr each, then incubated at room temperature with AlexaFluor-488-conjugated goat anti-mouse IgG antibody (Invitrogen cat. A11029, 1:800 dilution) for 2 hr. The samples were washed with PBS twice for 1 hr each, and Vectashield anti-fade mounting medium with DAPI (Vector Laboratories cat. H-1200) was added to each SDC and ATC. The MFCs were stored in a parafilm-sealed 1000mm dish wrapped in aluminum foil at 4°C until confocal imaging.

3.2.13 Confocal microscopy

Following immunocytochemistry, neurons in the SDC located within the frame bordering the microchannels were imaged by confocal microscopy using Zeiss LSM710 confocal microscope with 40x oil immersion. All images were obtained within the same session, and the acquisition and post-acquisition settings were kept identical for all images.

3.2.14 Quantification of QD-BDNF accumulation

To quantify the amount of QD-BDNF in SDC, images were converted to 8-bit with identical threshold, and particle analysis plug-in in ImageJ was used to quantify the number of QD-BDNF particles in each image (particle definition: size = 0-infinity, circularity = 0-1). The number of QD-BDNF particles in each image was normalized to the

number of neuronal cell body determined by DAPI and βIII-tubulin staining. Confocal imaging and quantification of QD-BDNF accumulation was performed by an experimenter blinded to experimental groups.

3.2.15 Statistical analysis

Any data point above 1.5 interquartile ranges (IQRs) below the first quartile or above the third quartile were defined as outliers and removed from the analysis. The amount of QD-accumulation in three different experimental groups (BDNF, BDNF+k252a, BDNF+CRT 0066101) was analyzed by one-way ANOVA using GraphPad Prism 7. Following one-way ANOVA, Tukey's post-hoc analysis was performed to reveal statistically significant group comparisons.

3.3 Results

3.3.1 Phosphorylation of DIC upon BDNF stimulation

We first confirmed the phosphorylation of dynein intermediate chain (DIC) upon BDNF stimulation in rat primary cortical neurons using two different methods. First, auto-radiographic analysis of dynein complex immunoprecipitated from 3 days *in vitro* (DIV) rat primary cortical neurons metabolically labeled with ^{32}P was used to show a selective increase in phosphorylation of DIC subunit of dynein complex with BDNF stimulation **(Figure 17A)**. Second, Phos-Tag SDS-PAGE was used to separate phosphorylated proteins from non-phosphorylated proteins by decreasing the mobility of phosphoproteins on SDS-PAGE gels with a phosphate-binding tag (Phos-Tag) (Kinoshita et al. 2006). Using Phos-Tag eliminates the need for radiometric labeling and immunoprecipitation of dynein complex as phosphorylation of DIC can be examined by running the whole cell lysates on Phos-Tag-containing gels followed by immunoblotting with an antibody against total DIC. To confirm autoradiography results using the Phos-Tag system, whole cell lysates from rat primary cortical neurons treated with BDNF were separated on Phos-Tag gel, then immunoblotted with 74.1 antibody, which recognizes DIC regardless of phosphorylation status. Similar to the autoradiography results, Phos-Tag SDS-PAGE method also showed increased levels of phosphorylated DIC in primary neurons treated with BDNF compared to untreated control **(Figure 17B)**. Technical issues in the electrotransfer of proteins from Phos-Tag SDS-PAGE gels to the PVDF membrane resulted in uneven transfer of phosphoproteins compared to non-phosphorylated proteins where BDNF treated samples appeared to have higher amount of total DIC. Conventional SDS-PAGE was used to verify that the total level of DIC did not change upon BDNF

stimulation in the same samples **(Figure 17C)**. Using Phos-Tag gels, we demonstrated that the phosphorylation of DIC by BDNF simulation occurs rapidly, within 5 minutes of stimulation **(Figure 18A),** and persists even up to 2 hours following a 20-minute pulse of BDNF stimulation **(Figure 18B)** in this treatment paradigm.

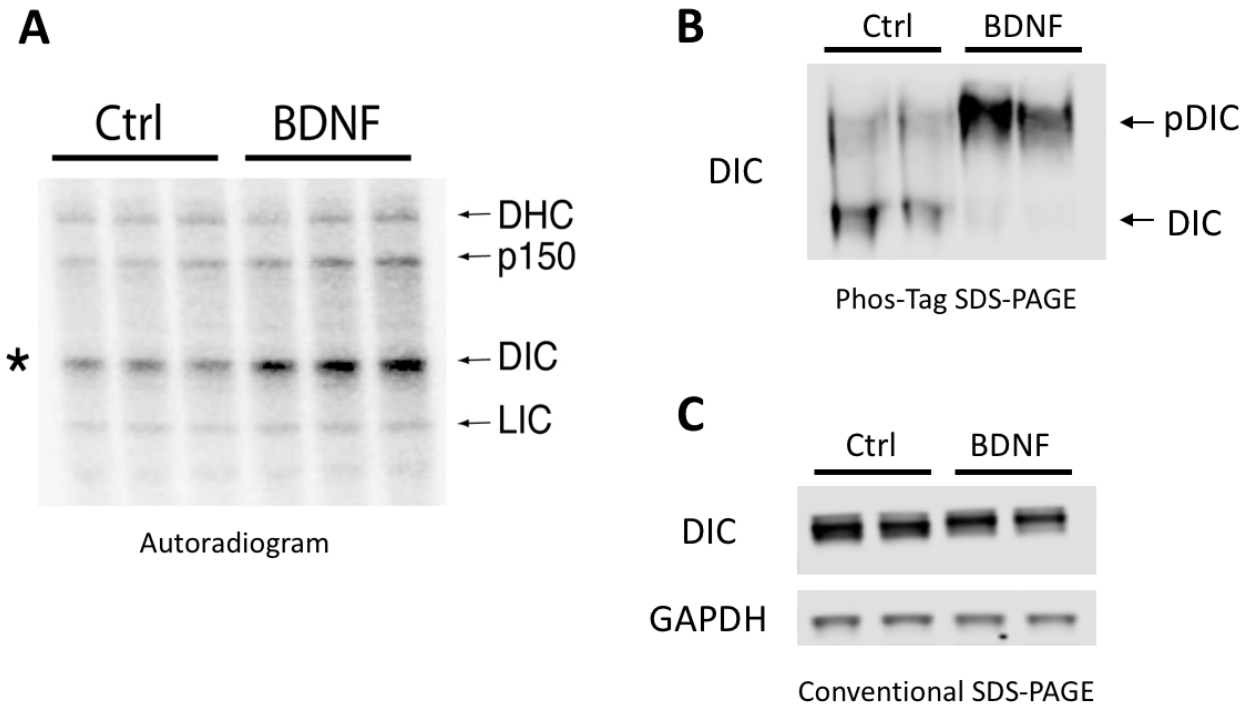

Figure 17. Phosphorylation of DIC upon BDNF stimulation. (A) Autoradiogram of dynein complex immunoprecipitated from 3DIV rat primary cortical neurons either untreated (control) or treated with BDNF (25ng/mL) in the presence of ^{32}P. BDNF treatment selectively increase the phosphorylation of DIC subunit. **(B)** BDNF treatment of 3DIV rat primary neurons increases phosphorylation of DIC that can be examined by Phos-Tag SDS-PAGE. **(C)** Total level of DIC does not change with BDNF stimulation. GAPDH is provided as a loading control.

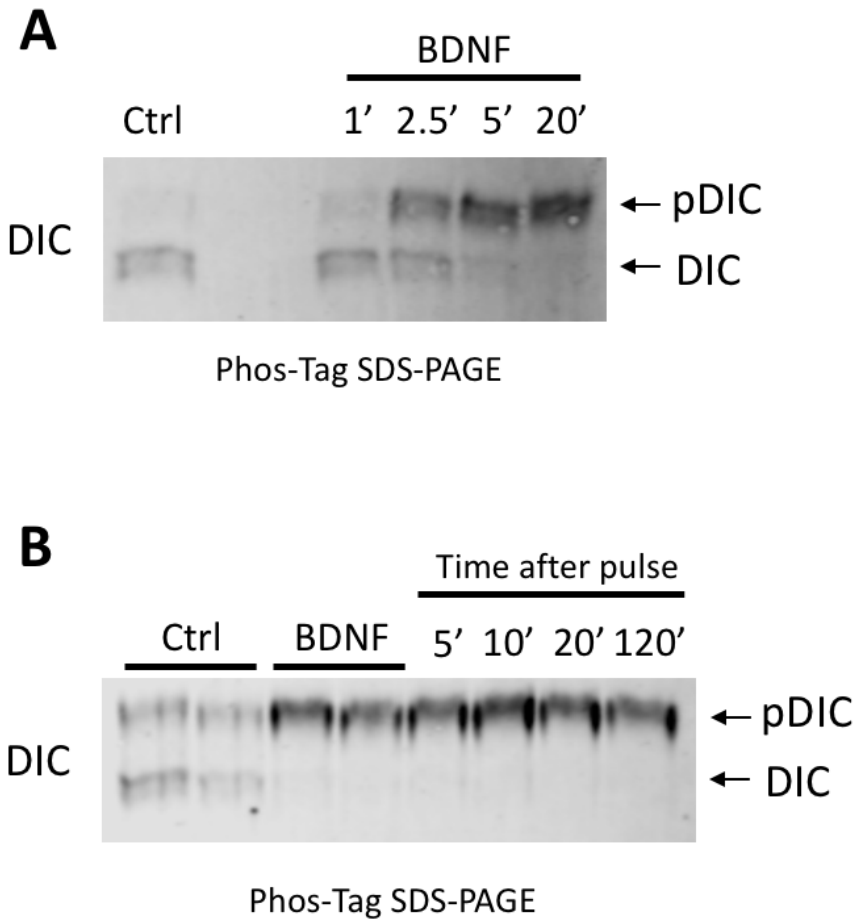

Figure 18. Characterization of BDNF-induced DIC phosphorylation. (A) Time course of DIC phosphorylation following BDNF (25ng/mL) stimulation from 1 minute to 20 minutes. Maximum DIC phosphorylation is achieved as early as 5 minutes of BDNF stimulation. **(B)** On 3DIV, rat cortical neurons were treated with BDNF (25ng/mL) for 20 minutes. Following 20-minute BDNF pulse, the media was changed to fresh media without BDNF in order to remove extracellular BDNF. Cells were collected at varying time points following the media change up to 120 minutes. Phosphorylation of DIC persisted for 120 minutes, indicating that this BDNF stimulation paradigm results in rapid and persistent phosphorylation of DIC.

3.3.2 BDNF-induced DIC phosphorylation requires activation of TrkB and SFKs

After confirming the phosphorylation of DIC following BDNF stimulation, we used various kinase inhibitors to identify kinase signaling cascade responsible for DIC phosphorylation. Rat primary cortical neurons were pre-treated with one of the following inhibitors for 20 minutes before BDNF stimulation: 200nM k252a, a pharmacological inhibitor of the tyrosine kinase activity of Trk receptors (Tapley et al. 1992), and two different inhibitors of Src family kinases (SFKs), 50μM SU6656 (Blake et al. 2000) and 30μM A419529 (Wilson et al. 2002). Inhibition of TrkB kinase activity by k252a prevents activation of all downstream kinase cascades, and as expected, also prevents phosphorylation of DIC upon BDNF stimulation **(Figure 19A)**. Interestingly, inhibitors of SFKs, which are among the downstream kinase pathways activated by TrkB, SU6656 and A419529, also prevented phosphorylation of DIC upon BDNF stimulation **(Figure 19A)**. Neither the BDNF nor inhibitor treatments altered the total level of DIC **(Figure 19B)**. These data indicate that BDNF-induced phosphorylation of DIC requires activation of TrkB and SFKs.

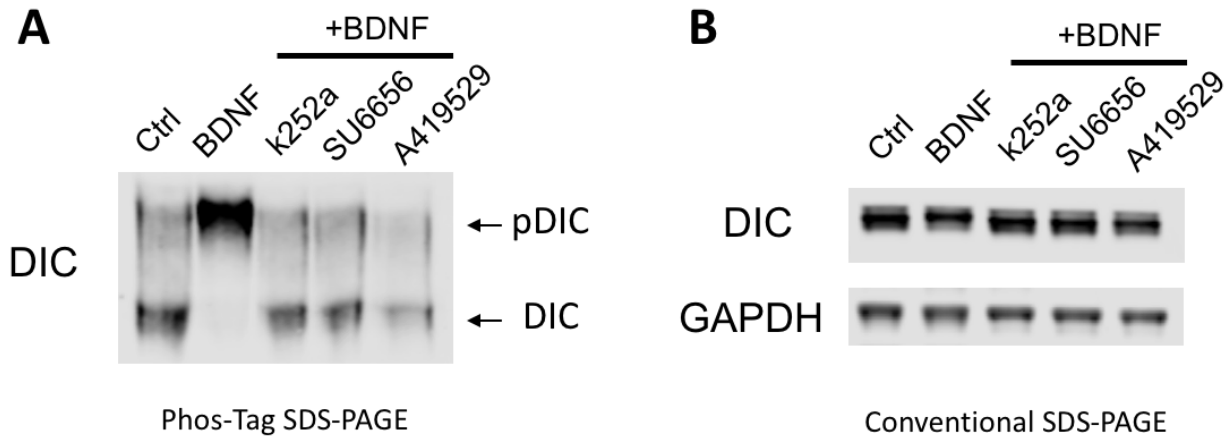

Figure 19. BDNF-induced DIC phosphorylation requires activation of TrkB and SFKs. (A) Pre-treatment with pharmacological inhibitors of TrkB (k252a) and SFKs (SU6656, A419529) prevented phosphorylation of DIC upon BDNF stimulation in 3DIV rat primary cortical neurons. **(B)** Total levels of DIC did not change with BDNF and inhibitor treatments. GAPDH is provided as a loading control.

3.3.3 SFKs activate retrograde fast axonal transport in isolated squid axoplasms

Since the activation of SFKs was necessary for BDNF-induced phosphorylation of DIC, we examined whether SFKs affect cytoplasmic dynein (CDyn), retrograde axonal transport of membrane-bound cargoes, in isolated squid axoplasms using vesicle motility assay described in **3.2**. As this preparation removes the plasma membrane from the axoplasm, the direct effect of BDNF stimulation, which requires a transmembrane receptor TrkB, cannot be studied in isolated axoplasms. In addition, whether or not squid expresses either Trk receptors or BDNF is not known. Therefore, we perfused active recombinant kinases downstream of TrkB to examine their effects on axonal transport.

SFKs are a family of non-receptor tyrosine kinases with several members including, but not limited to, Src, Fyn, Lyn, Hck, and Lck (Filippakopoulos et al. 2009). We perfused recombinant Src (70nM) and Fyn (70nM) in isolated squid axoplasms, and the vesicle motility assays following the perfusion revealed both Src and Fyn activated retrograde fast axonal transport while mildly inhibiting anterograde fast axonal transport **(Figure 20)**. These findings suggest that activation of SFKs upon BDNF stimulation, which is required for the BDNF-induced phosphorylation of DIC, may lead to activation of retrograde axonal transport of signaling endosomes.

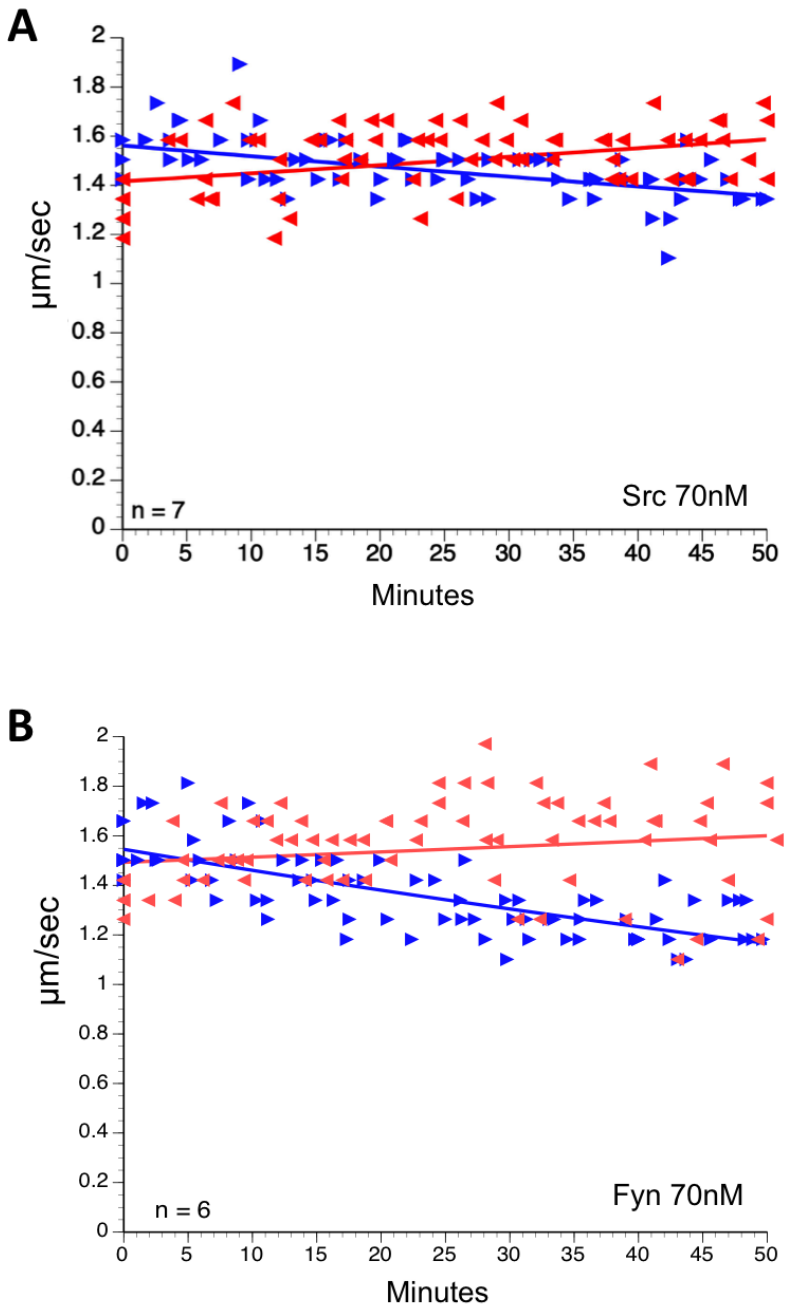

Figure 20. SFKs activate retrograde fast axonal transport in isolated squid axoplasms. Perfusions of recombinant Src **(A)** and Fyn **(B)** in isolated squid axoplasms activate CDyn-driven retrograde axonal transport (*red*) while mildly inhibiting kinesin-driven anterograde axonal transport (*blue*).

3.3.4 Dynein is not phosphorylated on tyrosine residues

The activation of a receptor tyrosine kinase TrkB and the downstream non-receptor tyrosine kinases SFKs is required for the phosphorylation of DIC upon BDNF stimulation, and active SFKs activate retrograde fast axonal transport in isolated squid axoplasms. However, previous report by Dillman and Pfister (1994) showed that DIC is phosphorylated on serine residues, but not tyrosine residues, using phosphoamino acid analysis. We tested whether DIC is phosphorylated on tyrosine residues using immuno-precipitation. Rat primary cortical neurons were cultured and stimulated with BDNF on 3DIV as described in **3.2**. Following BDNF stimulation, cells were scraped in a non-denaturing buffer (ROLB) and immunoprecipitated with either DIC antibody or pTyr antibody. Immunoprecipitation using DIC antibody, as expected, showed enriched DIC (74kDa) in the immunoprecipitates compared to input and post-immunoprecipitation supernatant (PIS), while mock immunoprecipitate that contained agarose beads and DIC antibody but not the cell lysates showed no DIC immunoreactivity. In the same samples, immunoblotting with pTyr antibody did not show any immunoreactive bands in the immunoprecipitates at 74kDa molecular weight **(Figure 21A)**. Some immunoreactivity was observed in all immunoprecipitates *including* the mock sample, indicating that there is some background from the antibody used for immunoprecipitation, which was generated from the same host (mouse) as the pTyr antibody used for immunoblotting. Immunoprecipitation using pTyr antibody showed enriched proteins with pTyr in the immunoprecipitates compared to input and PIS as expected. DIC was detected in input and PIS, but not in the pTyr immunoprecipitates **(Figure 21B)**. This result agrees with the previous report demonstrating that DIC is not phosphorylated on tyrosine residues.

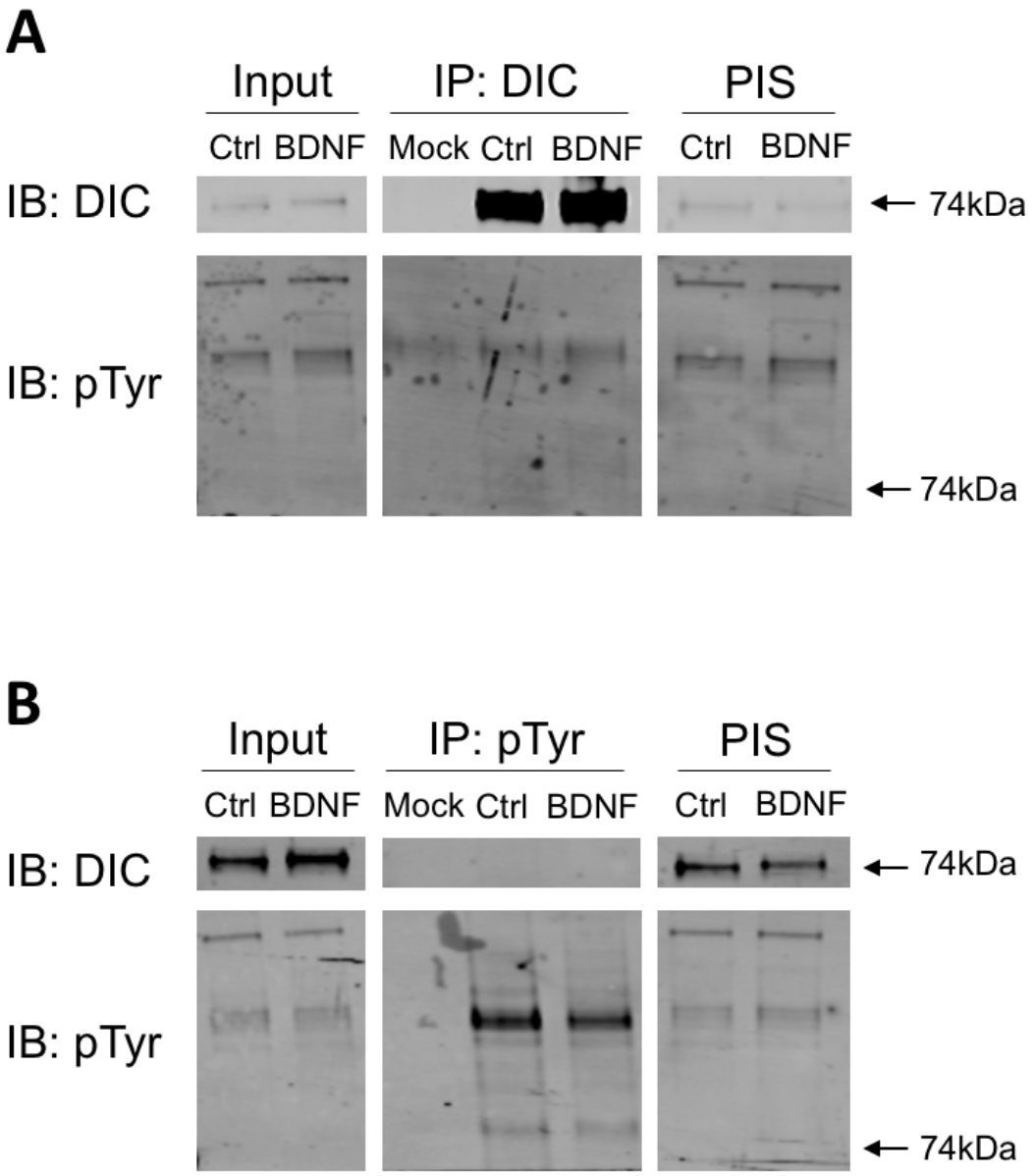

Figure 21. DIC is not phosphorylated on tyrosine residues. (A) DIC was immunoprecipitated from untreated (Ctrl) and BDNF-treated (BDNF) 3DIV rat primary cortical neurons, then immunoblotted with antibodies against DIC and pTyr. DIC (74kDa) is enriched in the immunoprecipitates but there is no pTyr immunoreactive band at 74kDa molecular weight. **(B)** Proteins phosphorylated at pTyr residues were immunoprecipitated from untreated and BDNF-treated 3DIV rat primary cortical neurons, then immunoblotted with antibodies against DIC and pTyr. DIC is detected in the input and PIS, but not in the pTyr immunoprecipitates.

** "Mock" lanes indicate a control immunoprecipitate where agarose beads and the antibody used for immunoprecipitation were incubated without cell lysate.*
** "PIS" refers to post-immunoprecipitation supernatant.*

3.3.5 SFKs activate retrograde fast axonal transport by activation of a downstream serine/threonine kinase PKD1.

Activation of non-receptor tyrosine kinases SFKs is required for phosphorylation of DIC upon BDNF stimulation and SFKs activate retrograde fast axonal transport. However, DIC is phosphorylated on serine but not tyrosine residues. Thus, we reasoned that there may be an intermediate serine/threonine kinase between SFKs and phosphorylation of DIC. Previous work from our lab reported that members of the protein kinase C (PKC) family can also activate retrograde fast axonal transport in isolated squid axoplasms (G. Morfini et al. 2007). Previous studies (Senis et al. 2014; Murugappan et al. 2009) have reported that SFKs can activate one or more members of the PKC family. The PKC family can be categorized as including three subfamilies based on their regulatory domains: conventional (cPKCs), novel (nPKCs), and atypical PKCs (aPKCs) **(Table 4)** and we sought to determine whether any of these isoforms were downstream of SFKs.

To evaluate whether any PKC mediates the activation of retrograde fast axonal transport by SFKs in the squid axoplasms by co-perfusion of Fyn, a member of SFK family, with two pharmacological inhibitors of PKC, Gö6983 and Gö6976. Both compounds are inhibitors of PKC, but they inhibit different subsets of PKC isoforms. Gö6983 inhibits PKCα, PKCβ, PKCγ, PKCδ, and PKCζ (IC_{50}= 7nM, 7nM, 6nM, 10nM, 60nM, respectively as measured by *in vitro* kinase assay with 35μM ATP) but does not inhibit PKCμ/PKD1 (IC_{50} > 20μM) (Gschwendt et al. 1996). Gö6976 inhibits PKCα, PKCβ , and PKCμ/PKD1 (IC_{50}= 2nM, 6nM, 20nM, respectively) (Martiny-Baron et al. 1993; Gschwendt et al. 1996) **(Figure 22A)**. Co-perfusion of 70nM Fyn and 500nM Gö6983 did

not prevent activation of retrograde axonal transport by Fyn **(Figure 22B),** while co-perfusion of 70nM Fyn and 500nM Gö6976 blocked effects on both anterograde and retrograde FAT **(Figure 22C)**, suggesting that PKD1, but not PKCα, PKCβ, PKCγ, PKCδ, and PKCζ, could mediate the activation of retrograde axonal transport by SFKs. In addition, perfusion of active recombinant PKD1 to isolated squid axoplasms activates retrograde fast axonal transport, mimicking the effects of SFKs **(Figure 23)**. Together, these data strongly indicates that PKD1 mediates the activation of retrograde fast axonal transport by SFKs.

Subfamily	Isoforms	Require DAG for activation	Require Ca2+ for activation
Conventional	PKCα, PKCβ, PKCγ	✓	✓
Novel	PKCδ, PKCε, PKCη PKCθ	✓	✗
Atypical	PKCλ/ι, PKCζ, *PKCμ (PKD1)	✗	✗

Table 4. Classification of PKC isoforms. 1) Conventional PKCs (cPKCs) require both Ca^{2+} and diacylglycerol (DAG)/phorbol ester for activation; 2) novel PKCs (nPKCs) require DAG but not $Ca2^{+}$ for activation; and 3) atypical PKCs (aPKCs) do not require neither DAG nor Ca^{2+} for activation (Mackay and Twelves 2007).

**PKCμ was initially classified as a member of aPKCs then later re-classified to protein kinase D1 (PKD1) (Manning et al. 2002; Celil and Campbell 2005; Ren 2016).*

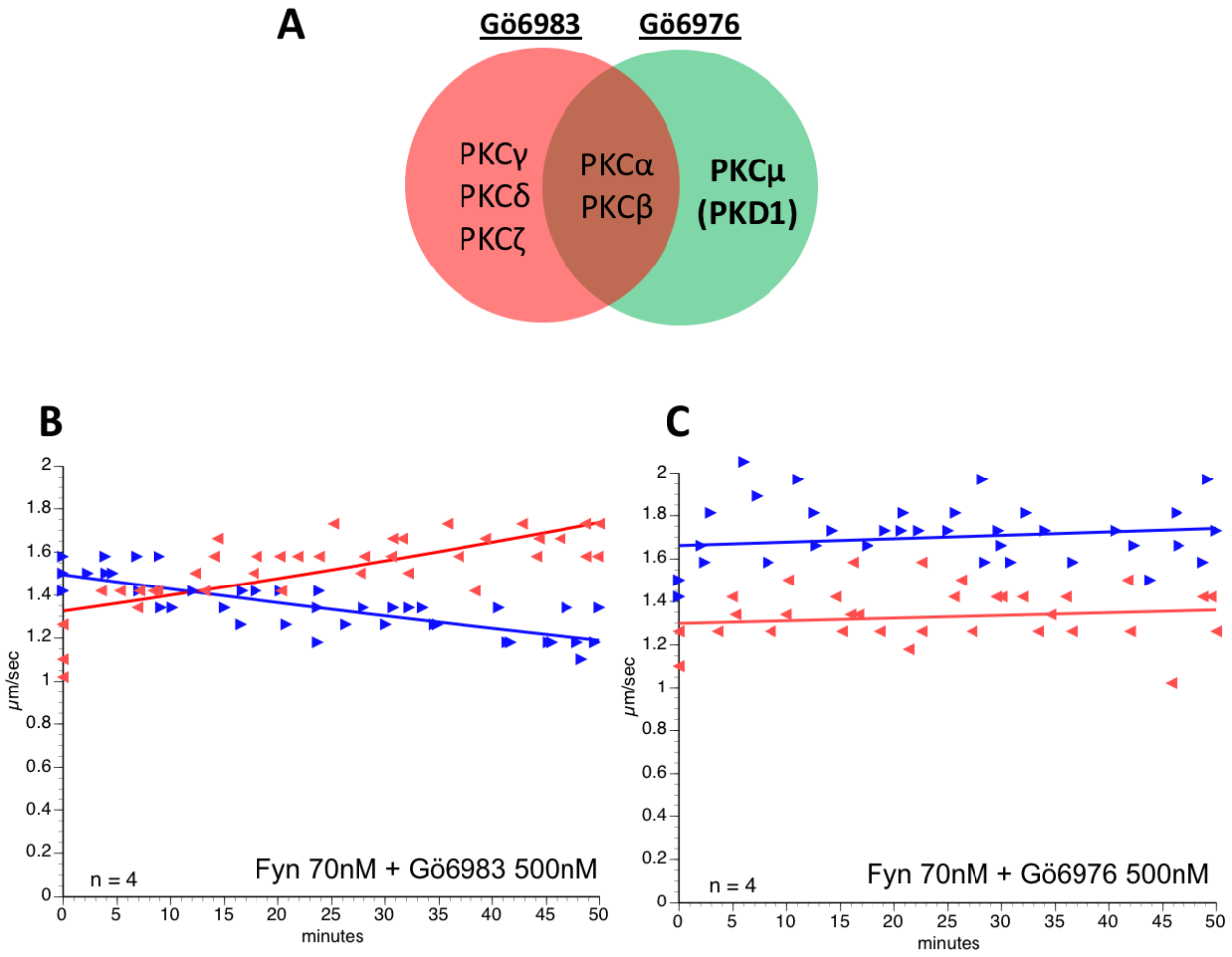

Figure 22. SFKs activate retrograde fast axonal transport via activation of a downstream kinase PKD1. (A) Isoform-selectivity of PKC inhibitors Gö6983 and Gö6976 (Martiny-Baron et al. 1993; Gschwendt et al. 1996). **(B)** Gö6983 does not prevent the activation of retrograde fast axonal transport by Fyn, a member of SFKs, in isolated squid axoplasms while **(C)** Gö6976 does. These data suggest that PKD1, which is inhibited by Gö6976 but not by Gö6983, is required for the activation of retrograde fast axonal transport by SFKs.

**Blue: kinesin-based anterograde fast axonal transport*
**Red: CDyn-based retrograde fast axonal transport*

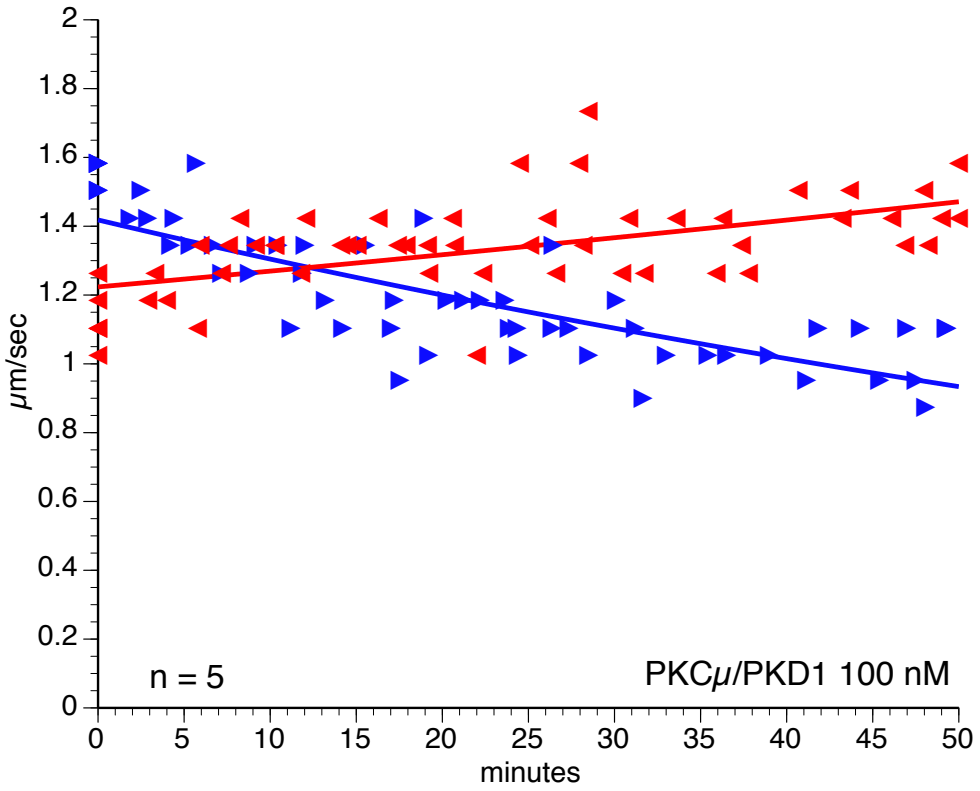

Figure 23. PKD1 activates retrograde fast axonal transport in isolated squid axoplasms. Perfusion of active recombinant PKD1 in isolated squid axoplasms activates retrograde fast axonal transport (*red*) while inhibiting anterograde fast axonal transport (*blue*), mimicking the effects of SFKs **(Figure 20)**.

3.3.6 BDNF treatment leads to DIC phosphorylation in rat primary neurons via activation of PKD1

Our data from isolated squid axoplasms strongly suggest that PKD1 mediates the activation of retrograde axonal transport by SFKs, which are activated upon BDNF stimulation and subsequent TrkB activation. To test our hypothesis that PKD1 mediates DIC phosphorylation upon BDNF in mammalian neurons, we pre-treated rat primary cortical neurons with PKD1 inhibitors. The inhibitor of PKD1 used in isolated squid axoplasm, Gö6976, was found to also inhibit the kinase activity of TrkB in nanomolar concentration (Behrens et al. 1999). This off-target inhibition made Gö6976 unsuitable for the use in primary neurons although it can still be used in isolated squid axoplasms where the plasma membrane—which contains TrkB—is removed. Instead, we used a small-molecule inhibitor of PKD named CRT 0066101 (Harikumar et al. 2010; Ni et al. 2013), as well as PKD1 substrate peptide fused with modified HIV-TAT protein sequence (GRKKRRQRRRPP) in the N-terminus, which enables the peptide to cross the plasma membrane (Green and Loewenstein 1988; Frankel and Pabo 1988). PKCs have well-characterized consensus substrate sequences that are specific to different isoforms of PKCs (Nishikawa et al. 1997) **(Figure 24A)**, which can provide better isoform-specific inhibition of PKCs than chemical inhibitors. Excess amounts of exogenous substrates specific to a particular isoform of PKC prevent phosphorylation of the endogenous substrates by that PKC isoform through competitive inhibition. PKCδ substrate peptide with the same modification was used as a control for isoform-specificity of PKC inhibition.

BDNF stimulation of 3DIV rat primary cortical neurons increased PKD activation, shown as increased immunoreactivity using an antibody against phospho-PKD at Ser916

residue, which is a PKD1 autophosphorylation site **(Figure 24B)**. Although phosphorylation of S916 is not required for the activity of PKD1, it is a marker for PKD1 activation as PKD1 shows strong autophosphorylation at this site following activation (Matthews et al. 1999; Vertommen et al. 2000). Neurons pre-treated with 30μM CRT 0066101, which inhibits the kinase activity of PKD1, did not show an increase in phospho-PKD (S916) immunoreactivity as expected. Neurons pre-treated with 10μM PKD1 substrate peptide or 10μM PKCδ substrate peptide did still show phosphorylation of PKD1 at S916, indicating activation **(Figure 24B)**. Even though an excess amount of PKD1 substrate peptide inhibits phosphorylation of endogenous substrates by competitive inhibition, it cannot easily compete against the S916 autophosphorylation as this is an intramolecular interaction. Total levels of PKD1 and DIC do not significantly change with inhibitor treatments **(Figure 24B)**. Analysis of DIC phosphorylation in the same samples using Phos-Tag SDS-PAGE revealed that inhibition of PKD1 by either CRT 0066101 or PKD1 substrate peptide, but not PKCδ substrate peptide, prevents BDNF-induced phosphorylation of DIC **(Figure 24C)**. These results indicate that activation of PKD1, but not another PKC isoform, is required for BDNF-induced DIC phosphorylation.

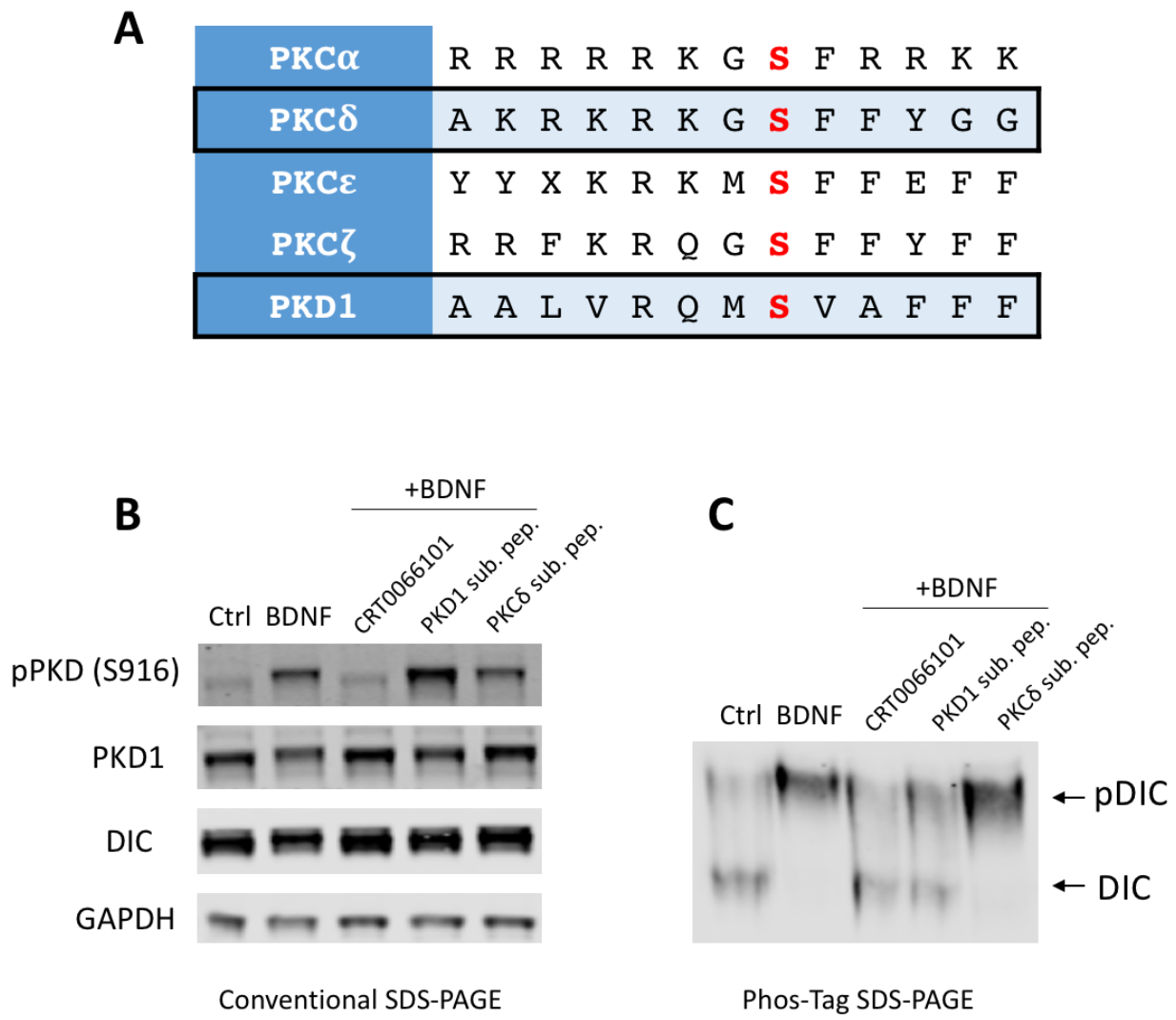

Figure 24. Activation of PKD1 is necessary for BDNF-induced DIC phosphorylation. (A) PKC isoforms have unique consensus substrate sequences (Nishikawa et al. 1997). Red "S" denotes the serine residue that gets phosphorylated by active PKCs. Two peptides used in this study, PKCδ and PKD1 substrate sequences, are highlighted. **(B)** BDNF treatment of rat primary cortical neurons increases activation of PKD1 measured by S916 autophosphorylation. A small-molecule PKD1 inhibitor CRT 0066101 blocks this activation. Cell-permeable PKD1 substrate peptide prevents phosphorylation of endogenous substrates by PKD1 but does not block PKD1 autophosphorylation. Cell-permeable PKCδ substrate peptide does not inhibit PKD1 activity. Total levels of PKD1 and DIC do not change with inhibitor treatments. GAPDH is provided as a loading control. **(C)** Phos-Tag SDS-PAGE of the same samples shows inhibition of BDNF-induced DIC phosphorylation by CRT 0066101 and PKD1 substrate peptide, but not by PKCδ substrate peptide, indicating that PKD1 is required for the phosphorylation of DIC upon BDNF stimulation.

3.3.7 Inhibition of PKD1 reduces retrograde axonal transport of QD-BDNF in rat primary cortical neurons

To determine the role of PKD1 on retrograde axonal transport in mammalian neurons, we cultured rat primary neurons in microfluidic chambers capable of fluidic isolation of the axon terminal compartment (ATC) from axonal (Ax) and somatodendritic compartment (SDC) as described in **3.2**. On 12 DIV, we observed that neuronal cell bodies were localized to SDC as the narrow width of microchannels in Ax does not allow neuronal cell body to migrate through. Axons originating from neuronal soma in the SDC successfully entered the microchannels of axonal compartment and reached the ATC where they arborize extensively **(Figure 25)**. Quantum-dot-labeled BDNF (QD-BDNF) was prepared as described in **3.2** and was added to the ATC for a 20-minute pulse at 1nM final concentration. Neurons in control group only received the QD-BDNF treatment while those in experimental groups received a 20-minute pre-treatment of either 200nM k252a (+k252a) or 30μM of CRT 0066101 (+CRT 0066101) prior to QD-BDNF treatment. Following the treatment, neurons were incubated for additional 30 minutes at 37˚C before fixation and immunocytochemistry. After fixation and immunocytochemistry, the accumulation of QD-BDNF in the SDC was quantified from confocal images of neurons in SDC as described in **3.2** and normalized to the number of neuronal cell bodies in each image. 53-66 images were taken from 6 separate MFCs (2 MFCs per experimental condition) and analyzed by an experimenter blinded to experimental conditions of each MFC. One-way ANOVA revealed a statistically significant difference among control (n=53), +252a (n=66), and +CRT 0066101 (n=56) group means ($F(2, 172)=9.909$, $p=0.0004$). Tukey's post-hoc analysis revealed a statistically significant reduction in the

amount of QD-BDNF accumulation in the SDC of +k252a compared to control ($P<0.01$) as well as CRT 0066101 compared to control ($P<0.001$), but not between +k252a and +CRT 0066101 **(Figure 26)**. This result is consistent with our previous finding in the squid axoplasm perfused with SFKs and PKD1 (**Figures 20, 22, and 23**) that activation of retrograde axonal transport upon neurotrophin signaling requires activation of PKD1, suggesting that phosphorylation of DIC by PKD1 has biological function of retrograde axonal transport activation.

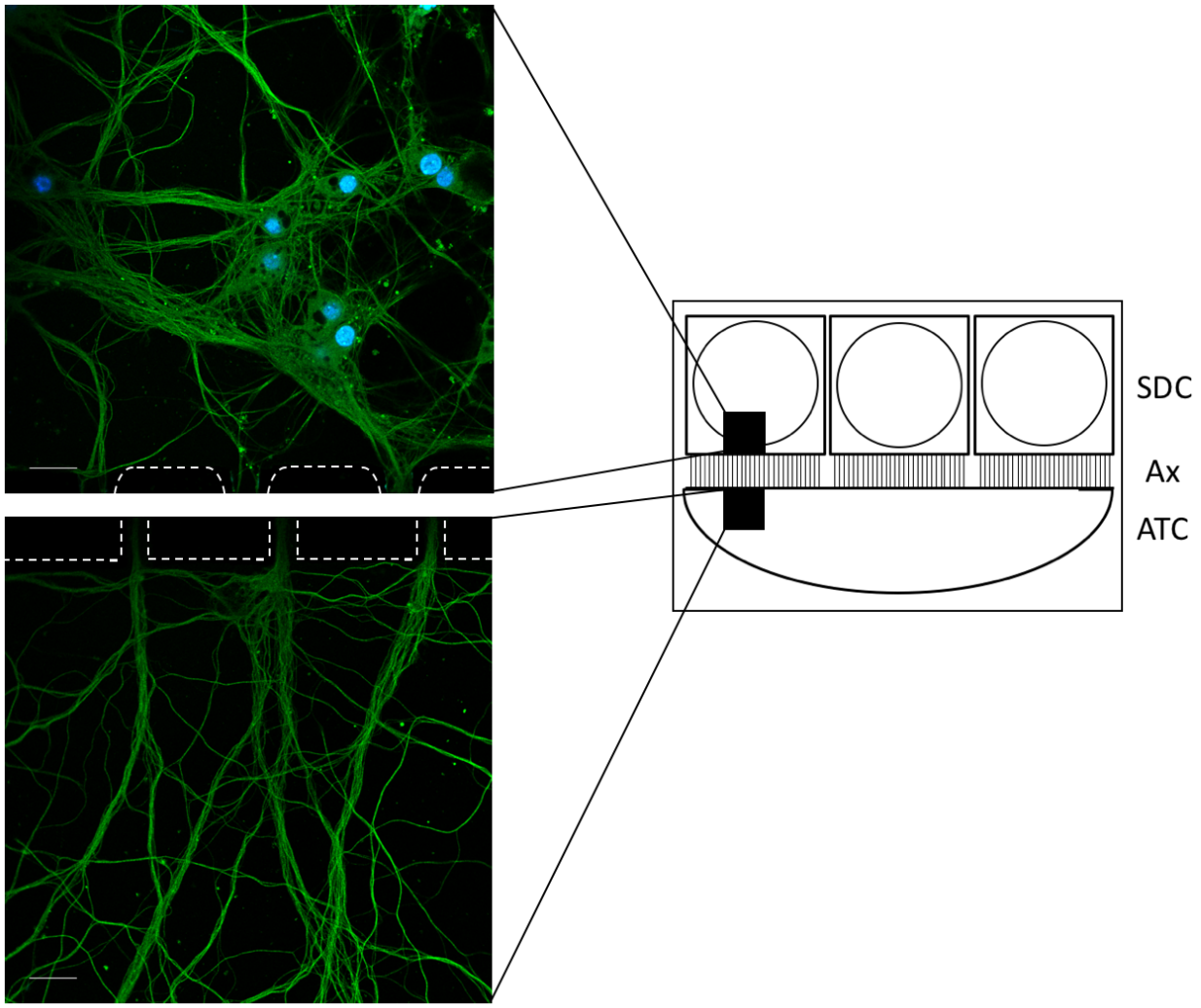

Figure 25. Culturing of rat primary cortical neurons in microfluidic chamber. Confocal images of 12DIV rat primary cortical neurons cultured in microfluidic chamber. Neuronal cell bodies are localized to the somatodendritic compartment (SDC) and axons grew through the axonal compartment (Ax) where cell bodies cannot enter, and reached the axon terminal compartment (ATC) where extensive axonal arborization is observed. Fluidic isolation of was maintained to prevent diffusion of materials from ATC to SDC and Ax. The white dotted lines represent the edges of axonal compartment. Blue: DAPI, Green: βIII-tubulin. Scale bar = 200μm

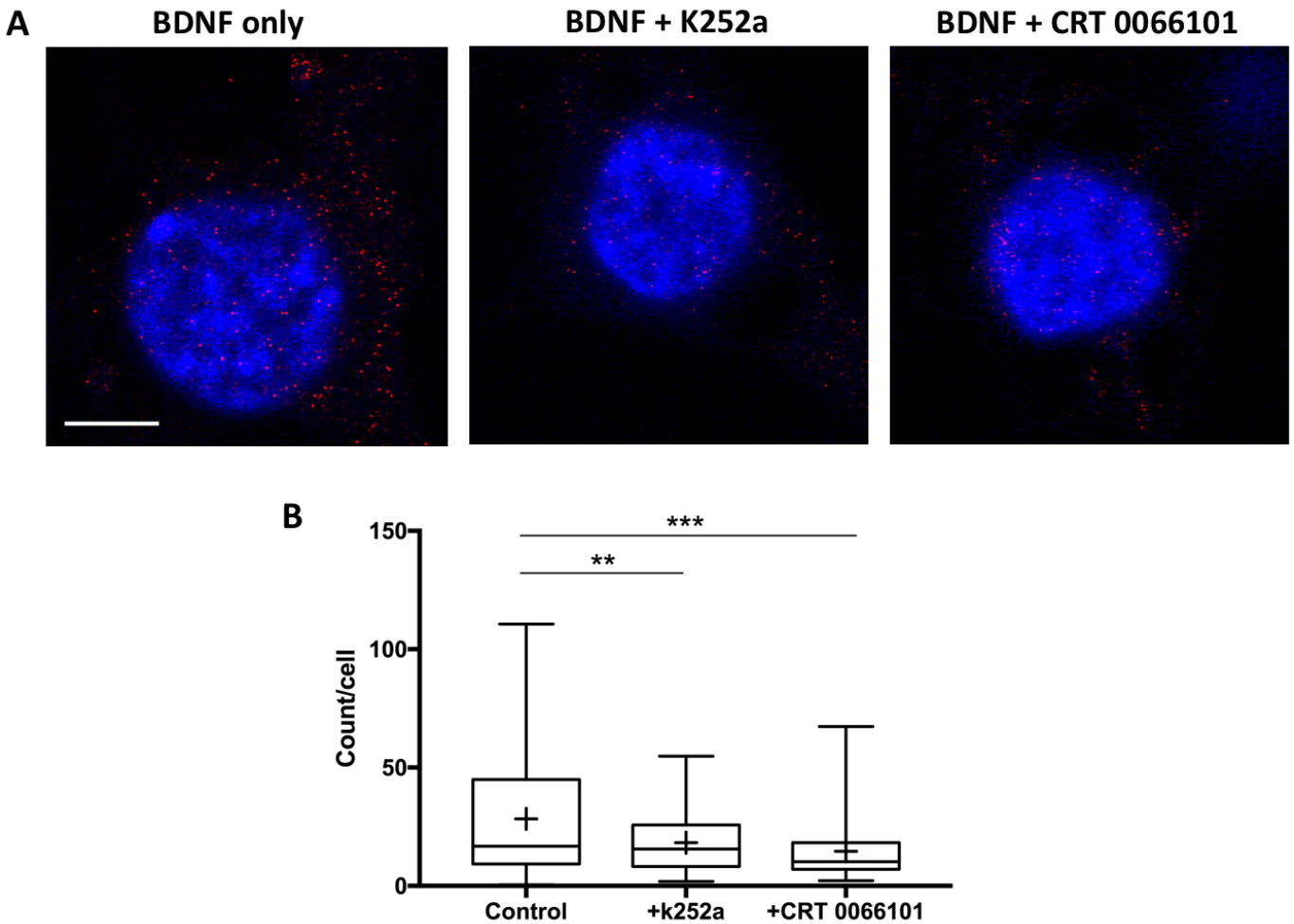

Figure 26. Inhibition of PKD1 reduces retrograde axonal transport of QD-BDNF in rat primary cortical neurons. (A) confocal images of neuronal cell bodies in the SDC compartments of BDNF only control, BDNF + k252a, and BDNF + CRT 0066101 group following the QD-BDNF pulse. *Blue: DAPI, Red: QD-BDNF. Scale bar = 100µm*. **(B)** Box plots of QD-BDNF accumulation in the SDC of BDNF control (Control), BDNF + k252a (+k252a), and BDNF + CRT (+CRT 0066101). One-way ANOVA and Tukey's post-hoc analysis revealed a statistically significant reduction in QD-BDNF accumulation in neurons pre-treated with k252a (**, $p<0.01$) and CRT 0066101 (***, $p<0.001$). *Line: median, +: mean*.

3.3.8 PKD1 phosphorylates DIC *in vitro*

To test if PKD1 can directly phosphorylate DIC, an *in vitro* kinase assay using recombinant PKD1 and recombinant full-length rat DIC 1A was performed in the presence of radiolabeled ATP [γ-^{32}P] as described in **3.2**. Unfortunately, the recombinant DIC had a significant amount of degradation from long-term storage as shown with Coomassie blue stain **(Figure 27A)**. Nevertheless, the autoradiogram of the Coomassie blue-stained gel clearly shows phosphorylation of full-length DIC and degraded fragments of DIC by recombinant PKD1, as well as autophosphorylation of PKD1 **(Figure 27B)**, identifying PKD1 as a serine/threonine kinase capable of phosphorylating DIC.

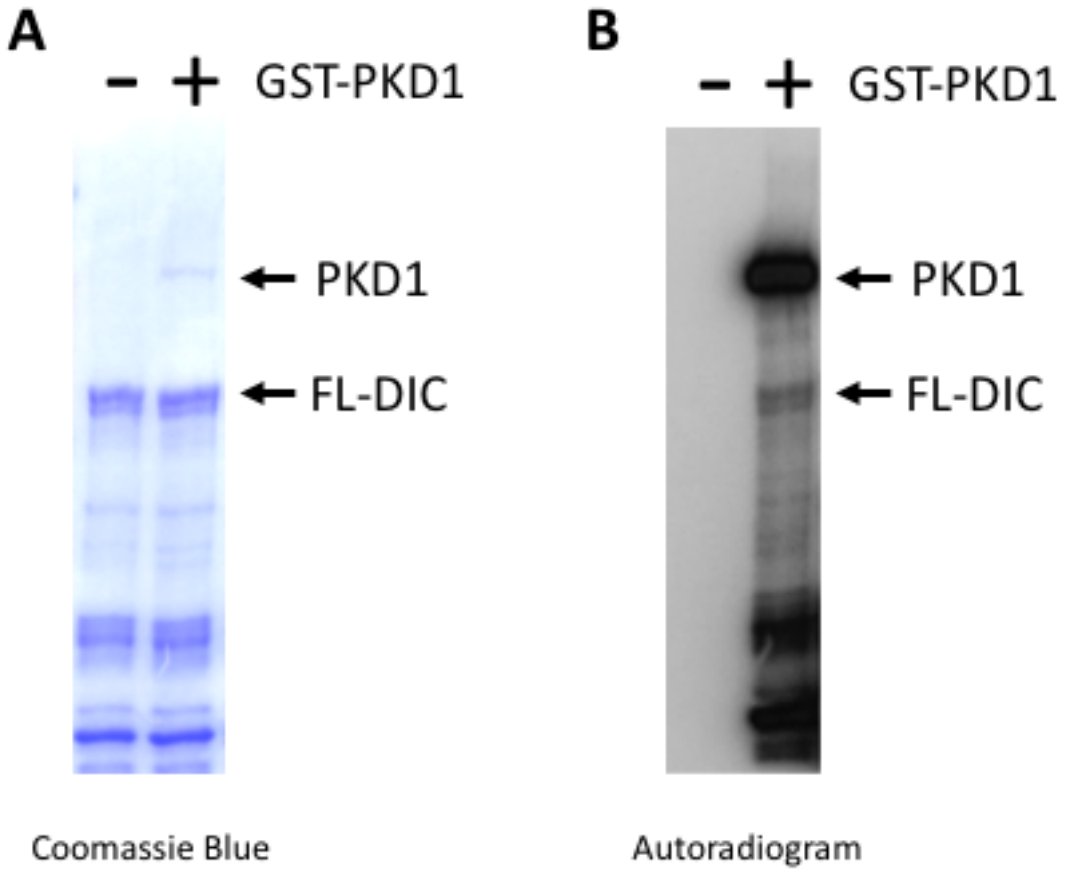

Figure 27. PKD1 directly phosphorylates DIC *in vitro*. Recombinant full-length rat DIC 1A was phosphorylated by recombinant GST-PKD1 *in vitro* in the presence of radiolabeled ATP [γ-32P]. Following in vitro phosphorylation, samples were electrophoresed on an SDS-PAGE gel. **(A)** Coomassie blue stain of SDS-PAGE gel shows recombinant GST-PKD1 (PKD1), recombinant full-length DIC 1A (FL-DIC), and degraded recombinant DIC. **(B)** Autoradiogram shows phosphorylation of FL-DIC and degraded DIC, as well as autophosphorylation of PKD1.

3.3.9 PKD1 phosphorylates DIC at different residues than ERK1/2

Mitchell et al. (2012) reported that DIC can be phosphorylated at Ser80/81 (DIC-1/2 isoforms, respectively) by extracellular signal-regulated protein kinase 1/2 (ERK1/2) and that this phosphorylation stimulates recruitment of CDyn to signaling endosomes for retrograde axonal transport. We obtained a polyclonal antibody raised against DIC-1/2 phosphorylated at Ser80/81 from Dr. K. Kevin Pfister (D. J. Mitchell et al. 2012), and tested whether this phosphorylation site was relevant to TrkB-SFKs-PKD1 pathway we identified. Western blot analysis of rat primary cortical neurons showed that inhibitors of TrkB, SFKs, and PKD1 diminished the increase in phosphorylation of DIC at Ser80/81 by BDNF treatment **(Figure 28)**, suggesting that PKD1 may also phosphorylate DIC at Ser80/81. In addition, the antibody against phospho-DIC (Sr80/81) shows fairly high level of immunoreactivity even in untreated control **(Figure 28)**, and BDNF treatment leads to a smaller magnitude of increase in phosphorylation at this site compared to the magnitude of increase in DIC phosphorylation detected by Phos-Tag. In order to test whether PKD1 and ERK1/2 truly phosphorylate DIC at the same residue, we treated rat primary cortical neurons with phorbol 12-myristate 13-acetate (PMA) **(Figure 29A)**. PMA is a phorbol ester that activates conventional and novel PKCs (i.e. PKCα, PKCδ), but not atypical PKCs (i.e. PKD1) **(Table 4)** (Tatin et al. 2006; Mackay and Twelves 2007), and leads to downstream activation of ERK (Lallemend et al. 2005). PMA treatment of rat primary neurons resulted in increases in phosphorylation of DIC at Ser80/81 **(Figure 29B)** and activation of ERK **(Figure 29C)** similar to BDNF treatment. However, PMA treatment did not show increase in phosphorylation of DIC detected by Phos-Tag SDS-PAGE **(Figure 29D)**. Phos-Tag separates phosphorylated proteins from unphosphorylated proteins by

decreasing the gel mobility of phosphorylated proteins. However, Phos-Tag does not separate all phosphorylated proteins, and the separation of phosphoproteins varies depending on phosphorylated residues (Kinoshita and Kinoshita-Kikuta 2011). This suggests that PKD1 phosphorylates DIC at additional residues other than Ser80/81 that can be detected by Phos-Tag SDS-PAGE. Consistent with this, vesicle motility assay in isolated squid axoplasms perfused with active recombinant ERK2 showed no effect of ERK2 on retrograde axonal transport **(Figure 30)**, which is in contrast to activation of retrograde axonal transport by PKD1. Taken together, our data suggest that PKD1 phosphorylates DIC at different residues than ERK1/2, and that phosphorylation by PKD1, but not ERK1/2, has biological function of activating CDyn-based retrograde axonal transport.

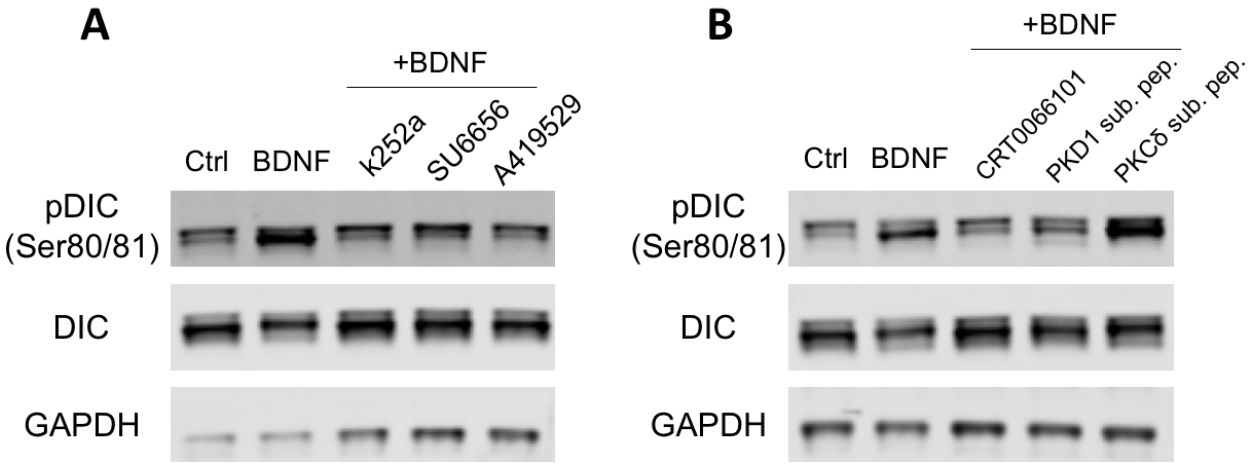

Figure 28. Inhibition of TrkB-SFKs-PKD1 pathway prevents phosphorylation of DIC at Ser80/81 upon BDNF stimulation. (A) Inhibitors of TrkB (k252a) and SFKs (SU6656 and A419529) prevents BDNF-induced phosphorylation of DIC at Ser80/81. **(B)** Inhibitors of PKD1 (CRT 0066101 and PKD1 substrate peptide), but not inhibitor of PKCδ (PKCδ substrate peptide), prevents BDNF-induced phosphorylation of DIC at Ser80/81.

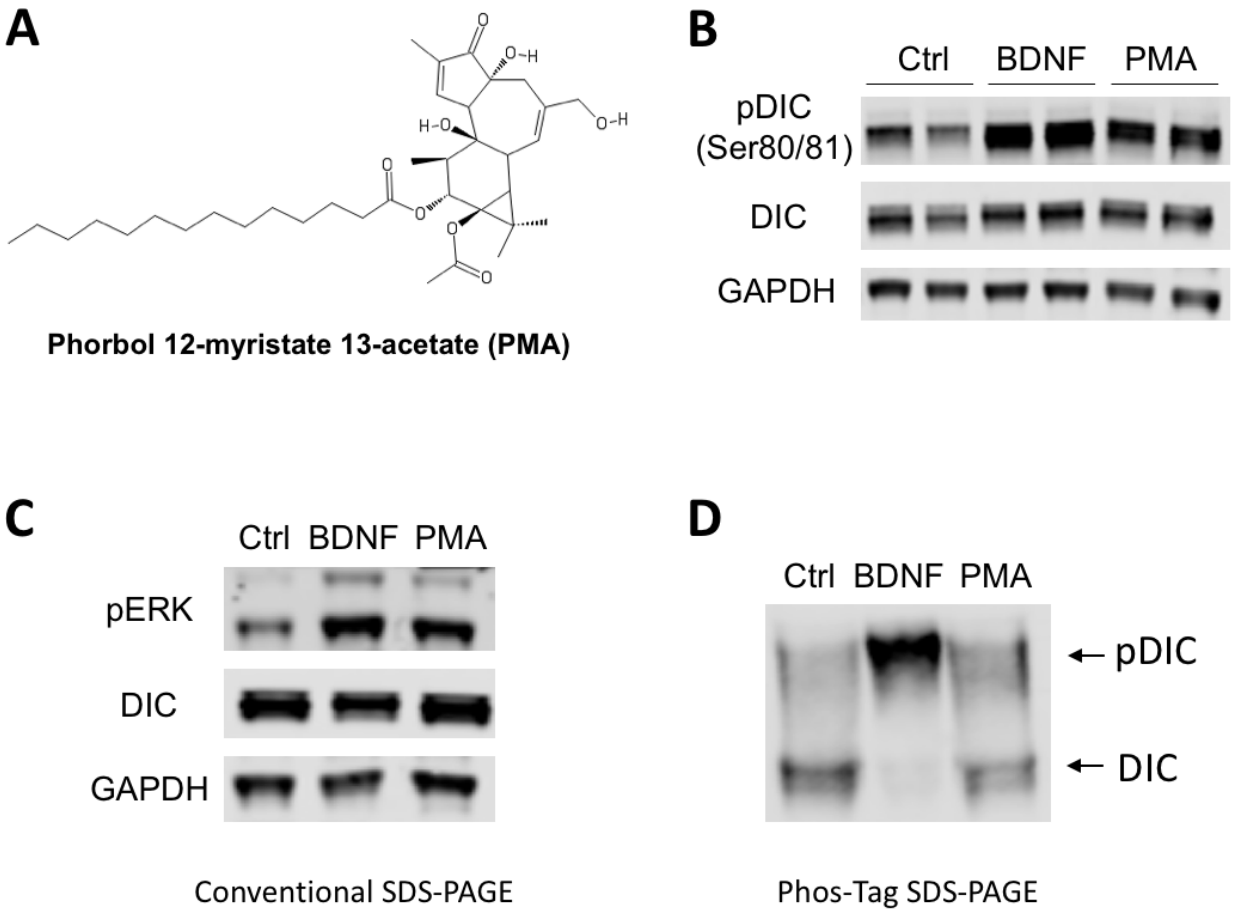

Figure 29. Phos-Tag SDS-PAGE separates DIC that is phosphorylated at residues other than Ser80/81 by gel mobility shift. (A) Chemical structure of phorbol 12-myristate 13-acetate (PMA). PMA is a phorbol ester that activates conventional and novel PKCs (i.e. PKCα, PKCδ) but not atypical PKCs (i.e. PKD1) **(Table 4)**. **(B)** Both BDNF and PMA treatments in 3DIV rat primary cortical neurons increase phosphorylation of DIC at Ser80/81, which is phosphorylated by ERK1/2 (Mitchell et al. 2012). **(C)** Both BDNF and PMA treatments activate ERK, measured by phosphorylation of ERK (pERK). **(D)** Gel mobility shift in Phos-Tag SDS-PAGE separated phosphorylated DIC (pDIC) from unphosphorylated DIC with BDNF treatment, but not with PMA treatment even though PMA treatment leads to phosphorylation of DIC at Ser80/81. Gel mobility shift in Phos-Tag SDS-PAGE varies depending on the phosphorylated residues, some phospho-proteins are not separated by Phos-Tag SDS-PAGE. This indicates that BDNF treatment increases phosphorylation of DIC at residues other than Ser80/81.

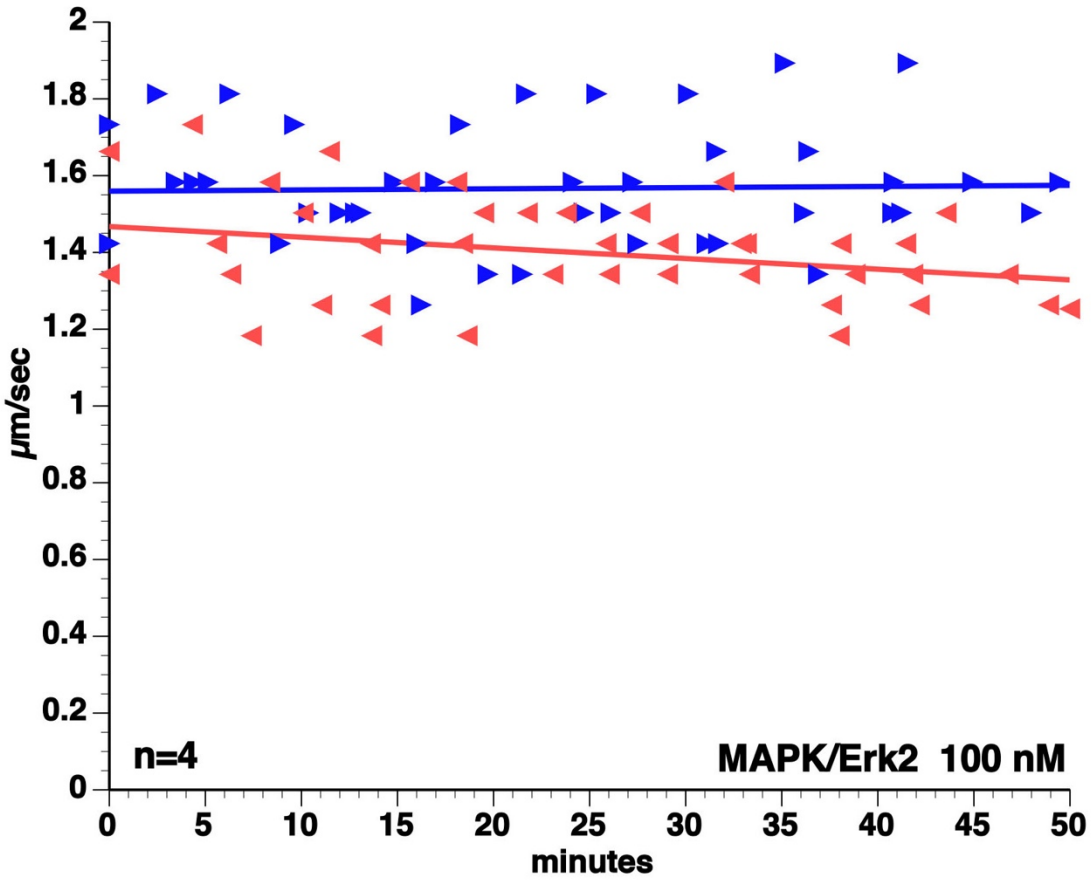

Figure 30. ERK2 does not activate retrograde fast axonal transport in isolated squid axoplasms. Vesicle motility assay in isolated squid axoplasms perfused with active recombinant ERK2 (100nM) shows no activation of retrograde fast axonal transport by ERK2.

3.4 Discussion

3.4.1 Activation of retrograde fast axonal transport by neurotrophin signaling

Work presented in this chapter identified a novel kinase pathway that activates retrograde fast axonal transport through phosphorylation of intermediate chain subunit (DIC) of cytoplasmic dynein (CDyn) upon neurotrophin (i.e. BDNF) signaling. We found that BDNF stimulation of primary rat cortical neurons leads to rapid and long-lasting phosphorylation of DIC **(Figure 18)**. From several kinase pathways activated by BDNF signaling **(Figure 16)**, we provided evidence that activation of TrkB-SFK-PKD1 pathway is required for phosphorylation of DIC upon BDNF stimulation in primary rat cortical neurons using a variety of pharmacological and peptide-based kinase inhibitors **(Figures 19 and 24)**, as well as showing that PKD1 can directly phosphorylate DIC **(Figure 27)**. From vesicle motility assays in the squid axoplasms, we discovered that SFKs, a subfamily of non-receptor tyrosine kinases, activate CDyn-based retrograde fast axonal transport by activating a downstream serine/threonine kinase PKD1 **(Figures 20, 22, and 23)**. In addition, the role of PKD1 in activation of retrograde axonal transport by neurotrophin was further demonstrated in mammalian neurons by examining the accumulation of QD-BDNF in rat primary cortical neurons cultured in microfluidic chambers. Inhibition of TrkB with k252a or PKD1 with CRT 0066101 reduced the amount of QD-BDNF accumulation in the cell body, indicating that activation of retrograde transport of signaling endosomes containing TrkB-BDNF is mediated by PKD1. Taken together, the data presented in this chapter indicate that phosphorylation of DIC by PKD1 upon neurotrophin signaling has biological function of activating retrograde fast axonal transport **(Figure 31)**.

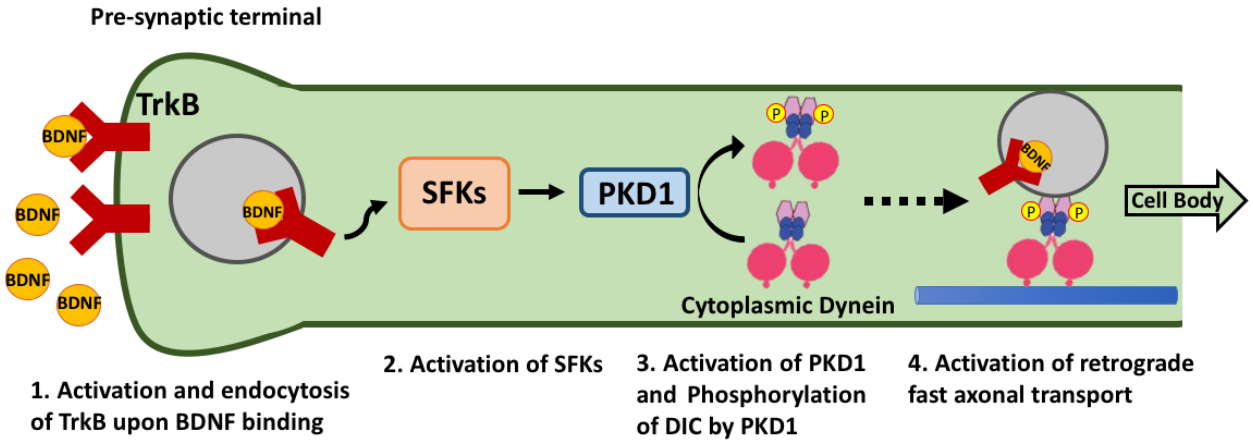

Figure 31. Proposed mechanism of activation of retrograde axonal transport by neurotrophin signaling. (1) Neurotrophins (i.e. BDNF) bind to their respective receptors (i.e. TrkB), triggering downstream signaling cascades and endocytosis of active receptors. **(2)** TrkB activates SFKs, a subgroup of non-receptor tyrosine kinase family. **(3)** SFKs activate PKD1, a serine/threonine kinase. **(4)** PKD1 phosphorylates dynein intermediate chain (DIC) on serine residue(s) and activation of retrograde fast axonal transport of signaling endosomes.

3.4.2 Phosphorylation of DIC by ERK1/2 and PKD1

Previously, ERK1/2 has been shown to phosphorylate DIC at Ser80/81 (DIC-1/2 isoforms, respectively), and phosphorylation of DIC at this residue was proposed to recruit CDyn to signaling endosomes for retrograde axonal transport based on experiments using rat primary neurons overexpressing pseudophosphorylated DIC (D. J. Mitchell et al. 2012). However, ERK2 had no effect on retrograde fast axonal transport in isolated squid axoplasms **(Figure 30)** while SFKs and their downstream kinase PKD1 activated retrograde fast axonal transport **(Figures 20, 22, and 23)**. We further investigated these seemingly conflicting results using PMA which activates conventional and novel PKCs as well as ERK without activating PKD1. Previously, we have shown that the constitutively active catalytic subunit of conventional PKCs such as PKCα and PKCβ can activate retrograde axonal transport in isolated squid axoplasms In rat primary cortical neurons (G. Morfini et al. 2007). However, data presented in this chapter clearly demonstrates that the activation of retrograde axonal transport by SFKs are mediated by PKD1, suggesting that activation of retrograde axonal transport by PKCs requires a complex mechanism beyond simple activation of the kinase. Nonetheless, PMA treatment increased activation of ERK and subsequent phosphorylation of DIC at Ser80/81 based on immunoblotting, but it did not lead to DIC phosphorylation that is discernable by Phos-Tag SDS-PAGE **(Figure 29)**. As separation of phosphoproteins by gel mobility shift in Phos-Tag SDS-PAGE varies depending on proteins and phospho-residues, we concluded that BDNF treatment leads to phosphorylation of DIC on residues distinct from Ser80/81 as well as Ser80/81 in a TrkB-SFKs-PKD1-dependent manner. Although we report that PKD1 can directly phosphorylate DIC **(Figure 27)**, further study using mass spectrometry is needed

to identify the exact residues on DIC that are phosphorylated by PKD1. Interestingly, inhibition of PKD1 by a chemical inhibitor CRT0066101 and even by a specific PKD1 substrate peptide reduced the increase in phosphorylation of DIC on Ser80/81 residues **(Figure 28B)** upon BDNF stimulation, suggesting a potential interaction between phosphorylation of DIC by ERK1/2 and PKD1. One such potential interaction is priming phosphorylation where phosphorylation of a substrate by one kinase enables another kinase to recognize and phosphorylate the same substrate at different residues, which has been shown in several kinases (Cho and Johnson 2003, 2004; Hergovich et al. 2006; G. Huang et al. 2007; St-Denis et al. 2015; Aoki and Yoshida 2017). Although it is not explicitly addressed in this study, it is plausible that PKD1 primes DIC for phosphorylation by ERK1/2 in the context of neurotrophin signaling based on our results. Considering phosphorylation of DIC by PKD1 and ERK1/2 as separate events, our data from isolated squid axoplasm indicate that PKD1, but not ERK1/2, activate retrograde axonal transport while they do not exclude the possibility of different biological consequences of DIC phosphorylation by ERK1/2.

3.4.3 Complexity of signaling cascades in different biological contexts

Here, we identified a novel kinase signaling cascade that activates retrograde fast axonal transport upon neurotrophin signaling through activation of TrkB-SFK-PKD1. While this pathway seems hierarchal and linear, that may be true only in the context of neurotrophin signaling. Some signaling cascades such as MAPKKK[17]-MAPKK[18]-MAPK[19]

[17] MAPKKK: mitogen-activated protein kinase kinase kinase
[18] MAPKK: mitogen-activated protein kinase kinase
[19] MAPK: mitogen-activate protein kinase

seem to be hierarchal while others may not be. For instance, activation of TrkB by BDNF in rat primary cortical neurons recruits SFKs via interaction between the auto-phosphorylated tyrosine residues of TrkB and Src-homology domain 2 (SH2) of SFKs, suggesting that there are SFKs downstream of TrkB (Iwasaki et al. 1998). Our data, also in the context of BDNF stimulation of rat primary cortical neurons, support this claim. However, influx of zinc from glutamatergic synaptic transmission has been shown to activate TrkB in an activity-dependent, but neurotrophin-independent, manner by activation of SFKs through inhibition of C-terminal Src kinase (CSK) in rat primary cortical neurons (Huang et al. 2008), indicating that SFKs can be upstream of TrkB in the context of zinc-mediated activation of TrkB in primary cortical neurons **(Figure 32A)**. Another example of multiple signaling pathway sequences dependent on the specific cellular context is activation of SFKs and PKCs. PKCs have been shown to be upstream of SFKs in the context of platelet activation (Senis et al. 2014), podosome formation (Tatin et al. 2006), and conjunctiva goblet cell proliferation (Li et al. 2013), while PKCs appear to be downstream of SFKs in the context of reactive oxygen species (ROS) signaling (Mu and Liu 2017) and development of primordial follicles (Du et al. 2012) **(Figure 32B)**. In addition, SFKs can activate PKD1 in oxidative stress signaling (Steinberg 2012) while activation of PKD1 appears to be dependent on PKC activation by DAG in cardiomyocytes (Guo et al. 2011) and in vascular endothelial growth factor (VEGF)-mediated angiogenesis (Ha and Jin 2009) **(Figure 32C)**, indicating that mechanisms of kinase activation differ based on cell types and contexts.

In addition to differential hierarchy of signaling cascades, activation of the same kinase can have different physiological outcomes depending on the context in which it is

activated. For instance, our lab has shown that 1-metyhl-4-phenylpyridinium (MPP+), a neurotoxin that causes Parkinsonian symptoms, activates retrograde fast axonal transport by activation of caspase-3 and subsequent proteolytic activation of PKCδ. This MPP+-mediated activation of retrograde axonal transport can be blocked by competitive inhibition with the PKCδ substrate peptide, but not with PKD1 substrate peptide (G. Morfini et al. 2007) even though caspase-3 has also been reported to activate PKD1 by proteolysis of its regulatory domain similarly to PKCδ (Rybin et al. 2012; Steinberg 2012). Data from our current work indicated that activation of SFKs as a result of neurotrophin signaling leads to activation of retrograde axonal transport in a PKD1-dependent manner even though SFKs can also activate PKCδ (Senis et al. 2014; Murugappan et al. 2009) in different contexts **(Figure 33)**.

These results suggest that the hierarchy of kinase signaling cascades can vary depending on the cell types and biological contexts, and even the activation of same kinase may result in different biological outcomes based on the context in which it is activated. The mechanisms of these differential signaling cascades remain elusive, but mapping out signaling pathways in various biological contexts provides us with some evidence that differences in localization, post-translational modifications such as ubiquitination (Mohapatra et al. 2013; Ball et al. 2016; Yang et al. 2010; Giannini and Bijlmakers 2004) and SUMOlyation (Simpson-Lavy and Johnston 2013; Wen et al. 2017; Knittle et al. 2017), and interacting scaffolding proteins may explain the differential biological outcome of intracellular signaling. Deeper understanding of these mechanisms is essential when manipulating kinase signaling in therapeutic interventions.

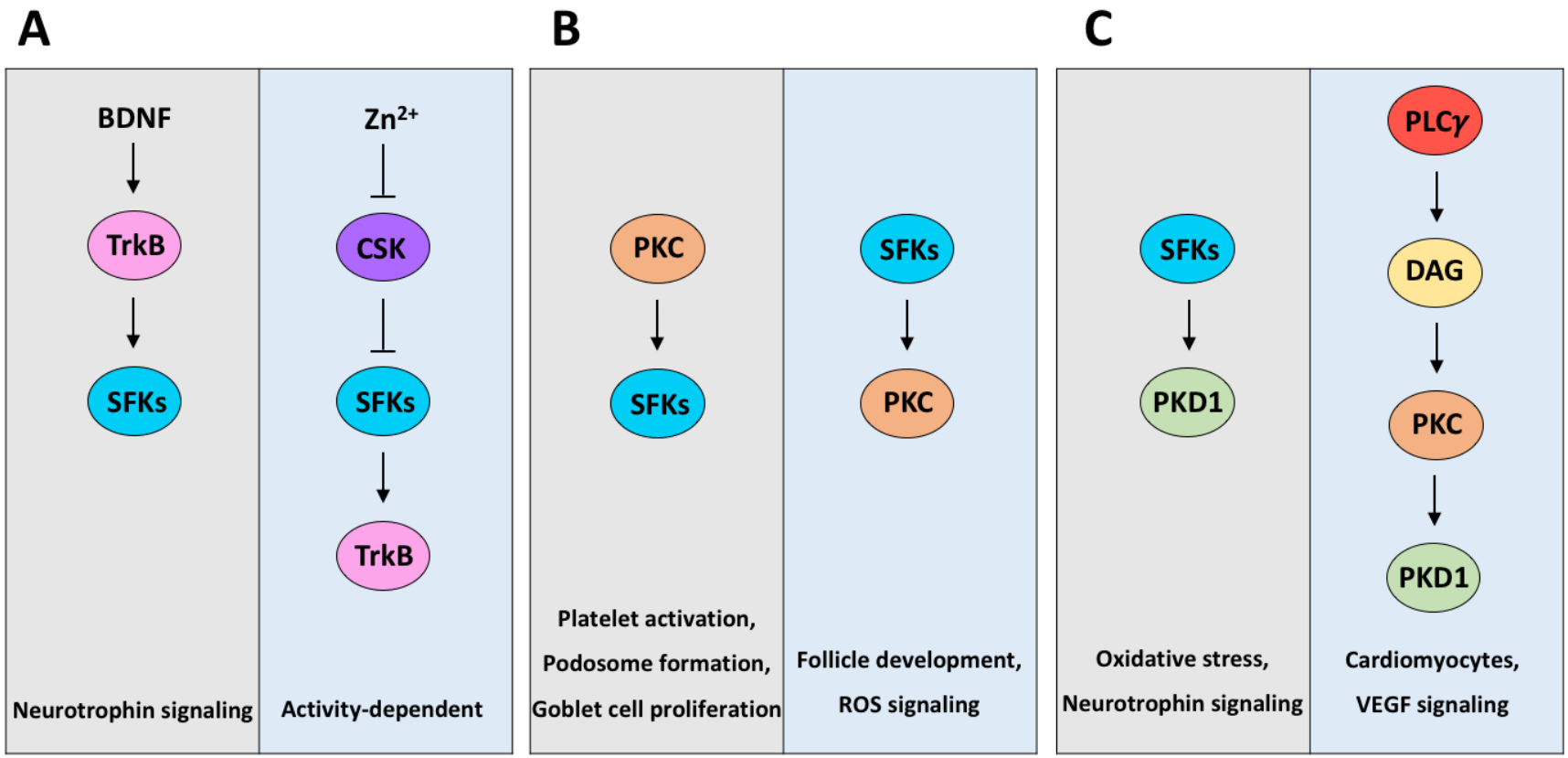

Figure 32. Differential hierarchy of signaling cascades in different biological contexts. The sequence of signaling cascades can differ based on the cell types and biological contexts in which they are activated. **(A)** In the context of BDNF signaling, TrkB activates SFKs while SFKs activate TrkB in the context of glutamatergic synaptic transmission-mediated Zn^{2+} influx (Huang et al. 2008). **(B)** PKCs activate SFKs in the context of platelet activation (Senis et al. 2014), podosome formation (Tatin et al. 2006), and goblet cell proliferation (Li et al. 2013) while SFKs activate PKCs in the context of follicle development (Du et al. 2012) and ROS signaling (Mu and Liu 2017). **(C)** SFKs activate PKD1 in the context of oxidative stress (Steinberg 2012) and neurotrophin signaling (Figures 19 and 22) while PKD1 is activated by PLC-DAG-PKC pathway in cardiomyocytes (Guo et al. 2011) and VEGF-mediated angiogenesis (Ha and Jin 2009).

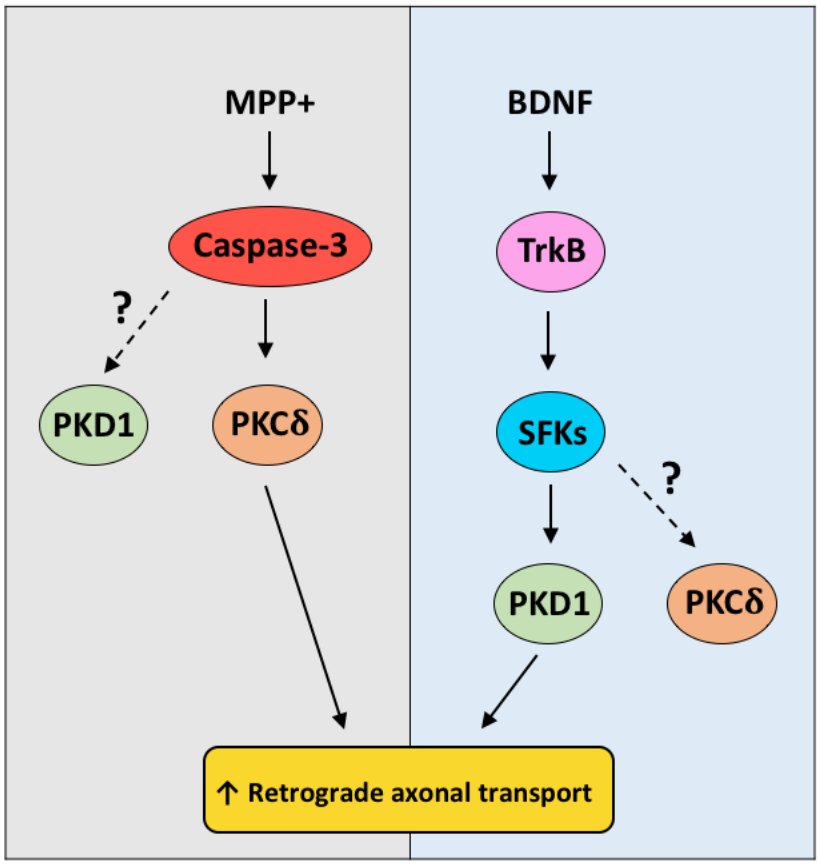

Figure 33. Differential biological consequences of identical kinases in specific biological contexts. PKCδ mediates activation of retrograde axonal transport by MPP+ Although caspase-3 has been shown to proteolytically activate PKD1 similarly to PKCδ, it is not known whether caspase-3 activates PKD1 upon MPP+ intoxication. On the other hand, PKD1 mediates activation of retrograde axonal transport by BDNF-TrkB-SFKs signaling cascade. While SFKs have been shown to activate PKCδ, it is not known whether a signaling cascade involving SFKs is required for activation of PKCδ upon BDNF stimulation.

3.4.4 Future directions

Our results identified a novel kinase signaling pathway that activates retrograde fast axonal transport through activation of TrkB-SFK-PKD1 and phosphorylation of DIC by PKD1. Although this work was done in the context of BDNF, neurotrophins have overlapping functions and downstream signaling cascades. Therefore, it is likely that the same pathway may be activated by other neurotrophins. Preliminary data from our lab shows phosphorylation of DIC following NT-3 treatment (data not shown) in rat primary cortical neurons although further experiments with kinase inhibitors and other neurotrophins are needed to test whether they share the same downstream signaling pathway leading to phosphorylation of DIC.

Furthermore, the molecular mechanisms underlying the activation of retrograde axonal transport have not been addressed by the present study. Based on the proposed role of DIC in cargo-binding, we hypothesize that phosphorylation of DIC by PKD1 commits signaling endosomes in the synaptic terminal for retrograde transport to the neuronal soma by recruiting cDyn to the endosome. Identifying the exact residues on DIC that are phosphorylated by PKD1 will facilitate analysis of the effects of DIC phosphorylation by PKD1 and ERK1/2. Introducing phosphomimetic and non-phosphorylatable mutations at those residues may be helpful in studying the molecular mechanisms underlying the retrograde fast axonal transport upon phosphorylation of DIC by PKD1. Understanding such mechanisms may also have relevance to pathological conditions. Aberrant activation of retrograde axonal transport of membrane-bound cargoes by MPP+ depletes membranous profiles from the presynaptic terminal by disturbing the lipid membrane equilibrium, leading to synaptic dysfunctions and dying-

back degeneration (Morfini et al. 2007; Serulle et al. 2007). Furthermore, neuroinvasive viruses such as poliovirus, rabies virus, and herpes simples virus may be capable of hijacking this pathway to enhance their retrograde axonal transport to the neuronal cell body for replication. Thus, inhibiting the activation of retrograde axonal transport during neuroinvasive viral infections may be helpful in preventing or minimizing the extend of the infections.

CHAPTER IV.

IV. CONCLUSIONS

Data presented in this work supports the main hypothesis in our lab that phosphorylation of motor proteins like conventional kinesin (kinesin) and cytoplasmic dynein (CDyn) regulates fast axonal transport of molecular cargoes. Normal phosphorylation can determine what is transported and where a cargo is delivered. Aberrant phosphorylation of motor proteins and subsequent axonal transport defects in neurodegenerative diseases such as Huntington's disease (HD) leads to dying-back neuropathy of affected neurons. Previous work from our lab has demonstrated that the CAG repeat expansion mutation in huntingtin that causes HD may be a hypermorphic gain-of-function for huntingtin, which results in abnormal activation c-Jun N-terminal kinase 3 (JNK3) and subsequent axonal transport defects. Work described in **Chapter II** extends this finding by addressing the role of JNK3 in HD pathology using the R6/2 mouse model of Huntington's disease with genetic deletion of JNK3. Our results indicate that deletion of JNK3 ameliorates HD pathology in R6/2 mice by preserving neuronal connectivity, suggesting that inhibition of JNK3 is a promising therapeutic approach for HD. Work presented in **Chapter III** identified a novel kinase signaling pathway that activates retrograde fast axonal transport by neurotrophin signaling. Brain-derived neurotrophic factor (BDNF), a member of neurotrophin family, triggers a signaling cascade involving activation of TrkB-SFK-PKD1. Active PKD1 then phosphorylates

dynein intermediate chain (DIC) subunit of CDyn to activate retrograde fast axonal transport.

The morphology of neurons places a unique challenge on neurons to maintain proper delivery of molecular cargoes to specific subcellular compartments. This evidence suggests that 1) there is a normal physiological role of kinase signaling in regulation of motor proteins and axonal transport; 2) neuropathogenic proteins disrupt this regulation by aberrant activation of specific kinase signaling pathways; and 3) correcting the abnormal kinase signaling can ameliorate axonal transport defects and dying-back neurodegeneration caused by neuropathogenic proteins.

Neurodegenerative diseases inflict immense pain and suffering on patients and their families, and place astronomical burdens on society. Unlike diseases such as cancer, very little success has been achieved in treating neurodegenerative diseases despite tremendous effort by many groups. Recent studies indicate that unrelated neurodegenerative diseases such as Alzheimer's disease, Huntington's disease, and amyotrophic lateral sclerosis may share a common pathology disruption of normal axonal transport function, leading to degeneration of neurons with long axons. I believe that defective axonal transport, termed "dysferopathy", represents a critical pathogenic event in neurodegenerative diseases. Expanding our knowledge on the regulation of axonal transport and the disruptions associated with specific disease mechanisms will help us develop effective therapeutic interventions for these diseases.

CITED LITERATURE

Al-Qudah, Mohammad A., and Ahmed Al-Dwairi. 2016. “Mechanisms and Regulation of Neurotrophin Synthesis and Secretion.” *Neurosciences*. https://doi.org/10.17712/nsj.2016.4.20160080.

Albin, Roger L., Anne B. Young, John B. Penney, Barbara Handelin, Rosemary Balfour, Keith D. Anderson, Dorene S. Markel, Wallace W. Tourtellotte, and Anton Reiner. 1990. “Abnormalities of Striatal Projection Neurons and N-Methyl-D-Aspartate Receptors in Presymptomatic Huntington’s Disease.” *New England Journal of Medicine* 322 (18): 1293–98. https://doi.org/10.1056/NEJM199005033221807.

Allaman, Igor, and Pierre J. Magistretti. 2013. “Brain Energy Metabolism.” In *Fundamental Neuroscience*, edited by L.R. Squire, D. Berg, F.E. Bloom, S. du Lac, A. Ghosh, and N.C. Spitzer, 261–84. San Diego: Academic Press.

Aoki, Katsuhiko, and Kiyotsugu Yoshida. 2017. “Biological Consequences of Priming Phosphorylation in Cancer Development.” In *Protein Phosphorylation*. InTech. https://doi.org/10.5772/intechopen.70039.

Apostol, Barbara L., Katalin Illes, Judit Pallos, Laszlo Bodai, Jun Wu, Andrew Strand, Erik S. Schweitzer, et al. 2006. “Mutant Huntingtin Alters MAPK Signaling Pathways in PC12 and Striatal Cells: ERK1/2 Protects against Mutant Hungtingtin-Associated Toxicity.” *Human Molecular Genetics* 15 (2): 273–85. https://doi.org/10.1093/hmg/ddi443.

Balkowiec, Agnieszka, Diana L Kunze, and David M Katz. 2000. “Brain-Derived Neurotrophic Factor Acutely Inhibits AMPA-Mediated Currents in Developing Sensory Relay Neurons.” *The Journal of Neuroscience* 20 (5): 1904–11. http://www.jneurosci.org/content/20/5/1904.abstract.

Ball, K. Aurelia, Jeffrey R. Johnson, Mary K. Lewinski, John Guatelli, Erik Verschueren, Nevan J. Krogan, and Matthew P. Jacobson. 2016. “Non-Degradative Ubiquitination of Protein Kinases.” *PLoS Computational Biology* 12 (6). https://doi.org/10.1371/journal.pcbi.1004898.

Barde, Y A, D Edgar, and H Thoenen. 1982. “Purification of a New Neurotrophic Factor from Mammalian Brain.” *The EMBO Journal* 1 (5): 549–53. https://doi.org/0261-4189/82/0105-0549.

Beattie, Michael S., Anthony W. Harrington, Ramee Lee, Ju Young Kim, Sheri L. Boyce, Frank M. Longo, Jacqueline C. Bresnahan, Barbara L. Hempstead, and Sung Ok Yoon. 2002. “ProNGF Induces p75-Mediated Death of Oligodendrocytes Following Spinal Cord Injury.” *Neuron* 36 (3): 375–86. https://doi.org/10.1016/S0896-6273(02)01005-X.

Beglinger, L J, P C Nopoulos, R E Jorge, D R Langbehn, A E Mikos, D J Moser, K Duff, R G Robinson, and J S Paulsen. 2005. “White Matter Volume and Cognitive Dysfunction in Early Huntington’s Disease.” *Cogn Behav Neurol* 18 (2): 102–7. https://doi.org/00146965-200506000-00004 [pii].

Behrens, M. Margarita, Uta Strasser, and D. W. Choi. 1999. “Go 6976 Is a Potent Inhibitor of Neurotrophin-Receptor Intrinsic Tyrosine Kinase.” *Journal of Neurochemistry* 72 (3): 919–24. https://doi.org/10.1046/j.1471-4159.1999.0720919.x.

Bell, K. F S, and a. Claudio Cuello. 2006. “Altered Synaptic Function in Alzheimer’s Disease.” *European Journal of Pharmacology* 545 (1): 11–21. https://doi.org/10.1016/j.ejphar.2006.06.045.

Berkelaar, M, D B Clarke, Y-C Wang, G M Bray, and A J Aguayo. 1994. “Axotomy Ganglion Results in Delayed Death and Apoptosis of Retinal Cells in Adult Rats.” *The Journal of Neuroscience* 14 (7): 4368–74.

Bibb, J A, Z Yan, P Svenningsson, G L Snyder, V A Pieribone, A Horiuchi, A C Nairn, A Messer, and P Greengard. 2000. “Severe Deficiencies in Dopamine Signaling in Presymptomatic Huntington’s Disease Mice.” *Proceedings of the National Academy of Sciences of the United States of America* 97 (12): 6809–14. https://doi.org/10.1073/pnas.120166397.

Binder, Devin K., and Helen E. Scharfman. 2004. “Brain-Derived Neurotrophic Factor.” *Growth Factors* 22 (3): 123–31. https://doi.org/10.1080/08977190410001723308.

Blake, R A, M A Broome, X Liu, J Wu, M Gishizky, L Sun, and S A Courtneidge. 2000. “SU6656, a Selective Src Family Kinase Inhibitor, Used to Probe Growth Factor Signaling.” *Molecular and Cellular Biology* 20 (23): 9018–27. https://doi.org/10.1128/MCB.20.23.9018-9027.2000.

Boltaev, Umed, Yves Meyer, Farangis Tolibzoda, Teresa Jacques, Madalee Gassaway, Qihong Xu, Florence Wagner, et al. 2017. “Multiplex Quantitative Assays Indicate a Need for Reevaluating Reported Small-Molecule TrkB Agonists.” *Science Signaling* 10 (493): eaal1670. https://doi.org/10.1126/scisignal.aal1670.

Bosco, Daryl A., Gerardo Morfini, N. Murat Karabacak, Yuyu Song, Francois Gros-Louis, Piera Pasinelli, Holly Goolsby, et al. 2010. “Wild-Type and Mutant SOD1 Share an Aberrant Conformation and a Common Pathogenic Pathway in ALS.” *Nature Neuroscience* 13 (11): 1396–1403. https://doi.org/10.1038/nn.2660.

Boyette-Davis, Jessica A, Edgar T Walters, and Patrick M Dougherty. 2015. “Mechanisms Involved in the Development of Chemotherapy-Induced Neuropathy.” *Pain Management* 5 (4): 285–96. https://doi.org/10.2217/pmt.15.19.

Brady, Scott T., and Gerardo A. Morfini. 2017. “Regulation of Motor Proteins, Axonal Transport Deficits and Adult-Onset Neurodegenerative Diseases.” *Neurobiology of Disease*. https://doi.org/10.1016/j.nbd.2017.04.010.

Carter, A. P. 2013. “Crystal Clear Insights into How the Dynein Motor Moves.” *Journal of Cell Science* 126 (3): 705–13. https://doi.org/10.1242/jcs.120725.

Caviston, Juliane P., and Erika L.F. Holzbaur. 2009. “Huntingtin as an Essential Integrator of Intracellular Vesicular Trafficking.” *Trends in Cell Biology*. https://doi.org/10.1016/j.tcb.2009.01.005.

Caviston, Juliane P., Jennifer L. Ross, Sheila M. Antony, Mariko Tokito, and Erika L.F. Holzbaur. 2007. “Huntingtin Facilitates Dynein/dynactin-Mediated Vesicle Transport.” *Proceedings of the National Academy of Sciences* 104 (24): 10045–50.

Celil, Ayse B., and Phil G. Campbell. 2005. “BMP-2 and Insulin-like Growth Factor-I Mediate Osterix (Osx) Expression in Human Mesenchymal Stem Cells via the MAPK and Protein Kinase D Signaling Pathways.” *Journal of Biological Chemistry* 280 (36): 31353–59. https://doi.org/10.1074/jbc.M503845200.

Cepeda, C, M A Ariano, C R Calvert, J Flores-Hernández, S H Chandler, B R Leavitt, M R Hayden, and M S Levine. 2001. “NMDA Receptor Function in Mouse Models of Huntington Disease.” *Journal of Neuroscience Research* 66 (4): 525–39. http://www.ncbi.nlm.nih.gov/pubmed/11746372.

Chang, Diane T.W., Gordon L. Rintoul, Sruthi Pandipati, and Ian J. Reynolds. 2006. “Mutant Huntingtin Aggregates Impair Mitochondrial Movement and Trafficking in Cortical Neurons.” *Neurobiology of Disease* 22 (2): 388–400. https://doi.org/10.1016/j.nbd.2005.12.007.

Chao, Moses V. 2003. “Neurotrophins and Their Receptors: A Convergence Point for Many Signalling Pathways.” *Nature Reviews Neuroscience* 4 (4): 299–309. https://doi.org/10.1038/nrn1078.

Chen, M, V O Ona, M Li, R J Ferrante, K B Fink, S Zhu, J Bian, et al. 2000. “Minocycline Inhibits Caspase-1 and Caspase-3 Expression and Delays Mortality in a Transgenic Mouse Model of Huntington Disease.” *Nature Medicine* 6: 797–801. https://doi.org/10.1038/77528.

Cho, Jae-Hyeon, and Gail V W Johnson. 2003. “Glycogen Synthase Kinase 3beta Phosphorylates Tau at Both Primed and Unprimed Sites. Differential Impact on Microtubule Binding.” *The Journal of Biological Chemistry* 278 (1): 187–93. https://doi.org/10.1074/jbc.M206236200.

Cho, J., and Johnson, G. V. 2004. “Primed Phosphorylation of Tau at Thr231 by Glycogen Synthase Kinase 3beta (GSK3beta) Plays a Critical Role in Regulating

Tau's Ability to Bind and Stabilize Microtubules." *Journal of Neurochemistry* 88 (2): 349–58. http://www.ncbi.nlm.nih.gov/pubmed/14690523.

Coffey, Eleanor T. 2014. "Nuclear and Cytosolic JNK Signalling in Neurons." *Nature Reviews. Neuroscience* 15 (5). Nature Publishing Group: 285–99. https://doi.org/10.1038/nrn3729.

Coffey, Eleanor T, Giedre Smiciene, Vesa Hongisto, Jiong Cao, Stephan Brecht, Thomas Herdegen, and Michael J Courtney. 2002. "C-Jun N-Terminal Protein Kinase (JNK) 2/3 Is Specifically Activated by Stress, Mediating c-Jun Activation, in the Presence of Constitutive JNK1 Activity in Cerebellar Neurons." *The Journal of Neuroscience : The Official Journal of the Society for Neuroscience* 22 (11): 4335–45. https://doi.org/20026401.

Colin, Emilie, Diana Zala, Geraldine Liot, Helene Rangone, Maria Borrell-Pages, Xiao-Jiang Li, Frederic Saudou, and Sandrine Humbert. 2008. "Huntingtin Phosphorylation Acts as a Molecular Switch for Anterograde/retrograde Transport in Neurons." *European Molecular Biology Organization Journal* 27: 2124–34. https://doi.org/10.1038/emboj.2008.133.

Crimella, C., C. Baschirotto, A. Arnoldi, A. Tonelli, E. Tenderini, G. Airoldi, A. Martinuzzi, et al. 2012. "Mutations in the Motor and Stalk Domains of KIF5A in Spastic Paraplegia Type 10 and in Axonal Charcot-Marie-Tooth Type 2." *Clinical Genetics* 82 (2): 157–64. https://doi.org/10.1111/j.1399-0004.2011.01717.x.

Cudkowicz, Merit, and Neil W. Kowall. 1990. "Degeneration of Pyramidal Projection Neurons in Huntington's Disease Cortex." *Annals of Neurology* 27 (2): 200–204. https://doi.org/10.1002/ana.410270217.

Cunha. 2010. "A Simple Role for BDNF in Learning and Memory?" *Frontiers in Molecular Neuroscience*. https://doi.org/10.3389/neuro.02.001.2010.

Dale, Scott M., Rong Zhen Kuang, Xin Wei, and Silvio Varon. 1995. "Corticospinal Motor Neurons in the Adult Rat: Degeneration after Intracortical Axotomy and Protection by Ciliary Neurotrophic Factor (CNTF)." *Experimental Neurology* 135 (1): 67–73. https://doi.org/10.1006/exnr.1995.1066.

Dauer, William, and Serge Przedborski. 2003. "Parkinson's Disease: Mechanisms and Models." *Neuron*. https://doi.org/10.1016/S0896-6273(03)00568-3.

Davies, A M, A Horton, L E Burton, C Schmelzer, R Vandlen, and A Rosenthal. 1993. "Neurotrophin-4/5 Is a Mammalian-Specific Survival Factor for Distinct Populations of Sensory Neurons." *J Neurosci* 13 (11): 4961–67. http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids=8229208.

Dawbarn, D., M. E. De Quidt, and P. C. Emson. 1985. "Survival of Basal Ganglia

Neuropeptide Y-Somatostatin Neurones in Huntington's Disease." *Brain Research* 340 (2): 251–60. https://doi.org/10.1016/0006-8993(85)90921-7.

DeBoer, Scott R., YiMei You, Anita Szodorai, Agnieszka Kaminska, Gustavo Pigino, Evelyn Nwabuisi, Bin Wang, et al. 2008. "Conventional Kinesin Holoenzymes Are Composed of Heavy and Light Chain Homodimers." *Biochemistry* 47 (15): 4535–43. https://doi.org/10.1021/bi702445j.

Delcroix, Jean Dominique, David R. Tomlinson, and Paul Fernyhough. 1997. "Diabetes and Axotomy-Induced Deficits in Retrograde Axonal Transport of Nerve Growth Factor Correlate with Decreased Levels of p75(LNTR) Protein in Lumbar Dorsal Root Ganglia." *Molecular Brain Research* 51 (1–2): 82–90. https://doi.org/10.1016/S0169-328X(97)00215-5.

Deng, Y. P., R. L. Albin, J. B. Penney, A. B. Young, K. D. Anderson, and A. Reiner. 2004. "Differential Loss of Striatal Projection Systems in Huntington's Disease: A Quantitative Immunohistochemical Study." *Journal of Chemical Neuroanatomy* 27 (3): 143–64. https://doi.org/10.1016/j.jchemneu.2004.02.005.

Deng, Yun-Ping, Ting Wong, Jim Y Wan, and Anton Reiner. 2014. "Differential Loss of Thalamostriatal and Corticostriatal Input to Striatal Projection Neuron Types prior to Overt Motor Symptoms in the Q140 Knock-in Mouse Model of Huntington's Disease." *Frontiers in Systems Neuroscience* 8 (October): 198. https://doi.org/10.3389/fnsys.2014.00198.

DiFiglia, M, E Sapp, K Chase, C Schwarz, A Meloni, C Young, E Martin, J P Vonsattel, R Carraway, and S a Reeves. 1995. "Huntingtin Is a Cytoplasmic Protein Associated with Vesicles in Human and Rat Brain Neurons." *Neuron* 14 (5): 1075–81. https://doi.org/10.1016/0896-6273(95)90346-1.

Dillman, James F., and K. Kevin Pfister. 1994. "Differential Phosphorylation in Vivo of Cytoplasmic Dynein Associated with Anterogradely Moving Organelles." *Journal of Cell Biology* 127 (6 I): 1671–81. https://doi.org/10.1083/jcb.127.6.1671.

Diprospero, Nicholas A., Er Yun Chen, Vinod Charles, Markus Plomann, Jeffrey H. Kordower, and Danilo A. Tagle. 2004. "Early Changes in Huntington's Disease Patient Brains Involve Alterations in Cytoskeletal and Synaptic Elements." *Journal of Neurocytology* 33 (5): 517–33. https://doi.org/10.1007/s11068-004-0514-8.

Du, Xiao-Yu, Jian Huang, Liang-Quan Xu, Dan-Feng Tang, Lei Wu, Li-Xia Zhang, Xiao-Ling Pan, Wei-Yun Chen, Li-Ping Zheng, and Yue-Hui Zheng. 2012. "The Proto-Oncogene c-Src Is Involved in Primordial Follicle Activation through the PI3K, PKC and MAPK Signaling Pathways." *Reproductive Biology and Endocrinology* 10 (1): 58. https://doi.org/10.1186/1477-7827-10-58.

Duman, Ronald S., and Bhavya Voleti. 2012. "Signaling Pathways Underlying the

Pathophysiology and Treatment of Depression: Novel Mechanisms for Rapid-Acting Agents." *Trends in Neurosciences*. https://doi.org/10.1016/j.tins.2011.11.004.

Ehrnhoefer, Dagmar E, Stefanie L Butland, Mahmoud a Pouladi, and Michael R Hayden. 2014. "Mouse Models of Huntington Disease: Variations on a Theme." *Disease Models & Mechanisms* 2 (3–4): 123–29. https://doi.org/10.1242/dmm.002451.

Elluru, R G, G S Bloom, and S T Brady. 1995. "Fast Axonal Transport of Kinesin in the Rat Visual System: Functionality of Kinesin Heavy Chain Isoforms." *Molecular Biology of the Cell* 6 (1): 21–40. https://doi.org/10.1091/mbc.6.1.21.

Estrada Sánchez, Ana María, Jana Mejía-Toiber, and Lourdes Massieu. 2008. "Excitotoxic Neuronal Death and the Pathogenesis of Huntington's Disease." *Archives of Medical Research*. https://doi.org/10.1016/j.arcmed.2007.11.011.

Fariñas, I, G A Wilkinson, C Backus, L F Reichardt, and A Patapoutian. 1998. "Characterization of Neurotrophin and Trk Receptor Functions in Developing Sensory Ganglia: Direct NT-3 Activation of TrkB Neurons in Vivo." *Neuron* 21: 325–34. https://doi.org/10.1016/S0896-6273(00)80542-5.

Farrer, Matthew J, Mary M Hulihan, Jennifer M Kachergus, Justus C Dächsel, A Jon Stoessl, Linda L Grantier, Susan Calne, et al. 2009. "DCTN1 Mutations in Perry Syndrome." *Nature Genetics* 41 (2): 163–65. https://doi.org/10.1038/ng.293.

Ferrante, R J, N W Kowall, M F Beal, J B Martin, E D Bird, and E P Jr Richardson. 1987. "Morphologic and Histochemical Characteristics of a Spared Subset of Striatal Neurons in Huntington's Disease." *Journal of Neuropathology and Experimental Neurology* 46 (1): 12–27. https://doi.org/10.1097/00005072-198701000-00002.

Ferrante, R J, N W Kowall, M F Beal, E P Richardson, E D Bird, and J B Martin. 1985. "Selective Sparing of a Class of Striatal Neurons in Huntington's Disease." *Science (New York, N.Y.)* 230 (4725): 561–63. https://doi.org/10.1126/science.2931802.

Ferrante, Robert J., M. Flint Beal, Neil W. Kowall, Edward P. Richardson, and Joseph B. Martin. 1987. "Sparing of Acetylcholinesterase-Containing Striatal Neurons in Huntington's Disease." *Brain Research* 411 (1): 162–66. https://doi.org/10.1016/0006-8993(87)90694-9.

Ferrante, Robert J., Neil W. Kowall, Edward P. Richardson, Edward D. Bird, and Joseph B. Martin. 1986. "Topography of Enkephalin, Substance P and Acetylcholinesterase Staining in Huntington's Disease Striatum." *Neuroscience Letters* 71 (3): 283–88. https://doi.org/10.1016/0304-3940(86)90634-8.

Ferrer, I., E. Goutan, C. Marín, M. J. Rey, and T. Ribalta. 2000. “Brain-Derived Neurotrophic Factor in Huntington Disease.” *Brain Research* 866 (1–2): 257–61. https://doi.org/10.1016/S0006-8993(00)02237-X.

Filippakopoulos, Panagis, Susanne Müller, and Stefan Knapp. 2009. “SH2 Domains: Modulators of Nonreceptor Tyrosine Kinase Activity.” *Current Opinion in Structural Biology*. https://doi.org/10.1016/j.sbi.2009.10.001.

Fischer, Lindsey R., Deborah G. Culver, Philip Tennant, Albert A. Davis, Minsheng Wang, Amilcar Castellano-Sanchez, Jaffar Khan, Meraida A. Polak, and Jonathan D. Glass. 2004. “Amyotrophic Lateral Sclerosis Is a Distal Axonopathy: Evidence in Mice and Man.” *Experimental Neurology* 185 (2): 232–40. https://doi.org/10.1016/j.expneurol.2003.10.004.

Fishman, P. S., and D. A. Parks. 1998. “Death of Transcallosal Neurons after Close Axotomy.” *Brain Research* 779 (1–2): 231–39. https://doi.org/10.1016/S0006-8993(97)01125-6.

Frankel, Alan D., and Carl O. Pabo. 1988. “Cellular Uptake of the Tat Protein from Human Immunodeficiency Virus.” *Cell* 55 (6): 1189–93. https://doi.org/10.1016/0092-8674(88)90263-2.

Garland, Huw, Nigel I. Wood, Elizabeth A. Skillings, Peter J. Detloff, A. Jennifer Morton, and Robyn A. Grant. 2016. “Characterisation of Progressive Motor Deficits in Whisker Movements in R6/2, Q175 and Hdh Knock-in Mouse Models of Huntington’s Disease.” *Journal of Neuroscience Methods*, 2016. https://doi.org/10.1016/j.jneumeth.2017.04.020.

Gatto, Rodolfo G., Yaping Chu, Allen Q. Ye, Steven D. Price, Ehsan Tavassoli, Andrea Buenaventura, Scott T. Brady, Richard L. Magin, Jeffrey H. Kordower, and Gerardo A. Morfini. 2015. “Analysis of YFP(J16)-R6/2 Reporter Mice and Postmortem Brains Reveals Early Pathology and Increased Vulnerability of Callosal Axons in Huntington’s Disease.” *Human Molecular Genetics* 24 (18): 5285–98. https://doi.org/10.1093/hmg/ddv248.

Gauthier, Laurent R., Bénédicte C. Charrin, Maria Borrell-Pagès, Jim P. Dompierre, Hélène Rangone, Fabrice P. Cordelières, Jan De Mey, et al. 2004. “Huntingtin Controls Neurotrophic Support and Survival of Neurons by Enhancing BDNF Vesicular Transport along Microtubules.” *Cell* 118 (1): 127–38. https://doi.org/10.1016/j.cell.2004.06.018.

Gharami, Kusumika, Yuxiang Xie, Juan Ji An, Susumu Tonegawa, and Baoji Xu. 2008. “Brain-Derived Neurotrophic Factor over-Expression in the Forebrain Ameliorates Huntington’s Disease Phenotypes in Mice.” *Journal of Neurochemistry* 105 (2): 369–79. https://doi.org/10.1111/j.1471-4159.2007.05137.x.

Giamp??, Carmela, Elena Montagna, Clemente Dato, Mariarosa A B Melone, Giorgio Bernardi, and Francesca Romana Fusco. 2013. “Systemic Delivery of Recombinant Brain Derived Neurotrophic Factor (BDNF) in the R6/2 Mouse Model of Huntington’s Disease.” *PLoS ONE* 8 (5). https://doi.org/10.1371/journal.pone.0064037.

Giannini, Ana, and Marie-José Bijlmakers. 2004. “Regulation of the Src Family Kinase Lck by Hsp90 and Ubiquitination.” *Molecular and Cellular Biology* 24 (13): 5667–76. https://doi.org/10.1128/MCB.24.13.5667-5676.2004.

Goshgarian, Harry G., Jeanne M. Koistinen, and Eric R. Schmidt. 1983. “Cell Death and Changes in the Retrograde Transport of Horseradish Peroxidase in Rubrospinal Neurons Following Spinal Cord Hemisection in the Adult Rat.” *The Journal of Comparative Neurology* 214 (3): 251–57. https://doi.org/10.1002/cne.902140303.

Green, Maurice, and Paul M. Loewenstein. 1988. “Autonomous Functional Domains of Chemically Synthesized Human Immunodeficiency Virus Tat Trans-Activator Protein.” *Cell* 55 (6): 1179–88. https://doi.org/10.1016/0092-8674(88)90262-0.

Gschwendt, Michael, Sabine Dieterich, Joerg Rennecke, Walter Kittstein, Hans-Joachim Mueller, and Franz-Josef Johannes. 1996. “Inhibition of Protein Kinase C μ by Various Inhibitors. Inhibition from Protein Kinase c Isoenzymes.” *FEBS Letters* 392 (2): 77–80. https://doi.org/10.1016/0014-5793(96)00785-5.

Gunawardena, Shermali, Lu Shiun Her, Richard G. Brusch, Robert A. Laymon, Ingrid R. Niesman, Beth Gordesky-Gold, Louis Sintasath, Nancy M. Bonini, and Lawrence S B Goldstein. 2003. “Disruption of Axonal Transport by Loss of Huntingtin or Expression of Pathogenic polyQ Proteins in Drosophila.” *Neuron* 40 (1): 25–40. https://doi.org/10.1016/S0896-6273(03)00594-4.

Guo, Jianfen, Zoya Gertsberg, Nazira Ozgen, Abdelkarim Sabri, and Susan F Steinberg. 2011. “Protein Kinase D Isoforms Are Activated in an Agonist-Specific Manner in Cardiomyocytes.” *The Journal of Biological Chemistry* 286 (8): 6500–6509. https://doi.org/10.1074/jbc.M110.208058.

Ha, Chang Hoon, and Zheng Gen Jin. 2009. “Protein Kinase D1, a New Molecular Player in VEGF Signaling and Angiogenesis.” *Molecules and Cells* 28 (1): 1–5. https://doi.org/10.1007/s10059-009-0109-9.

Hafezparast, M. 2003. “Mutations in Dynein Link Motor Neuron Degeneration to Defects in Retrograde Transport.” *Science* 300 (5620): 808–12. https://doi.org/10.1126/science.1083129.

Hallböök, Finn, Carlos F. Ibáñez, and Håkan Persson. 1991. “Evolutionary Studies of the Nerve Growth Factor Family Reveal a Novel Member Abundantly Expressed in Xenopus Ovary.” *Neuron* 6 (5): 845–58. https://doi.org/10.1016/0896-

6273(91)90180-8.

Hamburger, Viktor. 1934. “The Effects of Wing Bud Extirpation on the Development of the Central Nervous System in Chick Embryos.” *Journal of Experimental Zoology* 68 (3): 449–94. https://doi.org/10.1002/jez.1400680305.

Han, Ina, Yimei You, Jeffrey H. Kordower, Scott T. Brady, and Gerardo A. Morfini. 2010. “Differential Vulnerability of Neurons in Huntington’s Disease: The Role of Cell Type-Specific Features.” *Journal of Neurochemistry*. https://doi.org/10.1111/j.1471-4159.2010.06672.x.

Harikumar, Kuzhuvelil B, Ajaikumar B Kunnumakkara, Nobuo Ochi, Zhimin Tong, Amit Deorukhkar, Bokyung Sung, Lloyd Kelland, et al. 2010. “A Novel Small-Molecule Inhibitor of Protein Kinase D Blocks Pancreatic Cancer Growth in Vitro and in Vivo.” *Molecular Cancer Therapeutics* 9 (5): 1136–46. https://doi.org/10.1158/1535-7163.MCT-09-1145.

Harjes, Phoebe, and Erich E. Wanker. 2003. “The Hunt for Huntingtin Function: Interaction Partners Tell Many Different Stories.” *Trends in Biochemical Sciences*. https://doi.org/10.1016/S0968-0004(03)00168-3.

Harper, P.S. 1996. *Huntington’s Disease*. 2nd ed. London: WP Saunders.

Hedreen, John C., Carol E. Peyser, Susan E. Folstein, and Christopher A. Ross. 1991. “Neuronal Loss in Layers V and VI of Cerebral Cortex in Huntington’s Disease.” *Neuroscience Letters* 133 (2): 257–61. https://doi.org/10.1016/0304-3940(91)90583-F.

Heerssen, Heather M., Maria F. Pazyra, and Rosalind A. Segal. 2004. “Dynein Motors Transport Activated Trks to Promote Survival of Target-Dependent Neurons.” *Nature Neuroscience* 7 (6): 596–604. https://doi.org/10.1038/nn1242.

Hefti, Franz. 1997. “PHARMACOLOGY OF NEUROTROPHIC FACTORS.” *Annual Review of Pharmacology and Toxicology* 37 (1): 239–67. https://doi.org/10.1146/annurev.pharmtox.37.1.239.

Hemachandra Reddy, P., V. Charles, M. Williams, G. Miller, W. O. Whetsell, and D. A. Tagle. 1999. “Transgenic Mice Expressing Mutated Full-Length HD cDNA: A Paradigm for Locomotor Changes and Selective Neuronal Loss in Huntington’s Disease.” *Philosophical Transactions of the Royal Society B: Biological Sciences* 354 (1386): 1035–45. https://doi.org/10.1098/rstb.1999.0456.

Hemachandra Reddy, P., Maya Williams, Vinod Charles, Lisa Garrett, Lisa Pike-Buchanan, William O. Whetsell, Georgina Miller, and Danilo A. Tagle. 1998. “Behavioural Abnormalities and Selective Neuronal Loss in HD Transgenic Mice Expressing Mutated Full-Length HD cDNA.” *Nature Genetics* 20 (2): 198–202.

https://doi.org/10.1038/2510.

Heng, Mary Y., Peter J. Detloff, and Roger L. Albin. 2008. “Rodent Genetic Models of Huntington Disease.” *Neurobiology of Disease*. https://doi.org/10.1016/j.nbd.2008.06.005.

Hergovich, A., J. Lisztwan, C. R. Thoma, C. Wirbelauer, R. E. Barry, and W. Krek. 2006. “Priming-Dependent Phosphorylation and Regulation of the Tumor Suppressor pVHL by Glycogen Synthase Kinase 3.” *Molecular and Cellular Biology* 26 (15): 5784–96. https://doi.org/10.1128/MCB.00232-06.

Hickey, Miriam A., and M. F. Chesselet. 2003. “Apoptosis in Huntington’s Disease.” *Progress in Neuro-Psychopharmacology and Biological Psychiatry*. https://doi.org/10.1016/S0278-5846(03)00021-6.

Hirokawa, Nobutaka, Shinsuke Niwa, and Yosuke Tanaka. 2010. “Molecular Motors in Neurons: Transport Mechanisms and Roles in Brain Function, Development, and Disease.” *Neuron* 68 (4). Elsevier Inc.: 610–38. https://doi.org/10.1016/j.neuron.2010.09.039.

Hirokawa, Nobutaka, and Reiko Takemura. 2005. “Molecular Motors and Mechanisms of Directional Transport in Neurons.” *Nature Reviews Neuroscience*. https://doi.org/10.1038/nrn1624.

Hodgson, J. Graeme, Nadia Agopyan, Claire Anne Gutekunst, Blair R. Leavitt, Fred Lepiane, Roshni Singaraja, Desmond J. Smith, et al. 1999. “A YAC Mouse Model for Huntington’s Disease with Full-Length Mutant Huntingtin, Cytoplasmic Toxicity, and Selective Striatal Neurodegeneration.” *Neuron* 23 (1): 181–92. https://doi.org/10.1016/S0896-6273(00)80764-3.

Hollenbeck, Peter J. 1993. “Phosphorylation of Neuronal Kinesin Heavy and Light Chains In Vivo.” *Journal of Neurochemistry* 60 (6): 2265–75. https://doi.org/10.1111/j.1471-4159.1993.tb03513.x.

Huang, Eric J, and Louis F Reichardt. 2001. “Neurotrophins: Roles in Neuronal Development and Function.” *Annual Review of Neuroscience* 24 (1): 677–736. https://doi.org/10.1146/annurev.neuro.24.1.677.

Huang, G., S. Chen, S. Li, J. Cha, C. Long, L. Li, Q. He, and Y. Liu. 2007. “Protein Kinase A and Casein Kinases Mediate Sequential Phosphorylation Events in the Circadian Negative Feedback Loop.” *Genes & Development* 21 (24): 3283–95. https://doi.org/10.1101/gad.1610207.

Huang, Yang Z., and James O. McNamara. 2010. “Mutual Regulation of Src Family Kinases and the Neurotrophin Receptor TrkB.” *Journal of Biological Chemistry* 285 (11): 8207–17. https://doi.org/10.1074/jbc.M109.091041.

Huang, Yang Z, Enhui Pan, Zhi-Qi Xiong, and James O McNamara. 2008. “Zinc-Mediated Transactivation of TrkB Potentiates the Hippocampal Mossy Fiber-CA3 Pyramid Synapse.” *Neuron* 57 (4): 546–58. https://doi.org/10.1016/j.neuron.2007.11.026.

Ihara, M, T M Polvikoski, R Hall, J Y Slade, R H Perry, A E Oakley, E Englund, J T O’Brien, P G Ince, and R N Kalaria. 2010. “Quantification of Myelin Loss in Frontal Lobe White Matter in Vascular Dementia, Alzheimer’s Disease, and Dementia with Lewy Bodies.” *Acta Neuropathol* 119 (5): 579–89. https://doi.org/10.1007/s00401-009-0635-8.

Ip, N Y, C F Ibáñez, S H Nye, J McClain, P F Jones, D R Gies, L Belluscio, M M Le Beau, R Espinosa, and S P Squinto. 1992. “Mammalian Neurotrophin-4: Structure, Chromosomal Localization, Tissue Distribution, and Receptor Specificity.” *Proceedings of the National Academy of Sciences of the United States of America* 89 (7): 3060–64. https://doi.org/10.1073/pnas.89.7.3060.

Ito, Keisuke, and Hideki Enomoto. 2016. “Retrograde Transport of Neurotrophic Factor Signaling: Implications in Neuronal Development and Pathogenesis.” *Journal of Biochemistry*. https://doi.org/10.1093/jb/mvw037.

Iwasaki, Y, B Gay, K Wada, and S Koizumi. 1998. “Association of the Src Family Tyrosine Kinase Fyn with TrkB.” *Journal of Neurochemistry* 71 (1): 106–11. http://www.ncbi.nlm.nih.gov/pubmed/9648856.

Jakel, Rebekah J., and William F. Maragos. 2000. “Neuronal Cell Death in Huntington’s Disease: A Potential Role for Dopamine.” *Trends in Neurosciences*. https://doi.org/10.1016/S0166-2236(00)01568-X.

Jiang, Mali, Qi Peng, Xia Liu, Jing Jin, Zhipeng Hou, Jiangyang Zhang, Susumu Mori, Christopher A. Ross, Keqiang Ye, and Wenzhen Duan. 2013. “Small-Molecule TrKB Receptor Agonists Improve Motor Function and Extend Survival in a Mouse Model of Huntington’s Disease.” *Human Molecular Genetics* 22 (12): 2462–70. https://doi.org/10.1093/hmg/ddt098.

Jordan, M. 2012. “Mechanism of Action of Antitumor Drugs That Interact with Microtubules and Tubulin.” *Current Medicinal Chemistry-Anti-Cancer Agents* 2 (1): 1–17. https://doi.org/10.2174/1568011023354290.

Julien, Camille L, Jennifer C Thompson, Sue Wild, Pamela Yardumian, Julie S Snowden, Gwen Turner, and David Craufurd. 2007. “Psychiatric Disorders in Preclinical Huntington’s Disease.” *Journal of Neurology, Neurosurgery, and Psychiatry* 78 (9): 939–43. https://doi.org/10.1136/jnnp.2006.103309.

Jungbluth, S, K Bailey, and Y a Barde. 1994. “Purification and Characterisation of a Brain-Derived Neurotrophic Factor/neurotrophin-3 (BDNF/NT-3) Heterodimer.”

European Journal of Biochemistry / FEBS 221 (2): 677–85. http://www.ncbi.nlm.nih.gov/pubmed/8174548.

Kalinowska-Lyszczarz, Alicja, and Jacek Losy. 2012. “The Role of Neurotrophins in Multiple Sclerosis-Pathological and Clinical Implications.” *International Journal of Molecular Sciences*. https://doi.org/10.3390/ijms131013713.

Kanaan, N. M., G. A. Morfini, N. E. LaPointe, G. F. Pigino, K. R. Patterson, Y. Song, A. Andreadis, Y. Fu, S. T. Brady, and L. I. Binder. 2011. “Pathogenic Forms of Tau Inhibit Kinesin-Dependent Axonal Transport through a Mechanism Involving Activation of Axonal Phosphotransferases.” *Journal of Neuroscience* 31 (27): 9858–68. https://doi.org/10.1523/JNEUROSCI.0560-11.2011.

Kanaan, Nicholas M., Gustavo F. Pigino, Scott T. Brady, Orly Lazarov, Lester I. Binder, and Gerardo A. Morfini. 2013. “Axonal Degeneration in Alzheimer’s Disease: When Signaling Abnormalities Meet the Axonal Transport System.” *Experimental Neurology*. https://doi.org/10.1016/j.expneurol.2012.06.003.

Kaplan, David R., and Freda D. Miller. 2000. “Neurotrophin Signal Transduction in the Nervous System.” *Current Opinion in Neurobiology*. https://doi.org/10.1016/S0959-4388(00)00092-1.

Kardon, Julia R, and Ronald D Vale. 2009. “Regulators of the Cytoplasmic Dynein Motor.” *Nature Reviews Molecular Cell Biology* 10. https://doi.org/10.1038/nrm2804.

Karle, Kathrin N., Diana Möckel, Evan Reid, and Ludger Schöls. 2012. “Axonal Transport Deficit in a KIF5A -/- Mouse Model.” *Neurogenetics* 13 (2): 169–79. https://doi.org/10.1007/s10048-012-0324-y.

Kemi, Cecilia, Johan Grunewald, Anders Eklund, and Caroline Olgart Höglund. 2006. “Differential Regulation of Neurotrophin Expression in Human Bronchial Smooth Muscle Cells.” *Respiratory Research* 7 (1): 18. https://doi.org/10.1186/1465-9921-7-18.

Kinoshita, Eiji, and Emiko Kinoshita-Kikuta. 2011. “Improved Phos-Tag SDS-PAGE under Neutral pH Conditions for Advanced Protein Phosphorylation Profiling.” *Proteomics* 11 (2): 319–23. https://doi.org/10.1002/pmic.201000472.

Kinoshita, Eiji, Emiko Kinoshita-Kikuta, Kei Takiyama, and Tohru Koike. 2006. “Phosphate-Binding Tag, a New Tool to Visualize Phosphorylated Proteins.” *Molecular & Cellular Proteomics* 5 (4): 749–57. https://doi.org/10.1074/mcp.T500024-MCP200.

Klapstein, Gloria J., Robin S. Fisher, Hadi Zanjani, Carlos Cepeda, Eve S. Jokel, Marie-Francoise Chesselet, and Michael S. Levine. 2001. “Electrophysiological and

Morphological Changes in Striatal Spiny Neurons in R6/2 Huntington's Disease Transgenic Mice." *J Neurophysiol* 86 (6): 2667–77. http://jn.physiology.org/content/86/6/2667.short.

Knittle, Anna M., Maria Helkkula, Mark S. Johnson, Maria Sundvall, and Klaus Elenius. 2017. "SUMOylation Regulates Nuclear Accumulation and Signaling Activity of the Soluble Intracellular Domain of the ErbB4 Receptor Tyrosine Kinase." *Journal of Biological Chemistry* 292 (48): 19890–904. https://doi.org/10.1074/jbc.M117.794271.

Konno, Takuya, Owen A. Ross, Hélio A.G. Teive, Jarosław Sławek, Dennis W. Dickson, and Zbigniew K. Wszolek. 2017. "DCTN1-Related Neurodegeneration: Perry Syndrome and beyond." *Parkinsonism and Related Disorders*, 2017. https://doi.org/10.1016/j.parkreldis.2017.06.004.

Kordasiewicz, Holly B., Lisa M. Stanek, Edward V. Wancewicz, Curt Mazur, Melissa M. McAlonis, Kimberly A. Pytel, Jonathan W. Artates, et al. 2012. "Sustained Therapeutic Reversal of Huntington's Disease by Transient Repression of Huntingtin Synthesis." *Neuron* 74 (6): 1031–44. https://doi.org/10.1016/j.neuron.2012.05.009.

Kowall, Neil W., and Kenneth S. Kosik. 1987. "Axonal Disruption and Aberrant Localization of Tau Protein Characterize the Neuropil Pathology of Alzheimer's Disease." *Annals of Neurology* 22 (5): 639–43. https://doi.org/10.1002/ana.410220514.

Laforet, G a, E Sapp, K Chase, C McIntyre, F M Boyce, M Campbell, B a Cadigan, et al. 2001. "Changes in Cortical and Striatal Neurons Predict Behavioral and Electrophysiological Abnormalities in a Transgenic Murine Model of Huntington's Disease." *The Journal of Neuroscience : The Official Journal of the Society for Neuroscience* 21 (23): 9112–23. https://doi.org/21/23/9112 [pii].

Lallemend, François, Saïda Hadjab, Grégory Hans, Gustave Moonen, Philippe P Lefebvre, and Brigitte Malgrange. 2005. "Activation of Protein Kinase CbetaI Constitutes a New Neurotrophic Pathway for Deafferented Spiral Ganglion Neurons." *Journal of Cell Science* 118 (Pt 19): 4511–25. https://doi.org/10.1242/jcs.02572.

LaPointe, Nichole E., Gerardo Morfini, Scott T. Brady, Stuart C. Feinstein, Leslie Wilson, and Mary Ann Jordan. 2013. "Effects of Eribulin, Vincristine, Paclitaxel and Ixabepilone on Fast Axonal Transport and Kinesin-1 Driven Microtubule Gliding: Implications for Chemotherapy-Induced Peripheral Neuropathy." *NeuroToxicology* 37: 231–39. https://doi.org/10.1016/j.neuro.2013.05.008.

LaPointe, Nichole E., Gerardo Morfini, Gustavo Pigino, Irina N. Gaisina, Alan P. Kozikowski, Lester I. Binder, and Scott T. Brady. 2009. "The Amino Terminus of

Tau Inhibits Kinesin-Dependent Axonal Transport: Implications for Filament Toxicity." *Journal of Neuroscience Research* 87 (2): 440–51. https://doi.org/10.1002/jnr.21850.

Lee, R. 2001. "Regulation of Cell Survival by Secreted Proneurotrophins." *Science* 294 (5548): 1945–48. https://doi.org/10.1126/science.1065057.

Lee, Wyan-Ching Mimi, Motojiro Yoshihara, and J Troy Littleton. 2004. "Cytoplasmic Aggregates Trap Polyglutamine-Containing Proteins and Block Axonal Transport in a Drosophila Model of Huntington's Disease." *Proceedings of the National Academy of Sciences of the United States of America* 101 (9): 3224–29. https://doi.org/10.1073/pnas.0400243101.

Legha, Sewa S. 1986. "Vincristine Neurotoxicity: Pathophysiology and Management." *Medical Toxicology* 1 (6): 421–27. https://doi.org/10.1007/BF03259853.

Leone, J., and S. Ochs. 1978. "Anoxic Block and Recovery of Axoplasmic Transport and Electrical Excitability of Nerve." *Journal of Neurobiology* 9 (3): 229–45. https://doi.org/10.1002/neu.480090305.

Leopold, Philip L., Alasdair W. McDowall, K. Kevin Pfister, George S. Bloom, and Scott T. Brady. 1992. "Association of Kinesin with Characterized Membrane-bounded Organelles." *Cell Motility and the Cytoskeleton* 23 (1): 19–33. https://doi.org/10.1002/cm.970230104.

Levi-Montalcini, R. 1966. "The Nerve Growth Factor: Its Mode of Action on Sensory and Sympathetic Nerve Cells." *Harvey Lectures* 60: 217–59. http://www.ncbi.nlm.nih.gov/pubmed/5338067.

Levi-Montalcini, Rita, and Viktor Hamburger. 1951. "Selective Growth Stimulating Effects of Mouse Sarcoma on the Sensory and Sympathetic Nervous System of the Chick Embryo." *Journal of Experimental Zoology* 116 (2): 321–61. https://doi.org/10.1002/jez.1401160206.

Lewis, Tommy L., Gergely F. Turi, Seok Kyu Kwon, Attila Losonczy, and Franck Polleux. 2016. "Progressive Decrease of Mitochondrial Motility during Maturation of Cortical Axons In Vitro and In Vivo." *Current Biology* 26 (19): 2602–8. https://doi.org/10.1016/j.cub.2016.07.064.

Li, Dayu, Marie A. Shatos, Robin R. Hodges, and Darlene A. Dartt. 2013. "Role of PKCα Activation of Src, PI-3K/AKT, and ERK in EGF-Stimulated Proliferation of Rat and Human Conjunctival Goblet Cells." *Investigative Opthalmology & Visual Science* 54 (8): 5661. https://doi.org/10.1167/iovs.13-12473.

Li, H S, X Z Xu, and C Montell. 1999. "Activation of a TRPC3-Dependent Cation Current through the Neurotrophin BDNF." *Neuron* 24 (1): 261–73.

http://www.ncbi.nlm.nih.gov/pubmed/10677043.

Li, Jia Yi, Natalija Popovic, and Patrik Brundin. 2005. “The Use of the R6 Transgenic Mouse Models of Huntington’s Disease in Attempts to Develop Novel Therapeutic Strategies.” *NeuroRx : The Journal of the American Society for Experimental NeuroTherapeutics* 2 (3): 447–64. https://doi.org/10.1602/neurorx.2.3.447.

Liu, Jeh Ping, and Scott O. Zeitlin. 2017. “Is Huntingtin Dispensable in the Adult Brain?” *Journal of Huntington’s Disease*. https://doi.org/10.3233/JHD-170235.

Liu, Ya Fang. 1998. “Expression of Polyglutamine-Expanded Huntingtin Activates the SEK1-JNK Pathway and Induces Apoptosis in a Hippocampal Neuronal Cell Line.” *Journal of Biological Chemistry* 273 (44): 28873–77. https://doi.org/10.1074/jbc.273.44.28873.

Liu, Ya Fang, Donna Dorow, and John Marshall. 2000. “Activation of MLK2-Mediated Signaling Cascades by Polyglutamine-Expanded Huntingtin.” *Journal of Biological Chemistry* 275 (25): 19035–40. https://doi.org/10.1074/jbc.C000180200.

Lloret, Alejandro, Ella Dragileva, Allison Teed, Janice Espinola, Elisa Fossale, Tammy Gillis, Edith Lopez, Richard H. Myers, Marcy E. MacDonald, and Vanessa C. Wheeler. 2006. “Genetic Background Modifies Nuclear Mutant Huntingtin Accumulation and HD CAG Repeat Instability in Huntington’s Disease Knock-in Mice.” *Human Molecular Genetics* 15 (12): 2015–24. https://doi.org/10.1093/hmg/ddl125.

Lobert, Sharon, Bojana Vulevic, and John J. Correia. 1996. “Interaction of Vinca Alkaloids with Tubulin: A Comparison of Vinblastine, Vincristine, and Vinorelbine.” *Biochemistry* 35 (21): 6806–14. https://doi.org/10.1021/bi953037i.

López, Eva, Carlos Casasnovas, Javier Giménez, Raúl Santamaría, Jesús Ma Terrazas, and Víctor Volpini. 2015. “Identification of Two Novel KIF5A Mutations in Hereditary Spastic Paraplegia Associated with Mild Peripheral Neuropathy.” *Journal of the Neurological Sciences* 358 (1–2): 422–27. https://doi.org/10.1016/j.jns.2015.08.1529.

Macdonald, Virginia, and Glenda Halliday. 2002. “Pyramidal Cell Loss in Motor Cortices in Huntington’s Disease.” *Neurobiology of Disease* 10 (3): 378–86. https://doi.org/10.1006/nbdi.2002.0528.

Mackay, Helen J., and Christopher J. Twelves. 2007. “Targeting the Protein Kinase C Family: Are We There Yet?” *Nature Reviews Cancer*. https://doi.org/10.1038/nrc2168.

Maday, Sandra, Alison E. Twelvetrees, Armen J. Moughamian, and Erika L F Holzbaur. 2014. “Axonal Transport: Cargo-Specific Mechanisms of Motility and Regulation.” *Neuron*. https://doi.org/10.1016/j.neuron.2014.10.019.

Magistretti, Pierre J., and Igor Allaman. 2015. “A Cellular Perspective on Brain Energy Metabolism and Functional Imaging.” *Neuron*. https://doi.org/10.1016/j.neuron.2015.03.035.

Maisonpierre, P., L Belluscio, S Squinto, N. Ip, M. Furth, R. Lindsay, and G. Yancopoulos. 1990. “Neurotrophin-3: A Neurotrophic Factor Related to NGF and BDNF.” *Science* 247 (4949): 1446–51. https://doi.org/10.1126/science.2321006.

Malenka, R.C., E.J. Nestler, and S.E. Hyman. 2009. “Neurotrophic Factors Are Polypeptides or Small Proteins That Support the Growth, Differentiation, and Survival of Neurons. They Produce Their Effects by Activation of Tyrosine Kinases.” In *Molecular Neuropharmacology: A Foundation for Clinical Neuroscience*, edited by A. Sydor and R.Y. Brown, 2nd editio, 199–215. New York: McGraw-Hill Medical.

Mangiarini, Laura, Kirupa Sathasivam, Mary Seller, Barbara Cozens, Alex Harper, Colin Hetherington, Martin Lawton, et al. 1996. “Exon I of the HD Gene with an Expanded CAG Repeat Is Sufficient to Cause a Progressive Neurological Phenotype in Transgenic Mice.” *Cell* 87 (3): 493–506. https://doi.org/10.1016/S0092-8674(00)81369-0.

Mann, D M, R Oliver, and J S Snowden. 1993. “The Topographic Distribution of Brain Atrophy in Huntington’s Disease and Progressive Supranuclear Palsy.” *Acta Neuropathologica* 85 (5): 553–59. https://doi.org/10.1007/BF00230496.

Manning, G., D. B. Whyte, R. Martinez, T. Hunter, and S. Sudarsanam. 2002. “The Protein Kinase Complement of the Human Genome.” *Science*. https://doi.org/10.1126/science.1075762.

Marlin, M. Caleb, and Guangpu Li. 2015. “Biogenesis and Function of the NGF/TrkA Signaling Endosome.” *International Review of Cell and Molecular Biology* 314: 239–57. https://doi.org/10.1016/bs.ircmb.2014.10.002.

Martiny-Baron, G, M G Kazanietz, H Mischak, P M Blumberg, G Kochs, H Hug, D Marmé, and C Schächtele. 1993. “Selective Inhibition of Protein Kinase C Isozymes by the Indolocarbazole Gö 6976.” *The Journal of Biological Chemistry* 268 (13): 9194–97.

Massouh, Mireille, M. J. Wallman, Emmanuelle Pourcher, and André Parent. 2008. “The Fate of the Large Striatal Interneurons Expressing Calretinin in Huntington’s Disease.” *Neuroscience Research* 62 (4): 216–24. https://doi.org/10.1016/j.neures.2008.08.007.

Matthews, Sharon A., Enrique Rozengurt, and Doreen Cantrell. 1999. “Characterization of Serine 916 as an in Vivo Autophosphorylation Site for Protein Kinase D/protein Kinase Cμ.” *Journal of Biological Chemistry* 274 (37): 26543–49.

https://doi.org/10.1074/jbc.274.37.26543.

McGuire, John Russel, Juan Rong, Shi Hua Li, and Xiao Jiang Li. 2006. “Interaction of Huntingtin-Associated Protein-1 with Kinesin Light Chain: Implications in Intracellular Trafficking in Neurons.” *Journal of Biological Chemistry* 281 (6): 3552–59. https://doi.org/10.1074/jbc.M509806200.

McKenney, R. J., W. Huynh, M. E. Tanenbaum, G. Bhabha, and R. D. Vale. 2014. “Activation of Cytoplasmic Dynein Motility by Dynactin-Cargo Adapter Complexes.” *Science* 345 (6194). https://doi.org/10.1126/science.1254198.

McMahon, Stephen B., Mark P. Armanini, Lanway H. Ling, and Heidi S. Phillips. 1994. “Expression and Coexpression of Trk Receptors in Subpopulations of Adult Primary Sensory Neurons Projecting to Identified Peripheral Targets.” *Neuron* 12 (5): 1161–71. https://doi.org/10.1016/0896-6273(94)90323-9.

Menalled, Liliana B., and Marie-Françoise Chesselet. 2002. “Mouse Models of Huntington’s Disease.” *Trends Pharmacological Science* 23 (1): 32–39. https://doi.org/10.1242/dmm.002451.

Menalled, Liliana B., Andrea E. Kudwa, Sam Miller, Jon Fitzpatrick, Judy Watson-Johnson, Nicole Keating, Melinda Ruiz, et al. 2012. “Comprehensive Behavioral and Molecular Characterization of a New Knock-In Mouse Model of Huntington’s Disease: zQ175.” Edited by Hitoshi Okazawa. *PLoS ONE* 7 (12): e49838. https://doi.org/10.1371/journal.pone.0049838.

Menalled, Liliana B., Jessica D. Sison, Ioannis Dragatsis, Scott Zeitlin, and Marie-Fran□oise Chesselet. 2003. “Time Course of Early Motor and Neuropathological Anomalies in a Knock-in Mouse Model of Huntington’s Disease with 140 CAG Repeats.” *The Journal of Comparative Neurology* 465 (1): 11–26. https://doi.org/10.1002/cne.10776.

Mitchell, David J, Kiev R Blasier, Erin D Jeffery, Mitchell W Ross, Ashok K Pullikuth, Suo Dong, Park Juyeon, et al. 2012. “Trk Activation of the ERK1/2 Kinase Pathway Stimulates Intermediate Chain Phosphorylation and Recruits Cytoplasmic Dynein to Signaling Endosomes for Retrograde Axonal Transport.” *Journal of Neuroscience* 29 (44): 997–1003. https://doi.org/10.1016/j.biotechadv.2011.08.021.

Mitchell, I. J., A. J. Cooper, and M. R. Griffiths. 1999. “The Selective Vulnerability of Striatopallidal Neurons.” *Progress in Neurobiology* 59 (6): 691–719. https://doi.org/10.1016/S0301-0082(99)00019-2.

Modregger, Jan, Nicholas A DiProspero, Vinod Charles, Danilo A Tagle, and Markus Plomann. 2002. “PACSIN 1 Interacts with Huntingtin and Is Absent from Synaptic Varicosities in Presymptomatic Huntington’s Disease Brains.” *Human Molecular Genetics* 11 (21): 2547–58. https://doi.org/10.1093/hmg/11.21.2547.

Mohapatra, B, G Ahmad, S Nadeau, N Zutshi, W An, S Scheffe, L Dong, et al. 2013. "Protein Tyrosine Kinase Regulation by Ubiquitination: Critical Roles of Cbl-Family Ubiquitin Ligases." *Biochim Biophys Acta* 1833 (1): 122–39. https://doi.org/10.1016/j.bbamcr.2012.10.010.

Morfi, Gerardo a, Matthew R Burns, David L Stenoien, and Scott T Brady. 2012. "Axonal Transport." *Basic Neurochemistry*, 146–64. https://doi.org/10.1016/B978-0-12-374947-5.00008-0.

Morfini, G. A., M. Burns, L. I. Binder, N. M. Kanaan, N. LaPointe, D. A. Bosco, R. H. Brown, et al. 2009. "Axonal Transport Defects in Neurodegenerative Diseases." *Journal of Neuroscience* 29 (41): 12776–86. https://doi.org/10.1523/JNEUROSCI.3463-09.2009.

Morfini, G., G. Pigino, K. Opalach, Y. Serulle, J. E. Moreira, M. Sugimori, R. R. Llinas, and S. T. Brady. 2007. "1-Methyl-4-Phenylpyridinium Affects Fast Axonal Transport by Activation of Caspase and Protein Kinase C." *Proceedings of the National Academy of Sciences* 104 (7): 2442–47. https://doi.org/10.1073/pnas.0611231104.

Morfini, Gerardo. 2002. "Glycogen Synthase Kinase 3 Phosphorylates Kinesin Light Chains and Negatively Regulates Kinesin-Based Motility." *EMBO Journal* 21 (3): 281–93. https://doi.org/10.1093/emboj/21.3.281.

Morfini, Gerardo A., Daryl A. Bosco, Hannah Brown, Rodolfo Gatto, Agnieszka Kaminska, Yuyu Song, Linda Molla, et al. 2013. "Inhibition of Fast Axonal Transport by Pathogenic SOD1 Involves Activation of p38 MAP Kinase." *PLoS ONE* 8 (6). https://doi.org/10.1371/journal.pone.0065235.

Morfini, Gerardo A., Yi-Mei You, Sarah L. Pollema, Agnieszka Kaminska, Katherine Liu, Katsuji Yoshioka, Benny Björkblom, et al. 2009. "Pathogenic Huntingtin Inhibits Fast Axonal Transport by Activating JNK3 and Phosphorylating Kinesin." *Nature Neuroscience* 12 (7): 864–71. https://doi.org/10.1038/nn.2346.

Morfini, Gerardo A, Yi-Mei You, Sarah L Pollema, Agnieszka Kaminska, Katherine Liu, Katsuji Yoshioka, Benny Björkblom, et al. 2009. "Pathogenic Huntingtin Inhibits Fast Axonal Transport by Activating JNK3 and Phosphorylating Kinesin." *Nature Neuroscience* 12 (7): 864–71. https://doi.org/10.1038/nn.2346.

Morfini, Gerardo, Gustavo Pigino, Györgyi Szebenyi, Yimei You, Sarah Pollema, and Scott T. Brady. 2006. "JNK Mediates Pathogenic Effects of Polyglutamine-Expanded Androgen Receptor on Fast Axonal Transport." *Nature Neuroscience* 9 (7): 907–16. https://doi.org/10.1038/nn1717.

Morfini, Gerardo, Györgyi Szebenyi, Hannah Brown, Harish C. Pant, Gustavo Pigino, Scott DeBoer, Uwe Beffert, and Scott T. Brady. 2004. "A Novel CDK5-Dependent Pathway for Regulating GSK3 Activity and Kinesin-Driven Motility in Neurons."

EMBO Journal 23 (11): 2235–45. https://doi.org/10.1038/sj.emboj.7600237.

Morfini, Gerardo, Györgyi Szebenyi, Bruce Richards, and Scott T. Brady. 2001. "Regulation of Kinesin: Implications for Neuronal Development." *Developmental Neuroscience* 23 (4–5): 364–76. https://doi.org/10.1159/000048720.

Mu, Wei, and Ling-Zhi Liu. 2017. "Reactive Oxygen Species Signaling in Cancer Development." *Reactive Oxygen Species*. https://doi.org/10.20455/ros.2017.843.

Murer, M. G., Q. Yan, and R. Raisman-Vozari. 2001. "Brain-Derived Neurotrophic Factor in the Control Human Brain, and in Alzheimer's Disease and Parkinson's Disease." *Progress in Neurobiology*. https://doi.org/10.1016/S0301-0082(00)00014-9.

Murugappan, S, R Chari, V M Palli, J Jin, and S P Kunapuli. 2009. "Differential Regulation of Threonine and Tyrosine Phosphorylations on Protein Kinase Cδ by G-Protein-Mediated Pathways in Platelets." *Biochem J* 417 (1): 113–20. https://doi.org/10.1042/BJ20080235.

Nagano, T., Y. Yanagawa, K. Obata, M. Narisawa-Saito, H. Namba, Y. Otsu, N. Takei, and H. Nawa. 2003. "Brain-Derived Neurotrophic Factor Upregulates and Maintains AMPA Receptor Currents in Neocortical GABAergic Neurons." *Molecular and Cellular Neuroscience* 24 (2): 340–56. https://doi.org/10.1016/S1044-7431(03)00172-6.

Nagy, József. 2008. "Alcohol Related Changes in Regulation of NMDA Receptor Functions." *Current Neuropharmacology* 6 (1): 39–54. https://doi.org/10.2174/157015908783769662.

Nasir, J., S.B. Floresco, J.R. O'Kusky, V.M. Diewert, J.M. Richman, J. Zeisler, A. Borowski, J.D. Marth, A.G. Phillips, and M.R. Hayden. 1995. "Targeted Disruption of the Huntington's Disease Gene Results in Embryonic Lethality and Behavioral and Morphological Changes in Heterozygotes." *Cell* 81 (5): 811–23. https://doi.org/10.1016/0092-8674(95)90542-1.

Ni, Yang, James Sinnett-Smith, Steven H. Young, and Enrique Rozengurt. 2013. "PKD1 Mediates Negative Feedback of PI3K/Akt Activation in Response to G Protein-Coupled Receptors." *PLoS ONE* 8 (9). https://doi.org/10.1371/journal.pone.0073149.

Nishikawa, K, a Toker, F J Johannes, Z Songyang, and L C Cantley. 1997. "Determination of the Specific Substrate Sequence Motifs of Protein Kinase C Isozymes." *The Journal of Biological Chemistry* 272 (2): 952–60. https://doi.org/10.1074/jbc.272.2.952.

Ochs, Sidney. 1971. "Local Supply of Energy to the Fast Axoplasmic Transport

Mechanism." *Proceedings of the National Academy of Sciences* 68 (6): 1279–82. https://doi.org/10.1073/pnas.68.6.1279.

Orr, Adam L, Shihua Li, Chuan-En Wang, He Li, Jianjun Wang, Juan Rong, Xingshun Xu, Pier Giorgio Mastroberardino, J Timothy Greenamyre, and Xiao-Jiang Li. 2008. "N-Terminal Mutant Huntingtin Associates with Mitochondria and Impairs Mitochondrial Trafficking." *The Journal of Neuroscience : The Official Journal of the Society for Neuroscience* 28 (11): 2783–92. https://doi.org/10.1523/JNEUROSCI.0106-08.2008.

Park, H, and M M Poo. 2013. "Neurotrophin Regulation of Neural Circuit Development and Function." *Nat Rev Neurosci* 14 (1): 7–23. https://doi.org/10.1038/nrn3379.

Paulsen, Jane S. 2011. "Cognitive Impairment in Huntington Disease: Diagnosis and Treatment." *Current Neurology and Neuroscience Reports* 11 (5): 474–83. https://doi.org/10.1007/s11910-011-0215-x.

Paulsen, Jane S., Carissa Nehl, Karin Ferneyhough Hoth, Jason E. Kanz, Michelle Benjamin, Rachel Conybeare, Bradley McDowell, and Beth Turner. 2005. "Depression and Stages of Huntington's Disease." *The Journal of Neuropsychiatry and Clinical Neurosciences* 17 (4): 496–502. https://doi.org/10.1176/jnp.17.4.496.

Peng, Qi, Bin Wu, Mali Jiang, Jing Jin, Zhipeng Hou, Jennifer Zheng, Jiangyang Zhang, and Wenzhen Duan. 2016. "Characterization of Behavioral, Neuropathological, Brain Metabolic and Key Molecular Changes in zQ175 Knock-in Mouse Model of Huntington's Disease." *PLoS ONE* 11 (2). https://doi.org/10.1371/journal.pone.0148839.

Perrin, V., N. Dufour, C. Raoul, R. Hassig, E. Brouillet, P. Aebischer, R. Luthi-Carter, and N. Déglon. 2009. "Implication of the JNK Pathway in a Rat Model of Huntington's Disease." *Experimental Neurology* 215 (1): 191–200. https://doi.org/10.1016/j.expneurol.2008.10.008.

Pfister, K. Kevin. 2015. "Distinct Functional Roles of Cytoplasmic Dynein Defined by the Intermediate Chain Isoforms." *Experimental Cell Research*. https://doi.org/10.1016/j.yexcr.2014.12.013.

Phillips, Heidi S., Jeanne M. Hains, Mark Armanini, Gary R. Laramee, Steven A. Johnson, and John W. Winslow. 1991. "BDNF mRNA Is Decreased in the Hippocampus of Individuals with Alzheimer's Disease." *Neuron* 7 (5): 695–702. https://doi.org/10.1016/0896-6273(91)90273-3.

Pigino, G., G. Morfini, Y. Atagi, A. Deshpande, C. Yu, L. Jungbauer, M. LaDu, J. Busciglio, and S. Brady. 2009. "Disruption of Fast Axonal Transport Is a Pathogenic Mechanism for Intraneuronal Amyloid Beta." *Proceedings of the National Academy of Sciences* 106 (14): 5907–12. https://doi.org/10.1073/pnas.0901229106.

Pirvola, Ulla, and Jukka Ylikoski. 2003. “Neurotrophic Factors during Inner Ear Development.” *Current Topics in Developmental Biology* 57: 207–23. https://doi.org/10.1016/S0070-2153(03)57007-7.

Portera-Cailliau, C., J. C. Hedreen, D. L. Price, and V. E. Koliatsos. 1995. “Evidence for Apoptotic Cell Death in Huntington Disease and Excitotoxic Animal Models.” *The Journal of Neuroscience* 15 (5): 3775–87. http://www.jneurosci.org/content/15/5/3775%5Cnhttp://www.jneurosci.org/content/15/5/3775.full.pdf%5Cnhttp://www.jneurosci.org/content/15/5/3775.short%5Cnhttp://www.ncbi.nlm.nih.gov/pubmed/7751945.

Pouladi, Mahmoud A., A. Jennifer Morton, and Michael R. Hayden. 2013. “Choosing an Animal Model for the Study of Huntington’s Disease.” *Nature Reviews Neuroscience*. https://doi.org/10.1038/nrn3570.

Pringsheim, T., Wiltshire, K., Day, L., Dykeman, J., Steeves, T., and Jette, N. 2012. "The incidence and prevalence of Huntington's disease: a systematic review and meta-analysis." *Movement disorders.* 27(9):1083-91. https://doi.org/10.1002/mds.25075

Puls, Imke, Shin J. Oh, Charlotte J. Sumner, Karen E. Wallace, Mary Kay Floeter, Eric A. Mann, William R. Kennedy, et al. 2005. “Distal Spinal and Bulbar Muscular Atrophy Caused by Dynactin Mutation.” *Annals of Neurology* 57 (5): 687–94. https://doi.org/10.1002/ana.20468.

Ratner, N, G S Bloom, and S T Brady. 1998. “A Role for Cyclin-Dependent Kinase(s) in the Modulation of Fast Anterograde Axonal Transport: Effects Defined by Olomoucine and the APC Tumor Suppressor Protein.” *The Journal of Neuroscience : The Official Journal of the Society for Neuroscience* 18 (19): 7717–26. http://www.ncbi.nlm.nih.gov/pubmed/9742142.

Reading, Sarah A.J., Michael A. Yassa, Arnold Bakker, Adam C. Dziorny, Lisa M. Gourley, Venu Yallapragada, Adam Rosenblatt, et al. 2005. “Regional White Matter Change in Pre-Symptomatic Huntington’s Disease: A Diffusion Tensor Imaging Study.” *Psychiatry Research - Neuroimaging* 140 (1): 55–62. https://doi.org/10.1016/j.pscychresns.2005.05.011.

Reichardt, L. F. 2006. “Neurotrophin-Regulated Signalling Pathways.” *Philosophical Transactions of the Royal Society B: Biological Sciences* 361 (1473): 1545–64. https://doi.org/10.1098/rstb.2006.1894.

Reid, Evan, Mark Kloos, Allison Ashley-Koch, Lori Hughes, Simon Bevan, Ingrid K. Svenson, Felicia Lennon Graham, et al. 2002. “A Kinesin Heavy Chain (KIF5A) Mutation in Hereditary Spastic Paraplegia (SPG10).” *The American Journal of Human Genetics* 71 (5): 1189–94. https://doi.org/10.1086/344210.

Reilmann, R, F Kirsten, L Quinn, H Henningsen, K Marder, and a M Gordon. 2001. “Objective Assessment of Progression in Huntington’s Disease: A 3-Year Follow-up Study.” *Neurology* 57 (5): 920–24. https://doi.org/10.1212/WNL.57.5.920.

Reiner, A., R. L. Albin, K. D. Anderson, C. J. D’Amato, J. B. Penney, and A. B. Young. 1988. “Differential Loss of Striatal Projection Neurons in Huntington Disease.” *Proceedings of the National Academy of Sciences* 85 (15): 5733–37. https://doi.org/10.1073/pnas.85.15.5733.

Ren, Bin. 2016. “Protein Kinase D1 Signaling in Angiogenic Gene Expression and VEGF-Mediated Angiogenesis.” *Frontiers in Cell and Developmental Biology* 4. https://doi.org/10.3389/fcell.2016.00037.

Rising, Aaron C., Jia Xu, Aaron Carlson, Vincent V. Napoli, Eileen M. Denovan-Wright, and Ronald J. Mandel. 2011. “Longitudinal Behavioral, Cross-Sectional Transcriptional and Histopathological Characterization of a Knock-in Mouse Model of Huntington’s Disease with 140 CAG Repeats.” *Experimental Neurology* 228 (2): 173–82. https://doi.org/10.1016/j.expneurol.2010.12.017.

Rosas, H. Diana, Stephanie Y. Lee, Alexander C. Bender, Alexandra K. Zaleta, Mark Vangel, Peng Yu, Bruce Fischl, et al. 2010. “Altered White Matter Microstructure in the Corpus Callosum in Huntington’s Disease: Implications for Cortical ‘disconnection.’” *NeuroImage* 49 (4): 2995–3004. https://doi.org/10.1016/j.neuroimage.2009.10.015.

Ross, Christopher A., and Sarah J. Tabrizi. 2011. “Huntington’s Disease: From Molecular Pathogenesis to Clinical Treatment.” *The Lancet Neurology*. https://doi.org/10.1016/S1474-4422(10)70245-3.

Roy, Subhojit, Bin Zhang, Virginia M Y Lee, and John Q. Trojanowski. 2005. “Axonal Transport Defects: A Common Theme in Neurodegenerative Diseases.” *Acta Neuropathologica*. https://doi.org/10.1007/s00401-004-0952-x.

Rybin, Vitalyi O., Jianfen Guo, Erin Harleton, Fan Zhang, and Susan F. Steinberg. 2012. “Regulatory Domain Determinants That Control PKD1 Activity.” *Journal of Biological Chemistry* 287 (27): 22609–15. https://doi.org/10.1074/jbc.M112.379719.

Sathasivam, K., A. Neueder, T. A. Gipson, C. Landles, A. C. Benjamin, M. K. Bondulich, D. L. Smith, et al. 2013. “Aberrant Splicing of HTT Generates the Pathogenic Exon 1 Protein in Huntington Disease.” *Proceedings of the National Academy of Sciences* 110 (6): 2366–70. https://doi.org/10.1073/pnas.1221891110.

Sato, C. 2017. “Releasing Mechanism of Neurotrophic Factors via Polysialic Acid.” In *Vitamins and Hormones*, 104:89–112. https://doi.org/10.1016/bs.vh.2016.11.004.

Schilling, Gabriele, Mark W Becher, Alan H Sharp, Hyder A Jinnah, Kui Duan, Joyce A

Kotzuk, Hilda H Slunt, et al. 1999. “Intranuclear Inclusions and Neuritic Aggregates in Transgenic Mice Expressing a Mutant N-Terminal Fragment of Huntingtin.” *Human Molecular Genetics* 8 (3): 397–407. https://doi.org/10.1093/hmg/8.3.397.

Senis, Yotis A., Alexandra Mazharian, and Jun Mori. 2014. “Src Family Kinases: At the Forefront of Platelet Activation.” *Blood*. https://doi.org/10.1182/blood-2014-01-453134.

Serulle, Yafell, Gerardo Morfini, Gustavo Pigino, Jorge E Moreira, Mutsuyuki Sugimori, Scott T Brady, and Rodolfo R Llinás. 2007. “1-Methyl-4-Phenylpyridinium Induces Synaptic Dysfunction through a Pathway Involving Caspase and PKCdelta Enzymatic Activities.” *Proceedings of the National Academy of Sciences of the United States of America* 104 (7): 2437–41. https://doi.org/10.1073/pnas.0611227104.

Shankar, Ganesh M, and Dominic M Walsh. 2009. “Alzheimer’s Disease: Synaptic Dysfunction and Abeta.” *Molecular Neurodegeneration* 4 (1): 48. https://doi.org/10.1186/1750-1326-4-48.

Shao, Yufang, Wendy Akmentin, Juan Jose Toledo-Aral, Julie Rosenbaum, Gregorio Valdez, John B. Cabot, Brian S. Hilbush, and Simon Halegoua. 2002. “Pincher, a Pinocytic Chaperone for Nerve Growth factor/TrkA Signaling Endosomes.” *Journal of Cell Biology* 157 (4): 679–91. https://doi.org/10.1083/jcb.200201063.

Shirendeb, Ulziibat P., Marcus J. Calkins, Maria Manczak, Vishwanath Anekonda, Brett Dufour, Jodi L. McBride, Peizhong Mao, and P. Hemachandra Reddy. 2012. “Mutant Huntingtin’s Interaction with Mitochondrial Protein Drp1 Impairs Mitochondrial Biogenesis and Causes Defective Axonal Transport and Synaptic Degeneration in Huntington’s Disease.” *Human Molecular Genetics* 21 (2): 406–20. https://doi.org/10.1093/hmg/ddr475.

Simpson-Lavy, Kobi J, and Mark Johnston. 2013. “SUMOylation Regulates the SNF1 Protein Kinase.” *Proceedings of the National Academy of Sciences of the United States of America* 110 (43): 17432–37. https://doi.org/10.1073/pnas.1304839110/-/DCSupplemental.www.pnas.org/cgi/doi/10.1073/pnas.1304839110.

Sinadinos, C., T. Burbidge-King, D. Soh, L. M. Thompson, J. L. Marsh, A. Wyttenbach, and A. K. Mudher. 2009. “Live Axonal Transport Disruption by Mutant Huntingtin Fragments in Drosophila Motor Neuron Axons.” *Neurobiology of Disease* 34 (2): 389–95. https://doi.org/10.1016/j.nbd.2009.02.012.

Skaper, Stephen. 2008. “The Biology of Neurotrophins, Signalling Pathways, and Functional Peptide Mimetics of Neurotrophins and Their Receptors.” *CNS & Neurological Disorders - Drug Targets* 7 (1): 46–62. https://doi.org/10.2174/187152708783885174.

Slow, Elizabeth J., Jeremy van Raamsdonk, Daniel Rogers, Sarah H. Coleman, Rona K. Graham, Yu Deng, Rosemary Oh, et al. 2003. “Selective Striatal Neuronal Loss in a YAC128 Mouse Model of Huntington Disease.” *Human Molecular Genetics*. https://doi.org/10.1093/hmg/ddg169.

Smit-Rigter, Laura, Rajeev Rajendran, Catia A.P. Silva, Liselot Spierenburg, Femke Groeneweg, Emma M. Ruimschotel, Danielle van Versendaal, et al. 2016. “Mitochondrial Dynamics in Visual Cortex Are Limited In Vivo and Not Affected by Axonal Structural Plasticity.” *Current Biology* 26 (19): 2609–16. https://doi.org/10.1016/j.cub.2016.07.033.

Smith, Y., M. D. Bevan, E. Shink, and J. P. Bolam. 1998. “Microcircuitry of the Direct and Indirect Pathways of the Basal Ganglia.” *Neuroscience* 86 (2): 353–87. https://doi.org/10.1016/S0306-4522(98)00004-9.

Song, Insuk, and Richard L Huganir. 2002. “Regulation of AMPA Receptors during Synaptic Plasticity.” *Trends in Neurosciences* 25 (11): 578–88. https://doi.org/10.1016/S0166-2236(02)02270-1.

Song, Yuyu, Minsu Kang, Gerardo Morfini, and Scott T Brady. 2016. “Fast Axonal Transport in Isolated Axoplasm from the Squid Giant Axon.” *Methods in Cell Biology* 131: 331–48. https://doi.org/10.1016/bs.mcb.2015.07.004.

Sotrel, A, P A Paskevich, D K Kiely, E D Bird, R S Williams, and R H Myers. 1991. “Morphometric Analysis of the Prefrontal Cortex in Huntington’s Disease.” *Neurology* 41 (7): 1117–23. https://doi.org/10.1212/WNL.41.7.1117.

St-Denis, Nicole, Michelle Gabriel, Jacob P. Turowec, Gregory B. Gloor, Shawn S.-C. Li, Anne-Claude Gingras, and David W. Litchfield. 2015. “Systematic Investigation of Hierarchical Phosphorylation by Protein Kinase CK2.” *Journal of Proteomics* 118 (April): 49–62. https://doi.org/10.1016/j.jprot.2014.10.020.

Steinberg, S. F. 2012. “Regulation of Protein Kinase D1 Activity.” *Molecular Pharmacology* 81 (3): 284–91. https://doi.org/10.1124/mol.111.075986.

Stenoien, D L, and S T Brady. 1997. “Immunochemical Analysis of Kinesin Light Chain Function.” *Molecular Biology of the Cell* 8 (4): 675–89. https://doi.org/10.1186/1750-1326-5-52.

Szebenyi, Györgyi, Gerardo A. Morfini, Alyssa Babcock, Milena Gould, Kimberly Selkoe, David L. Stenoien, Maureen Young, et al. 2003. “Neuropathogenic Forms of Huntingtin and Androgen Receptor Inhibit Fast Axonal Transport.” *Neuron* 40 (1): 41–52. https://doi.org/10.1016/S0896-6273(03)00569-5.

Takasu, Mari A., Matthew B. Dalva, Richard E. Zigmond, and Michael E. Greenberg. 2002. “Modulation of NMDA Receptor - Dependent Calcium Influx and Gene

Expression through EphB Receptors." *Science* 295 (5554): 491–95. https://doi.org/10.1126/science.1065983.

Tapley, P, F Lamballe, and M Barbacid. 1992. "K252a Is a Selective Inhibitor of the Tyrosine Protein Kinase Activity of the Trk Family of Oncogenes and Neurotrophin Receptors." *Oncogene* 7 (2): 371–81. http://www.ncbi.nlm.nih.gov/pubmed/1312698.

Tatin, Florence, Christine Varon, Elisabeth Génot, and Violaine Moreau. 2006. "A Signalling Cascade Involving PKC, Src and Cdc42 Regulates Podosome Assembly in Cultured Endothelial Cells in Response to Phorbol Ester." *Journal of Cell Science* 119 (Pt 4): 769–81. https://doi.org/10.1242/jcs.02787.

Taylor, David M, Roger Moser, Etienne Régulier, Lionel Breuillaud, Meredith Dixon, Ayshe Ana Beesen, Linda Elliston, et al. 2013. "MAP Kinase Phosphatase 1 (MKP-1/DUSP1) Is Neuroprotective in Huntington's Disease via Additive Effects of JNK and p38 Inhibition." *The Journal of Neuroscience : The Official Journal of the Society for Neuroscience* 33 (6): 2313–25. https://doi.org/10.1523/JNEUROSCI.4965-11.2013.

Thoenen, Hans, and Michael Sendtner. 2002. "Neurotrophins: From Enthusiastic Expectations through Sobering Experiences to Rational Therapeutic Approaches." *Nature Neuroscience* 5 Suppl (november): 1046–50. https://doi.org/10.1038/nn938.

Tian, Jun, Ya Ping Yan, Rui Zhou, Hui Fang Lou, Ye Rong, and Bao Rong Zhang. 2014. "Soluble N-Terminal Fragment of Mutant Huntingtin Protein Impairs Mitochondrial Axonal Transport in Cultured Hippocampal Neurons." *Neuroscience Bulletin* 30 (1): 74–80. https://doi.org/10.1007/s12264-013-1393-0.

Toro, Daniel del, Josep M Canals, Silvia Ginés, Masami Kojima, Gustavo Egea, and Jordi Alberch. 2006. "Mutant Huntingtin Impairs the Post-Golgi Trafficking of Brain-Derived Neurotrophic Factor but Not Its Val66Met Polymorphism." *The Journal of Neuroscience : The Official Journal of the Society for Neuroscience* 26 (49): 12748–57. https://doi.org/10.1523/JNEUROSCI.3873-06.2006.

Trushina, E., R. B. Dyer, J. D. Badger, D. Ure, L. Eide, D. D. Tran, B. T. Vrieze, et al. 2004. "Mutant Huntingtin Impairs Axonal Trafficking in Mammalian Neurons In Vivo and In Vitro." *Molecular and Cellular Biology* 24 (18): 8195–8209. https://doi.org/10.1128/MCB.24.18.8195-8209.2004.

Valdez, Gregorio, Wendy Akmentin, Polyxeni Philippidou, Rejji Kuruvilla, David D Ginty, and Simon Halegoua. 2005. "Pincher-Mediated Macroendocytosis Underlies Retrograde Signaling by Neurotrophin Receptors." *The Journal of Neuroscience : The Official Journal of the Society for Neuroscience* 25 (21): 5236–47. https://doi.org/10.1523/JNEUROSCI.5104-04.2005.

Vaughan, Patricia S., John D. Leszyk, and Kevin T. Vaughan. 2001. “Cytoplasmic Dynein Intermediate Chain Phosphorylation Regulates Binding to Dynactin.” *Journal of Biological Chemistry* 276 (28): 26171–79. https://doi.org/10.1074/jbc.M102649200.

Velier, James, Manho Kim, Cordula Schwarz, Tae Wan Kim, Ellen Sapp, Kathryn Chase, Neil Aronin, and Marian DiFiglia. 1998. “Wild-Type and Mutant Huntingtins Function in Vesicle Trafficking in the Secretory and Endocytic Pathways.” *Experimental Neurology* 152 (1): 34–40. https://doi.org/10.1006/exnr.1998.6832.

Vertommen, Didier, Mark Rider, Youping Ni, Etienne Waelkens, Wilfried Merlevede, Jackie R. Vandenheede, and Johan Van Lint. 2000. “Regulation of Protein Kinase D by Multisite Phosphorylation.” *Journal of Biological Chemistry* 275 (26): 19567–76. https://doi.org/10.1074/jbc.M001357200.

Vila, Miquel, and Serge Przedborski. 2003. “Targeting Programmed Cell Death in Neurodegenerative Diseases.” *Nature Reviews. Neuroscience* 4 (5): 365–75. https://doi.org/10.1038/nrn1100.

Vonsattel, J P G, and M DiFiglia. 1998. “Huntington Disease.” *Journal of Neuropathology & ….* http://journals.lww.com/jneuropath/Citation/1998/05000/Huntington_Disease.1.aspx %5Cnpapers3://publication/uuid/60C4E9E5-20D2-451C-A30A-D515BD5F44BB.

Wagner, Mark C., K. Kevin Pfister, Scott T. Brady, and George S. Bloom. 1991. “Purification of Kinesin from Bovine Brain and Assay of Microtubule-Stimulated ATPase Activity.” *Methods in Enzymology* 196 (C): 157–75. https://doi.org/10.1016/0076-6879(91)96016-K.

Wang, Guohao, Xudong Liu, Marta A. Gaertig, Shihua Li, and Xiao-Jiang Li. 2016. “Ablation of Huntingtin in Adult Neurons Is Nondeleterious but Its Depletion in Young Mice Causes Acute Pancreatitis.” *Proceedings of the National Academy of Sciences* 113 (12): 3359–64. https://doi.org/10.1073/pnas.1524575113.

Waters, C. M., R. Peck, M. Rossor, G. P. Reynolds, and S. P. Hunt. 1988. “Immunocytochemical Studies on the Basal Ganglia and Substantia Nigra in Parkinson’s Disease and Huntington’s Chorea.” *Neuroscience* 25 (2): 419–38. https://doi.org/10.1016/0306-4522(88)90249-7.

Weaver, Kurt E., Todd L. Richards, Olivia Liang, Mercy Y. Laurino, Ali Samii, and Elizabeth H. Aylward. 2009. “Longitudinal Diffusion Tensor Imaging in Huntington’s Disease.” *Experimental Neurology* 216 (2): 525–29. https://doi.org/10.1016/j.expneurol.2008.12.026.

Wen, Donghua, Jianguo Wu, Lei Wang, and Zheng Fu. 2017. “SUMOylation Promotes Nuclear Import and Stabilization of Polo-like Kinase 1 to Support Its Mitotic

Function." *Cell Reports* 21 (8): 2147–59. https://doi.org/10.1016/j.celrep.2017.10.085.

Wheeler, Vanessa C., Wojtek Auerbach, Jacqueline K. White, Jayalakshmi Srinidhi, Anna Auerbach, Angela Ryan, Mabel P. Duyao, et al. 1999. "Length-Dependent Gametic CAG Repeat Instability in the Huntington's Disease Knock-in Mouse." *Human Molecular Genetics* 8 (1): 115–22. https://doi.org/10.1093/hmg/8.1.115.

White, Joseph A., Eric Anderson, Katherine Zimmerman, Kan Hong Zheng, Roza Rouhani, and Shermali Gunawardena. 2015. "Huntingtin Differentially Regulates the Axonal Transport of a Sub-Set of Rab-Containing Vesicles in Vivo." *Human Molecular Genetics* 24 (25): 7182–95. https://doi.org/10.1093/hmg/ddv415.

Wilson, Matthew B., Steven J. Schreiner, Hyun Jung Choi, Joanne Kamens, and Thomas E. Smithgall. 2002. "Selective Pyrrolo-Pyrimidine Inhibitors Reveal a Necessary Role for Src Family Kinases in Bcr-Abl Signal Transduction and Oncogenesis." *Oncogene* 21 (53): 8075–88. https://doi.org/10.1038/sj.onc.1206008.

Wong, Y. C., and E. L. F. Holzbaur. 2014. "The Regulation of Autophagosome Dynamics by Huntingtin and HAP1 Is Disrupted by Expression of Mutant Huntingtin, Leading to Defective Cargo Degradation." *Journal of Neuroscience* 34 (4): 1293–1305. https://doi.org/10.1523/JNEUROSCI.1870-13.2014.

Wu, C., and W. C. Mobley. 2010. "Retrograde Neurotrophic Signaling." In *Encyclopedia of Neuroscience*, 283–89. https://doi.org/10.1016/B978-008045046-9.00738-5.

Yang, W. L., X. Zhang, and H. K. Lin. 2010. "Emerging Role of Lys-63 Ubiquitination in Protein Kinase and Phosphatase Activation and Cancer Development." *Oncogene*. https://doi.org/10.1038/onc.2010.190.

Yarza, Ramon, Silvia Vela, Maite Solas, and Maria J. Ramirez. 2016. "C-Jun N-Terminal Kinase (JNK) Signaling as a Therapeutic Target for Alzheimer's Disease." *Frontiers in Pharmacology*. https://doi.org/10.3389/fphar.2015.00321.

Ye, Haihong, Rejji Kuruvilla, Larry S. Zweifel, and David D. Ginty. 2003. "Evidence in Support of Signaling Endosome-Based Retrograde Survival of Sympathetic Neurons." *Neuron* 39 (1): 57–68. https://doi.org/10.1016/S0896-6273(03)00266-6.

Ylikoski, J, U Pirvola, M Moshnyakov, J Palgi, U Arumae, and M Saarma. 1993. "Expression Patterns of Neurotrophin and Their Receptor mRNAs in the Rat Inner Ear." *Hear Res* 65 (1–2): 69–78. http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids=8080462.

Zala, Diana, Maria Victoria Hinckelmann, and Frédéric Saudou. 2013. "Huntingtin's

Function in Axonal Transport Is Conserved in Drosophila Melanogaster." *PLoS ONE* 8 (3): 1–10. https://doi.org/10.1371/journal.pone.0060162.

Zeitlin, S, J P Liu, D L Chapman, V E Papaioannou, and a Efstratiadis. 1995. "Increased Apoptosis and Early Embryonic Lethality in Mice Nullizygous for the Huntington's Disease Gene Homologue." *Nature Genetics* 11 (2): 155–63. https://doi.org/10.1038/ng1095-155.

Zeron, M M, N Chen, a Moshaver, a T Lee, C L Wellington, M R Hayden, and L a Raymond. 2001. "Mutant Huntingtin Enhances Excitotoxic Cell Death." *Molecular and Cellular Neurosciences* 17 (1): 41–53. https://doi.org/10.1006/mcne.2000.0909.

Zheng, Jing, Wan Hua Shen, Ting Jia Lu, Yang Zhou, Qian Chen, Zi Wang, Ting Xiang, et al. 2008. "Clathrin-Dependent Endocytosis Is Required for TrkB-Dependent Akt-Mediated Neuronal Protection and Dendritic Growth." *Journal of Biological Chemistry* 283 (19): 13280–88. https://doi.org/10.1074/jbc.M709930200.

Zuccato, Chiara, and Elena Cattaneo. 2009. "Brain-Derived Neurotrophic Factor in Neurodegenerative Diseases." *Nature Reviews Neurology* 5 (6): 311–22. https://doi.org/10.1038/nrneurol.2009.54.

Zuccato, Chiara, Andrea Ciammola, Dorotea Rigamonti, Blair R. Leavitt, Donato Goffredo, Luciano Conti, Marcy E MacDonald, Tonis Timmusk, Simonetta Sipione, and Elena Cattaneo. 2001. "Loss of Huntingtin-Mediated BDNF Gene Transcription in Huntington ' S Disease." *Science* 293 (July): 493–98. https://doi.org/10.1126/science.1059581.

Zuccato, Chiara, Daniel Liber, Catarina Ramos, Alessia Tarditi, Dorotea Rigamonti, Marzia Tartari, Marta Valenza, and Elena Cattaneo. 2005. "Progressive Loss of BDNF in a Mouse Model of Huntington's Disease and Rescue by BDNF Delivery." *Pharmacological Research* 52 (2): 133–39. https://doi.org/10.1016/j.phrs.2005.01.001.

Zweifel, Larry S., Rejji Kuruvilla, and David D. Ginty. 2005. "Functions and Mechanisms of Retrograde Neurotrophin Signalling." *Nature Reviews Neuroscience*. https://doi.org/10.1038/nrn1727.

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STM/ALPSP Statement, June 2006:
http://www.alpsp.org/Ebusiness/AboutALPSP/ALPSPStatements/Statementdetails.aspx?ID=55

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Appendix B. IACUC Approvals

UIC UNIVERSITY OF ILLINOIS AT CHICAGO

Office of Animal Care and
Institutional Biosafety Committees (MC 672)
Office of the Vice Chancellor for Research
206 Administrative Office Building
1737 West Polk Street
Chicago, Illinois 60612-7227

December 1, 2011 ***Revised for PAF***

Gerardo Morfini
Anatomy & Cell Biology
M/C 512

Dear Dr. Morfini:

The protocol indicated below was reviewed at a convened ACC meeting in accordance with the Animal Care Policies of the University of Illinois at Chicago on **10/18/2011**. *The protocol was not initiated until final clarifications were reviewed and approved on* **11/15/2011**. *The protocol is approved for a period of* **3** years with annual continuation.

Title of Application: Analysis of Axonal Transport and Kinase-based Signaling Mechanisms in Neurodegenerative Diseases

ACC Number: 11-180

Initial Approval Period: 11/15/2011 to 10/18/2012

Current Funding: ***Portions of this protocol are supported by the funding sources indicated in the table below.***
Number of funding sources: 3

Funding Agency	**Funding Title**			**Portion of Proposal Matched**
NIH	*Axonal Transport Deficits During Hereditary Spastic Paraplegia*			*Matched*
Funding Number	**Current Status**	**UIC PAF NO.**	**Performance Site**	**Funding PI**
RO1 NS066942 (A1 version)	*Funded*	*201000275*	*UIC*	*Gerardo Morfini*
Funding Agency	**Funding Title**			**Portion of Proposal Matched**
ALS Therapy Alliance (ALSTA)	*Mechanisms Underlying MAPK Activation and Axonal Transport Deficits in Familial ALS*			*Matched*
Funding Number	**Current Status**	**UIC PAF NO.**	**Performance Site**	**Funding PI**
N/A	*Funded*	*2010-02403*	*UIC*	*Gerardo Morfini*
Funding Agency	**Funding Title**			**Portion of Proposal Matched**

Appendix B. IACUC Approvals (continued)

Brain Research Foundation	*Effects of Mutant Huntingtin on Cytoplasmic Dynein Phosphorylation and Function*			*Matched*
Funding Number	**Current Status**	**UIC PAF NO.**	**Performance Site**	**Funding PI**
N/A	*Funded*	*201104500*	*UIC*	*Gerardo Morfini*

This institution has Animal Welfare Assurance Number A3460.01 on file with the Office of Laboratory Animal Welfare (OLAW), NIH. **This letter may only be provided as proof of IACUC approval for those specific funding sources listed above in which all portions of the funding proposal are matched to this ACC protocol.**

In addition, all investigators are responsible for ensuring compliance with all federal and institutional policies and regulations related to use of animals under this protocol and the funding sources listed on this protocol. Please use OLAW's "*What Investigators Need to Know about the Use of Animals*" (http://grants.nih.gov/grants/olaw/InvestigatorsNeed2Know.pdf) as a reference guide. Thank you for complying with the Animal Care Policies and Procedures of UIC.

Sincerely yours,

Richard D. Minshall, PhD
Chair, Animal Care Committee

RDM/*mbb*
cc: BRL, ACC File, Rodolfo Gatto, PAF *201000275, 200901463, 201104500*

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This institution has Animal Welfare Assurance Number A3460.01 on file with the Office of Laboratory Animal Welfare (OLAW), NIH. **This letter may only be provided as proof of IACUC approval for those specific funding sources listed above in which all portions of the funding proposal are matched to this ACC protocol.**

In addition, all investigators are responsible for ensuring compliance with all federal and institutional policies and regulations related to use of animals under this protocol and the funding sources listed on this protocol. Please use OLAW's "*What Investigators Need to Know about the Use of Animals*" (http://grants.nih.gov/grants/olaw/InvestigatorsNeed2Know.pdf) as a reference guide. Thank you for complying with the Animal Care Policies and Procedures of UIC.

Sincerely yours,

Bradley Merrill, PhD
Chair, Animal Care Committee

BM/*mbb*
cc: BRL, ACC File, Rodolfo Gatto, Scott T. Brady, PAF *201000275, 201400263*

Appendix B. IACUC Approvals (continued)

UIC UNIVERSITY OF ILLINOIS AT CHICAGO

Office of Animal Care and
Institutional Biosafety Committees (MC 672)
Office of the Vice Chancellor for Research
206 Administrative Office Building
1737 West Polk Street
Chicago, Illinois 60612-7227

September 11, 2017

Gerardo Morfini
Anatomy & Cell Biology
M/C 512

Dear Dr. Morfini:

The protocol indicated below was reviewed at a convened ACC meeting in accordance with the Animal Care Policies of the University of Illinois at Chicago on **8/15/2017**. *The protocol was not initiated until final clarifications were reviewed and approved on* **9/11/2017**. *The protocol is approved for a period of* **3** years with annual continuation.

Title of Application: Analysis of axonal transport and kinase-based signaling mechanisms in neurodegenerative diseases

ACC Number: 17-140

Initial Approval Period: 9/11/2017 to 8/15/2018

Current Funding: ***Portions of this protocol are supported by the funding sources indicated in the table below.***
Number of funding sources: 2

Funding Agency	**Funding Title**			**Portion of Proposal Matched**
CDHI Fdn. Inc.	*Role of the JNK pathway on HD pathogenesis (Institutional #00004208)*			*All matched*
Funding Number	**Current Status**	**UIC PAF NO.**	**Performance Site**	**Funding PI**
A-11014	*Funded*	*201606069*	*UIC*	*Gerardo Morfini*
Funding Agency	**Funding Title**			**Portion of Proposal Matched**
NIH	*Addressing the Contribution of JNK3 to Axonal Pathology in Huntington's Disease (Institutional #00026078)*			*All matched*
Funding Number	**Current Status**	**UIC PAF NO.**	**Performance Site**	**Funding PI**
R21NS096642 (years 1-2 A1)	*Funded*	*201605902*	*UIC*	*Gerardo Morfini*

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In addition, all investigators are responsible for ensuring compliance with all federal and institutional policies and regulations related to use of animals under this protocol and the funding sources listed on this protocol. Please use OLAW's "*What Investigators Need to Know about the Use of Animals*" (http://grants.nih.gov/grants/olaw/InvestigatorsNeed2Know.pdf) as a reference guide. Thank you for complying with the Animal Care Policies and Procedures of UIC.

Sincerely yours,

John P. O'Bryan, PhD
Chair, Animal Care Committee
JPO /*mbb*
cc: BRL, ACC File, Mercedes Priego, Leon Tai

VITA

MINSU KANG

EDUCATION

University of Illinois, Chicago 2013-2018
Ph.D., Anatomy and Cell Biology with a Concentration in Neuroscience

University of Illinois, Urbana-Champaign 2009-2013
B.S. with High Distinction, Individual Plan of Study – Neuroscience

TEACHING ACTIVITIES

University of Illinois, Chicago 2016-2017
Teaching Assistant, Human Neuroanatomy

University of Illinois, Urbana-Champaign 2010-2012
Tutor, General Chemistry, Organic Chemistry, Molecular and Cellular Biology

AWARDS AND HONORS

Harry Monsen and Mildred Monsen Award for Excellence 2018
Department of Anatomy and Cell Biology, University of Illinois at Chicago

CCTS-PECTS Fellowship 2016-2018
Center for Clinical and Translational Science, University of Illinois at Chicago

Graduate Student Poster Award 2016
Brain Research Foundation

Graduate Student Council Travel Award 2016
Graduate Student Council, University of Illinois at Chicago

Student Presenter Award 2015
Graduate College, University of Illinois at Chicago

Horace Wu Scholarship Award 2013
University of Illinois at Urbana-Champaign

PUBLICATIONS

Leo, L., Weissmann, C., Burns, M., **Kang, M**., Song, Y., Qiang, L., Brady, S. T., Baas, P. W., Morfini, G. Mutant spastin proteins promote deficits in axonal transport through an isoform-specific mechanism involving casein kinase 2 activation. 2017. *Hum Mol Genet*.

Kang, M., Baker, L., Brady, S. T., Morfini, G. A. Analysis of axon-specific phosphorylation events in isolated squid axoplasms. 2016. *Methods Cell Biol*.

Song, Y., **Kang, M.**, Morfini, G. A., Brady, S. T. Fast axonal transport in isolated axoplasm from the squid giant axon. 2016. *Methods Cell Biol*.

PRESENTATIONS

Axonal transport defects and dying-back neuropathy in neurodegenerative diseases. 2018
UIC Association for Neuropsychological Student Training Seminar
University of Illinois, Chicago, Illinois

Kinase-based regulation of retrograde fast axonal transport by neurotrophin signaling. 2017
Departmental Seminar
Department of Anatomy and Cell Biology
University of Illinois, Chicago, Illinois

Inhibition of fast axonal transport by mutant huntingtin. 2016
Departmental Seminar
Department of Anatomy and Cell Biology
University of Illinois, Chicago, Illinois

Contribution of JNK3 in Huntington's disease pathology. 2016
Summer Postdoc and Graduate Student Seminar
Marine Biological Laboratory, Woods Hole, Massachusetts

Fast axonal transport deficits in Huntington's disease. 2015
Summer Postdoc and Graduate Student Seminar
Marine Biological Laboratory, Woods Hole, Massachusetts

Fast axonal transport deficits induced by mutant huntingtin involves activation of a specific MAPK pathway. 2015
UIC Neuroscience Day
Graduate Program in Neuroscience
University of Illinois, Chicago, Illinois

ABSTRACTS

Kang, M., Klein, A., Morris, S., Ebenezer, K., Zhang, R., Brady, S. T., Morfini, G. A. BDNF activates retrograde fast axonal transport by phosphorylation of cytoplasmic dynein. 2017. Abstract for poster presentation, *Graduate Education in Medical Sciences Research Forum*, Chicago, Illinois, September 2017.

Kang, M., Gatto, R., Weissmann, C., Mesnard-Hoaglin, N., Brady, S. T., Morfini, G. A. Mechanisms underlying axonal transport deficits in Huntington's disease. 2016. Abstract for poster presentation, *Society for Neuroscience*, San Diego, California, September 2016.

Kang, M., Gatto, R., Weissmann, C., Brady, S. T., Morfini, G. A. The role of JNK3 in Huntington's disease pathology. 2016. Abstract for poster presentation, *Graduate Education in Medical Sciences Research Forum*, Chicago, Illinois, September 2016.

Kang, M., Gatto, R., Weissmann, C., Brady, S. T., Morfini, G. A. Genetic deletion of JNK3 ameliorates Huntington's disease pathology. 2015. Abstract for poster presentation. *American Society for Neurochemistry*, Denver, Colorado, March 2016.

Kang, M., Gatto, R., Friedeck, H., Kim, B., Shah, S., Brady, S. T., Morfini, G. A. Contribution of JNK3 in Huntington's Disease Pathology. 2016. Abstract for poster presentation, *Brain Research Foundation Neuroscience Day*, Chicago, Illinois, January 2016.

Gatto, R., Chu, Y., **Kang, M.**, Lopez-Rosas, A., Friedeck, H., Kim, B., Kordower, J., Morfini, G. Addressing the in-vivo contribution of JNK3 to Huntington's disease pathogenesis. 2015. Abstract for poster presentation. *Society for Neuroscience*, Chicago, Illinois, October 2015.

Kang, M., Brady, S. T., Morfini, G. A. Fast axonal transport deficits induced by mutant huntingtin involve activation of a specific MAPK pathway. 2015. Abstract for poster presentation. *Student Research Forum*, Chicago, Illinois, March 2015.

Kang, M., Brady, S. T., Morfini, G. A. Fast axonal transport deficits induced by mutant huntingtin involve activation of a specific MAPK pathway. 2015. Abstract for poster presentation. *American Society for Neurochemistry*, Atlanta, Georgia, March 2015.

Kang, M., Brady, S. T., Morfini, G. A. Fast axonal transport deficits induced by mutant huntingtin involve activation of a specific MAPK pathway. 2015. Abstract for poster presentation. *Society for Neuroscience Chicago Chapter*, Chicago, Illinois, March 2015.

Kang, M., Kriefall, N., Abdelmesih, B., MacVeigh-Fierro, D. Purks, J., Brady, S. T., Morfini, G. A. Fast axonal transport deficits in Huntington's disease. 2014. Abstract for poster presentation. *UIC Neuroscience Day*, Chicago, Illinois, September 2015.

Kang, M., Hedger, J., Krishnamani, T., O'Hearn, D., Sherrill, L. K., Wu, M., Gulley, J. M. Ethanol Oral Self-Administration and the Assessment of Ethanol Intoxication. 2013. Abstract for poster presentation. *Undergraduate Research Symposium*, University of Illinois, Urbana, Illinois, April 2013.

PROFESSIONAL MEMBERSHIP

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