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Role of calpains in the injury-induced dysfunction and degeneration of the mammalian axon

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Abstract

Axonal injury and degeneration, whether primary or secondary, contribute to the morbidity and mortality seen in many acquired and inherited central nervous system (CNS) and peripheral nervous system (PNS) disorders, such as traumatic brain injury, spinal cord injury, cerebral ischemia, neurodegenerative diseases, and peripheral neuropathies. The calpain family of proteases has been mechanistically linked to the dysfunction and degeneration of axons. While the direct mechanisms by which transection, mechanical strain, ischemia, or complement activation trigger intra-axonal calpain activity are likely different, the downstream effects of unregulated calpain activity may be similar in seemingly disparate diseases. In this review, a brief examination of axonal structure is followed by a focused overview of the calpain family. Finally, the mechanisms by which calpains may disrupt the axonal cytoskeleton, transport, and specialized domains (axon initial segment, nodes, and terminals) are discussed.

Keywords

axon; calcium; calpain; ischemia; neurofilament; node; protease; synapse; trauma; Wallerian degeneration

I. Introduction

A growing body of work over the past 20 years has causally linked the calpain family of Ca^{2+} dependent cysteine proteases to axonal dysfunction and degeneration. Axons, which may reach over 1 meter in length in humans, are elegantly designed for anterograde and retrograde transport, conduction of electrical impulses, and release of neurotransmitters at synapses. To assist in these critical functions, axons have specialized domains and a unique subaxolemmal and central cytoskeleton, which are disrupted following injury in various experimental models. Strategies to injure axons, such as transection, stretch, ischemia, and complement activation, are known to activate intra-axonal calpains. In this review, I describe the findings from disparate lines of investigations and present a more unified narrative of the pathologic roles that calpains play within the mammalian axon.

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II. Axonal dysfunction and degeneration underlie common neurologic diseases

A. Traumatic brain injury

Worldwide, traumatic brain injury (TBI) leading to hospitalization or death is estimated to afflict over 10 million people annually and is predicted to surpass many diseases as the major cause of death and disability by the year 2020 (Hyder et al., 2007). The high incidence does not include most patients with mild TBI, which comprises the majority of all brain injuries, as these patients typically do not seek medical treatment. There are no currently approved therapies that specifically target the molecular cascades acutely triggered by TBI. A significant contribution to the morbidity and mortality is from traumatic axonal injury (TAI; Gennarelli et al., 1982; Povlishock, 1992; Smith and Meaney, 2000). In blunt TBI, rotational and shearing forces disproportionately affect long white matter tracts. Except in the most severe cases, where axons tear at the moment of trauma (primary axotomy), injured axons initially show no overt disconnection. However, these injured axons may swell, which is likely due to a disruption of axonal transport, and/or subsequently lose continuity and degenerate (secondary axotomy; Jafari et al., 1997; Maxwell and Graham, 1997; Povlishock and Katz, 2005; Saatman et al., 2003). Better understanding of the molecular cascades that lead to axonal injury and secondary axotomy may allow for targeted therapies for TBI.

B. Spinal cord injury

In the United States, there are over 250,000 people living with spinal cord injury (SCI; Ray et al., 2011). Like TBI, the victims of SCI are mostly young adults. The only approved therapy for SCI is the steroid methylprednisolone, but its efficacy is highly controversial (Ray et al., 2011). The neurological deficits are predominantly caused by the disruption of ascending and descending tracts (Medana and Esiri, 2003). Immediately after SCI in humans, a proportion of injured axons are not transected (Kakulas, 1999). These axons are swollen and immunoreactive for β-amyloid precursor protein (β-APP), which indicates a disruption of fast anterograde transport. Interestingly, these findings are also commonly found after TBI and TAI.

C. Traumatic injury to peripheral nerves

Traumatic injury to peripheral nerves results in considerable worldwide disability (Robinson, 2000). Even though traumatic nerve injuries are commonly classified into three types (neuropraxia, axonotmesis, and neurotmesis), injury is probably often mixed; some axons are transected, while others suffer conduction block, which is likely due to focal ischemia and/or demyelination (Robinson, 2000). Similar to axons in the brain and spinal cord, a subset of peripheral nervous system (PNS) axons may not be physically disrupted when subjected to mechanical forces. While some axons may undergo repair, there may be molecular triggers or cascades that push some to subsequently degenerate. Some of these molecules may be intrinsic to axons and be shared by the PNS and central nervous system (CNS).

D. Cerebral ischemia

The worldwide incidence and burden of strokes and cardiac arrests, which cause global cerebral ischemia, are high (Berdowski et al., 2010; Bramlett and Dietrich, 2004; Gustavsson et al., 2011; Kim and Johnston, 2011). About 50% of the volume of the human brain is white matter, which is almost always injured to some degree in cardiac arrest and stroke (Goldberg and Ransom, 2003). Axons are sensitive to ischemia and hypoxia (Pantoni et al., 1996; Underhill and Goldberg, 2007) even when the ischemic injury does not involve their parental cell bodies (Medana and Esiri, 2003). Ischemia triggers multiple deleterious

pathways in axons, some of which are also prominent after mechanical injury to axons. Therefore, therapeutic interventions to mitigate disruption of central conducting pathways may positively impact morbidity and mortality after both ischemia and trauma.

E. Other diseases

Axonal dysfunction and degeneration are likely important contributors to the morbidity and mortality caused by many other human diseases such as multiple sclerosis, amyotrophic lateral sclerosis, infections (e.g., human immunodeficiency syndrome and cerebral malaria), and peripheral neuropathies (Coleman, 2005; Coleman and Perry, 2002; Glass, 2004; Medana and Esiri, 2003; Shy et al., 2002). A good example is multiple sclerosis, which was initially characterized by multifocal demyelination with relative preservation of axons (Medana and Esiri, 2003). Later studies demonstrated that axon loss occurs earlier than would be expected for a primarily demyelinating disease, and that axon loss, but not demyelination, strongly correlates with disability (Das et al., 2008; Medana and Esiri, 2003). If axon loss is independent of demyelination, strategies to delay demyelination or encourage remyelination may have limited efficacy (Medana and Esiri, 2003). Thus, understanding axon physiology and pathophysiology is critically important to the development of therapeutic strategies for a broad spectrum of diseases and disorders.

III. Composition of specialized membrane domains and axonal cytoskeleton

A. Overview of specialized domains and subaxolemmal cytoskeleton

Broadly, there are two types of axons—myelinated and unmyelinated (Fig. 1). In myelinated axons, the clustering of ion channels and various proteins at the axon initial segment (AIS), nodes, and terminals are critical for action potential initiation, saltatory conduction, and neurotransmission. Both the mature AIS and node have high densities of voltage-gated Na^+ channels (Nav). Nav1.1, Nav1.2 and Nav1.6 are found in the mature AIS, while Nav1.1 and Nav1.6 are found in the mature node (Debanne et al., 2011; Duflocq et al., 2008; Rasband, 2011). Developing nodes, however, also contain Nav1.2 (Rasband, 2011). Voltage-gated K^+ channels (Kv) and/or cell adhesion molecules (CAMs) are also enriched in the AIS, nodes, and paranodes. The two main CAMs at the AIS and nodes are neurofascin-186 and NrCAM (neuronal cell adhesion molecule), while contactin and Caspr (contactin-associated protein) are present at the paranodes (Peles and Salzer, 2000; Rasband, 2011; Sherman et al., 2005). In unmyelinated axons, which are generally smaller and lack nodal domains, action potentials are thought to be propagated by a homogenous distribution of Nav1.2 (Debanne et al., 2011). Nav1.2 is also distributed throughout the length of axons of cultured cortical neurons (Iwata et al., 2004). In chemical synapses, axonal propagation culminates with the opening of presynaptic voltage-gated Ca^{2+} channels (Cav) and release of neurotransmitter into the synaptic cleft.

The subaxolemmal cytoskeleton consists of a lattice-like organization of various proteins, which contributes to the formation and stability of specialized electrical domains (Ogawa et al., 2006; Schafer et al., 2009). Of particular interest are spectrin, actin, and ankyrin, which are known calpain substrates (Roberts-Lewis et al., 1994; Schafer et al., 2009; Yoshida et al., 1984). One important function of spectrin is to link the cytoskeletal filament system, particularly actin filaments, to transmembrane proteins (Löfvenberg and Backman, 1999). Spectrins are comprised of α- and β-subunits, which associate laterally to form antiparallel heterodimers, and the heterodimers, in turn, are assembled head-to-head to form heterotetramers (Bennett and Baines, 2001). In humans, there are two α-subunits (αI and αII), four β-subunits (βI, βII, βIII, and βIV), and a β-H subunit (also referred to as βV). αII and βII-spectrins are located diffusely along the length of rodent optic nerve axons except for their exclusion from nodes and enrichment at paranodes (Ogawa et al., 2006). Similarly,

in sciatic nerve axons, they are not detected at nodes but are enriched at paranodes. αII/βII-spectrin is also present in presynaptic terminals and may play a key role in synaptic transmission (Goodman, 1999). On the other hand, βIV-spectrin is restricted to the AIS and nodes (Berghs et al., 2000; Yang et al., 2004). Of the subunits, αII, βII, and βIV-spectrin are calpain substrates (Glantz et al., 2007; Harris and Morrow, 1990; Roberts-Lewis et al., 1994; Schafer et al., 2009).

The mammalian genome contains 6 distinct genes that encode different actin isoforms (Cheever and Ervasti, 2013). The 3 α-actin and γ–smooth isoforms are predominantly expressed in muscle. While α-actin is probably not in neurons, β-cytoplasmic and γ-cytoplasmic actins (or simply β- and γ-actins) are ubiquitous (Cheever and Ervasti, 2013). In large axons in adult animals, γ-actin is likely the predominant isoform, while β-actin is either absent or present at relatively low levels. β- and γ-actins differ at only 4 out of 375 amino acids. Under proper buffer conditions, actin monomers (G-actin) spontaneously assemble into filaments (F-actin) (Letourneau, 2009). F-actin forms ring-like structures that wrap around the circumference of axons and are uniformly spaced along the axon (Xu et al., 2013). In mature axons, polymerized actin contributes to the stability of axolemma and presynaptic membrane and serves as “tracks” for myosin motor proteins and their cargos (Letourneau, 2009).

The ankyrin family of proteins has a general role as an adapter between a variety of integral membrane proteins—particularly ion channels and CAMs—and the spectrin skeleton (Bennett and Baines, 2001). Of particular interest is ankyrinG (ankG). The 480-kD and/or 270-kDa isoforms of ankG are localized to the AIS and nodes (Kordeli et al., 1995), and, together with βIV-spectrin, are required for Na^+ channel clustering (Komada and Soriano, 2002; Zhou et al., 1998).

Of the proteins discussed in this section, Nav1.2, αII-spectrin, βII-spectrin, βIV-spectrin, actin, and ankG are calpain substrates (Glantz et al., 2007; Harris and Morrow, 1990; Roberts-Lewis et al., 1994; Schafer et al., 2009; von Reyn et al., 2009; Yoshida et al., 1984). Calpains are also mechanistically linked to the disruption of the domain localization of two other key nodal or paranodal proteins—Nav1.6 and Caspr (McGonigal et al., 2010). The mislocalization of these transmembrane proteins may be due to proteolysis of the underlying subaxolemmal cytoskeleton or the proteins themselves.

B. Overview of axonal central cytoskeleton

Cytoskeletal disruption contributes to much of the axonal dysfunction and pathology observed after mechanical deformation or transection of axons. The axonal cytoskeleton is principally composed of neurofilaments and microtubules, which are well known calpain substrates (Billger et al., 1988; Kamakura et al., 1985, 1983; Malik et al., 1983; Schlaepfer et al., 1985).

Neurofilaments are the most abundant cytoskeletal protein in large myelinated axons and are longitudinally oriented and regularly spaced (see review by Perrot et al., 2008). Neurofilaments are ~10 nm in diameter and consist of homopolymers and heteropolymers of subunits with predicted molecular weights of 61.5, 102.5, and 112.5 kDa (in humans), known respectively as neurofilament light (NFL), medium (NFM), and heavy (NFH) subunits (Perrot et al., 2008). Each subunit has a ~46 nm-long central α-helical core or rod domain, flanked by a lobular amino-terminal head and non α-helical carboxy-terminal sidearm domain. The two larger isoforms are heavily phosphorylated, mainly in the sidearm domain. Phosphorylation is topographically regulated, as neurofilaments in axons are intensely phosphorylated (Perrot et al., 2008). Phosphorylation of the sidearms may influence inter-neurofilament distance and axonal caliber, an important determinant of

conduction velocity. Dephosphorylation or proteolysis of the sidearms is a marker of TAI (Povlishock et al., 1997).

Microtubules are critically important for anterograde and retrograde transport in axons. Microtubules are hollow cylindrical structures with outer diameters of ~25 nm typically formed by 13 parallel protofilaments, each composed of alternating α- and β-tubulin molecules (Amos and Schlieper, 2005; Maccioni and Cambiazo, 1995; Stanton et al., 2011). Microtubule assembly, stability, and elongation are promoted by microtubule-associated proteins (MAPs; Amos and Schlieper, 2005; Maccioni and Cambiazo, 1995). The high molecular weight MAPs, particularly MAP1A and MAP1B, have widespread neuronal distribution and are present in both axons and dendrites (Bloom et al., 1984; Maccioni and Cambiazo, 1995; Noble et al., 1989). Tau, a low molecular weight MAP, is highly enriched in axons, while MAP2 is found only in dendrites and somata in the mature nervous system (Maccioni and Cambiazo, 1995). The disruption of anterograde and retrograde axonal transport commonly observed after TBI has been attributed to depolymerization or loss of microtubules (Maxwell, 1996; Maxwell and Graham, 1997; McCracken et al., 1999; Saatman et al., 2003).

IV. The Calpain/Calpastatin System

A. Overview

Calpains are a family of Ca^{2+}-dependent non-lysosomal cysteine proteases, whose substrates are involved in cytoskeletal remodeling, signal transduction, cell differentiation, embryonic development, vesicular trafficking, apoptosis, and necrosis (Zatz and Starling, 2005). Since the discovery of the first calpain in 1964 (Guroff, 1964), it is now recognized that the human genome contains 15 genes encoding calpain-like proteases (Sorimachi et al., 2010). The ubiquitously expressed μ- and m-calpain are a focus of investigation in the diseased nervous system (Bevers et al., 2010; Geddes and Saatman, 2010; Yu et al., 2013). μ- and m-calpain are heterodimers that consist of 80 kDa catalytic subunits sharing 55-65% sequence homology (calpain 1 and calpain 2, respectively) and a common 28 kDa regulatory subunit, known as calpain 4 or common small subunit 1 (CSS1; Goll et al., 2003). CSS1 is likely important for the activity of both μ- and m-calpain. In 2002, a novel small subunit, named CSS2, was identified (Schád et al., 2002). Sharing significant sequence similarity with CSS1, CSS2 could substitute for CSS1 for m-calpain activity, at least *in vitro*. According to current scientific nomenclature, μ- and m-calpain refer to the heterodimers, while calpain 1 and 2 refer to the individual catalytic subunits.

μ- and m-calpains cleave their substrates in a limited manner, often modifying, rather than terminating, substrate functions (Sorimachi et al., 2010; Tompa et al., 2004). Even though some preferences for calpain cleavage site sequences have been identified, the complete rules governing substrate specificity remain unclear (Ray, 2006; Sorimachi et al., 2010). Despite sharing nearly identical substrate specificity (Goll et al., 2003), μ- and m-calpain serve unique physiological functions. Constitutive knockout of calpain 2 or CSS1 is embryonically lethal (Arthur et al., 2000; Dutt et al., 2006), while calpain 1-knockout mice are viable and fertile (Azam et al., 2001). A major biochemical difference between μ- and m-calpain is the *in vitro* Ca^{2+} concentration required for half-maximal activity, 3-50 μM and 400-800 μM, respectively (Goll et al., 2003). In the various injury models described in this review, axoplasmic $[Ca^{2+}]$ is elevated, thereby activating μ-calpains, m-calpains, or both.

In addition to calpain 1 and 2, several other calpain catalytic subunit isoforms (3, 5, and 10) have been identified in brain (Bevers and Neumar, 2008), but much less is known about them. Mutations in the gene for calpain 3, once thought to be muscle-specific, cause limb-girdle muscular dystrophy type 2A (Zatz and Starling, 2005). Even though present in

neuron-like PC12 cells (Marcilhac et al., 2006), calpain 3 has been detected in astrocytes, but not neurons, in the rodent and primate brain (König et al., 2003). Its autoproteolytic activity appears to be Ca^{2+}-independent (Kinbara et al., 1998), whereas *in vitro* proteolysis of the abundantly expressed muscle protein PDLIM1 is Ca^{2+}-dependent (Bertipaglia et al., 2009). Calpain 5 mRNA has been identified in rat and human brain (Waghray et al., 2004), and the protein has been detected in photoreceptors, but not bipolar or retinal ganglion cells (Mahajan et al., 2012). Mutations in the calpain 5 gene result in an autosomal dominant neovascular inflammatory vitreoretinopathy, which can progress to complete blindness (Mahajan et al., 2012). Calpain 10 protein has been detected in rat brain homogenates (Ma et al., 2001) and is partially targeted to mitochondria, where it likely plays a pathologic role in mitochondrial dysfunction via cleavage of Complex 1 subunits and activation of mitochondrial permeability transition (Arrington et al., 2006). Evidence suggests that calpain 10 activity is regulated by Ca^{2+} (Arrington et al., 2006). In the various studies described in this review, it is not clear what pathologic roles, if any, these other calpain isoforms may concurrently play within the axon.

Calpastatin is the endogenous inhibitor of both μ- and m-calpain, and it is not known to inhibit proteases of any other family (Goll et al., 2003; Pietsch et al., 2010). Although there is a single calpastatin gene in humans, pigs, and cattle, alternative splicing and different promoters give rise to isoforms ranging in molecular mass from 17.5 to 84 kDa (Goll et al., 2003). Binding of calpastatin to both the catalytic and regulatory subunits is required for effective inhibition of μ- and m-calpains (Goll et al., 2003). Details about calpastatin inhibition of the other calpain isoforms found in brain are scarce. There is evidence to suggest that the autoproteolytic activity of calpain 3 is not inhibited by calpastatin (Kinbara et al., 1998). Calpains 3, 5, and 10 are not believed to associate with the small regulatory subunit (Sorimachi et al., 1995; Suzuki et al., 2004; Waghray et al., 2004), but the regulatory subunit is not absolutely required for inhibition by calpastatin (Hata et al., 2007). Because calpastatin has absolute specificity for calpains, its overexpression in neuronal populations is used to provide a mechanistic link between calpains and axonal dysfunction and degeneration (Ma et al., 2013, 2012b).

Currently, there are no pharmacologic inhibitors completely specific for calpains or individual calpain isoforms (Bevers et al., 2009; Goll et al., 2003). However, calpain specificity of protease inhibitors has been improved recently. A synthetic 27-residue peptide based on subdomain B of domain I of human calpastatin (calpastatin-derived peptide 1B or CP1B) inhibits μ- and m-calpain with IC_{50} values in the nanomolar range and weakly inhibits cathepsin L (K_i = 6 μM), but not trypsin, cathepsin B, 20S proteasome, caspase 3, or papain (Pietsch et al., 2010). Most of the studies linking calpains to axonal pathology, described herein, rely on ALLN (calpain inhibitor I; *N*-Ac-Leu-Leu-norleucinal), ALLM (calpain inhibitor II; *N*-Ac-Leu-Leu-methioninal), MDL-28170 (calpain inhibitor III; carbobenzylzoxy-Val-Phe-H), or AK295 (Z-Leu-aminobutyric acid-CONH$(CH_2)_3$-morpholine). The membrane permeable ALLN and ALLM also inhibit cathepsin B, cathepsin L, and proteasome (Goll et al., 2003). MDL-28170, one of the best studied and most utilized calpain inhibitors, readily crosses the blood-brain barrier and is somewhat more selective for calpains than cathepsin B (K_i = 25 and 10 nM for cathepsin B and calpain, respectively) (Markgraf et al., 1998). AK295 is membrane permeable, and significantly more selective for calpain (K_i = 27-42 nM) over other cysteine proteases, such as cathepsin B (K_i = 24 μM) (Saatman et al., 1996).

B. Localization of μ- and m-calpains in axons

Calpains 1 and 2 have been identified in axons. In the CNS, calpain 1 immunoreactivity is detectable in myelinated and unmyelinated axons and rarely in presynaptic terminals (Fig. 1;

Perlmutter et al., 1990, 1988; Siman et al., 1985). Some axons (e.g., corpus callosum) though are either lightly or not detectably immunoreactive (Perlmutter et al., 1988). Hamakubo et al. (1986) localized calpain 2, but not calpain 1, immunoreactivity to myelinated axons in the cerebellum using light microscopy. Axonal localization of the calpain antibodies in other brain regions was not reported. Using electron microscopy, calpain 1 immunoreactivity is observed in cerebellar axons (Perlmutter et al., 1988). This discordance of calpain 1 immunoreactivity in cerebellar axons could be attributed to increased sensitivity of immunoelectron microscopy over standard immunohistochemistry. It is also not clear if the examined axons originated from the same neuronal population. In the PNS, calpain 2 immunoreactivity is observed in myelinated sciatic nerve axons (Mata et al., 1991).

In the rat cortex and hippocampus, immunoreactivity for CSS1 is readily detectable in neuronal somata and/or dendrites, while CSS2 staining is detectable mainly in the axon fibers and terminals (Friedrich et al., 2004). Their axonal localization in CNS white matter tracts or PNS has not been reported. Nonetheless, calpain activity has been identified in axons and synaptic terminals (Ma et al., 2013; O'Hanlon et al., 2003; Saatman et al., 2003). Identification of calpains and their activity in axons suggest that these proteases may play an important role in axonal dysfunction and degeneration.

V. Pathologic role of calpains in axons

The remainder of this review focuses on intra-axonal effects of pathologic calpain activity and the efficacy of calpain inhibitory strategies on preserving axonal function and morphology. Due to space constraints, our discussion of substrates of μ- and m-calpains will be limited to those that are proteolyzed under pathologic conditions and are either preferentially localized to distinct axonal domains or are critical to unique axonal functions. The role of calpains in specific organelles, like the endoplasmic reticulum and mitochondria, are reviewed elsewhere (Bevers and Neumar, 2008; Kar et al., 2010). Complex injury models (e.g. spinal cord crush and multiple sclerosis; Das et al., 2008; Ray and Banik, 2003), in which pathologic calpain activity in extra-axonal neuronal compartments, glia, or inflammatory immune cells may obfuscate the role of intra-axonal calpains, are deemphasized. Section V will serve to clarify the role of intra-axonal calpains in dysfunction and degeneration, highlight important gaps in our knowledge, and delineate the potential benefits and limitations of therapeutic calpain inhibition.

A. Degeneration of the axonal cytoskeleton in Wallerian degeneration

A.1. Overview of Wallerian degeneration—In 1850, Augustus Waller observed that nerve transection results in progressive degeneration of the distal portion of the nerve (Waller, 1850). After an axon is transected *in vivo*, there is a latency period where the axon remains structurally quiescent and electrically excitable (Wang et al., 2012). This is followed by a rapid, irreversible process, termed granular disintegration of the axonal cytoskeleton (GDC), in which neurofilaments, microtubules, and other cytoskeletal components disintegrate or disassemble (Vargas and Barres, 2007). Wallerian degeneration has attracted much scientific interest as the underlying molecular mechanisms are thought to be shared, at least in part, by many human diseases, such as TBI, cerebral ischemia, Alzheimer's disease, Parkinson's disease, and toxic and other neuropathies (Coleman, 2005; Glass, 2004; Saxena and Caroni, 2007; Wang et al., 2000; Wang et al., 2012).

It was once believed that when axons were separated from their somata, the cessation of anterogradely-delivered trophic factors was responsible for their passive degeneration (Vargas and Barres, 2007). However, with the discovery of the Wallerian degeneration slow mouse (Wld^s), axonal degeneration is now established as an active process. The autosomal

dominant Wld^S mutation is spontaneously occurring. Located on mouse chromosome 4, the Wld^S gene mutation is an 85-kb tandem triplication containing the full coding region of nicotinamide mononucleotide adenyl transferase 1 (Nmat-1), the coding region for the amino-terminal 70 amino acids of the ubiquitination factor E4B (Ube4b), and a unique 18 amino acid linker. The precise mechanism by which Wld^S mediates axonal protection has not been clearly established, but the bulk of existing evidence favors a mitochondrial role of Nmnat-1 in preserving axons (Coleman and Freeman, 2010; Wang and Barres, 2012).

Additional compelling evidence that axon degeneration is a regulated and active process comes from the recent identification of the *Drosophila* Toll receptor adaptor dSarm (sterile *α*/Armadillo/Toll-Interleukin receptor homology domain protein) (Osterloh et al., 2012). Knockout of the mouse ortholog of *dSarm*, *Sarm1*, results in significant axonal and synaptic preservation after transection of sciatic nerves. These results provide convincing evidence that injured axons actively promote their own destruction and identify dSarm/Sarm1 as a member of this signaling pathway (Osterloh et al., 2012). Better understanding of the signals and mediators of Wallerian degeneration may reveal opportunities for therapeutic intervention in human disease.

A.2. Cytoskeletal components are calpain substrates—Early investigators implicated calpains as causative agents of GDC (Kamakura et al., 1985, 1983; Schlaepfer et al., 1984). All three neurofilament isoforms—NFL, NFM, and NFH—are calpain substrates (Fig. 1) (Kamakura et al., 1985, 1983; Malik et al., 1983; Schlaepfer et al., 1985). Of the three isoforms, NFM appears to be most susceptible to proteolysis (Kamakura et al., 1985). Except for two sites (after Lys^{467} and Lys^{516}) identified in bovine NFM (Shaw et al., 2004), the calpain cleavage sites for the mammalian neurofilament isoforms have not been established. Even though the properties of individual neurofilament fragments have not been systematically examined, evidence suggests that calpain proteolysis of the neurofilament cytoskeleton results in loss of filamentous structure (Ma et al., 2013).

Similar to neurofilaments, microtubules are critical components of the axonal cytoskeleton and disintegrate during GDC (Donat and Wiśniewski, 1973; Schlaepfer, 1974). The early disintegration or loss of microtubules may be due to depolymerization, proteolysis of microtubule components, or a combination of the two. Most studies implicate tubulin as a calpain substrate (Fig. 1; Billger et al., 1988; Malik et al., 1987, 1983). The lack of tubulin proteolysis observed in the study by Sandoval and Weber (1978) is likely due to the relatively low concentration or activity of calpains in crude brain extract that was used as a source of the protease. It has been reported that *in vitro* calpain proteolysis of tubulin results in a ~50 kDa fragment (Billger et al., 1988), even though the fragment would expectedly be difficult to discriminate from intact tubulin on one-dimensional electrophoresis. Purified tubulin subjected to calpain proteolysis forms large aggregates without any obvious ultrastructure (Billger et al., 1988). Microtubule depolymerization may also be due to proteolysis of MAPs (Sandoval and Weber, 1978). MAP1 and tau are calpain substrates, and their proteolysis generates multiple fragments (Fischer et al., 1991; Sato et al., 1986; Yang and Ksiezak-Reding, 1995). When a purified mixture of microtubules, MAP1 and MAP2, is incubated with calpain, both MAPs are degraded and no longer bind to microtubules (Billger et al., 1988). Adding further complexity, microtubule depolymerization may result directly from elevated Ca^{2+} or by Ca^{2+} activation of calmodulin (Gaskin et al., 1975; Job et al., 1981; Keith et al., 1983; O'Brien et al., 1997). Therefore, in injured axons *in vivo*, it is difficult to tease apart the role of calpains from the other effects of elevated Ca^{2+}. The mechanism of microtubule loss in GDC may be multifactorial.

A.3. Calpains and GDC in Wallerian degeneration—Evidence for the role of calpains in Wallerian degeneration initially came from *in vitro* studies (Table 1; Fig. 2).

Reducing extracellular Ca^{2+} or application of pharmacologic calpain inhibitors decrease degeneration of transected neurites in dorsal root ganglia (DRG) and sympathetic superior ganglia (SCG) explant cultures, as assessed by phase contrast microscopy, MAP1B immunostaining, neurofilament immunolabeling, and neurofilament western blotting (Finn et al., 2000; George et al., 1995; Wang et al., 2000; Zhai et al., 2003). However, ALLN does not reduce axolemmal degeneration (Finn et al., 2000) or microtubule fragmentation, which refers to gaps in tubulin immunostaining of neurites (Zhai et al., 2003). Exactly what microtubule fragmentation represents on the ultrastructural level is unclear, but it may represent focal depolymerization of microtubules. In contrast, the Ca^{2+} chelator EGTA (ethylene glycol-bis(2-aminoethylether)-*N,N,N',N'*-tetraacetic acid) and pharmacologic inhibitors to the ubiquitin-proteasome system (UPS) prevent both neurofilament degradation and microtubule fragmentation. Another study did not find statistically significant protection from degeneration of βIII-tubulin-labeled transected axons (MAP2-negative neurites) with ALLN (Kilinc et al., 2011), but the large variability in the treatment group may have obscured any drug effects. These two *in vitro* studies, together with a third one that strongly links E3 ubiquitin ligase ZNRF1 (zinc and ring finger 1) to *in vivo* Wallerian degeneration (Wakatsuki et al., 2011), suggest that certain features of Wallerian degeneration, such as microtubule fragmentation, may be mediated by the UPS, and not by calpains. Nonetheless, the evidence in total supports that calpains are important for key aspects of neurite degeneration, particularly the loss of neurofilament integrity.

Care must be exercised when extrapolating these experimental findings to axons *in vivo*. First, cultures consist of developmentally young neurons in a “simplified” extracellular environment. Second, even though neurites are comprised of dendrites and axons (Touma et al., 2007), differentiating axons from dendrites was not considered when studying explant cultures. Third, the *in vivo* correlates of *in vitro* measures of neurite degeneration are not clear. Does loss of neurofilament immunolabeling in neurites represent GDC seen *in vivo*? Does fragmentation of tubulin immunostaining represent microtubule depolymerization? Finally, all the pharmacologic calpain inhibitors that were used in these experiments inhibit other proteases (Goll et al., 2003; Saatman et al., 1996).

Two early *in vivo* studies provide inconclusive evidence for a role of calpains in Wallerian degeneration. Couto et al. (2004) applied a calpain inhibitor (Mu-F-HF-FMK) directly to the optic nerve crush site in opossums. Four days post-crush, there is a slight, but statistically significant, increase in axons with preserved cytoskeleton (mean±SD: 76±3.7% versus 67±4.2% nontreated injured) and reduction in degenerating axons compared to injured animals without treatment. Experimental interpretation is limited by the modest effects and limited specificity of the inhibitor (Ma et al., 2013). Glass et al. (2002) studied sciatic nerve transection in mice. One h after transection, there is increased calpain activity, as measured by Ab38, in nerve homogenates; Ab38 specifically detects the calpain-cleaved αII-spectrin fragment (for more details, see section V.C1). The increase in Ab38 signal is not present when significant loss of NFL and β-tubulin occurs (between 24 and 48 h post-axotomy). The inability to detect an increase in Ab38 signal beyond 1 h post-transection is possibly due to the low baseline level of αII-spectrin in sciatic nerves or the instability of the fragment in the setting of robust and prolonged calpain activity (Ma et al., 2013). Pretreatment with intraperitoneally administered calpain inhibitor leupeptin, which is continued throughout the survival period, does not prevent NFL degradation 3 days post-sciatic nerve transection (Glass et al., 2002). As leupeptin has poor cellular permeability, it is possible that there was insufficient intra-axonal concentration (Ma et al., 2013).

An *in vivo* study recently established that calpains are causally responsible for GDC (Ma et al., 2013). Based on the appearance of NFL fragments generated by calpain proteolysis, robust calpain activity first appears between 24 and 48 h after sciatic nerve transection and

between 48 and 72 h after optic nerve transection. Calpastatin overexpression in transgenic mice reduces the proteolysis of NFL after sciatic and optic nerve transection, as well as the loss of NFH in sciatic nerves. It also provides robust ultrastructural protection of the axonal cytoskeleton, particularly neurofilaments, after sciatic nerve transection (Fig. 3). Ultrastructural protection of optic nerve axons is not observed however, likely due to the presence of "dark degeneration," which obscures visualization of the cytoskeleton (Ma et al., 2013). "Dark degeneration" occurs when axons become filled with electron-dense material, and is commonly seen after transection of optic nerve axons (Bignami et al., 1981; Marques et al., 2003; Narciso et al., 2001). Neither the mechanism nor functional significance of "dark degeneration" is known. In this study, other cytoskeletal components were not examined. Nonetheless, this study confirms that GDC, in particular neurofilament proteolysis, is mediated by calpains.

In 2005, Kerschensteiner et al. (2005) coined the term "acute axonal degeneration" (AAD) to describe the rapid fragmentation of axons in regions immediately proximal and distal to the transection site. These investigators studied transgenic mice that express green fluorescent protein (GFP) in DRG axons traversing superficially through the spinal cord. After 10-20 min of quiescence, transection results in rapid (<5 min) fragmentation of 200-300 μm of axon segments at the cut ends, based on the fragmented appearance of intra-axonal GFP. Application of a calpain inhibitor cocktail to the transection site completely prevents this fragmentation (Kerschensteiner et al., 2005). Ultrastructural changes during AAD in optic nerves include condensation and misalignment of neurofilaments and fragmentation of microtubules (Knöferle et al., 2010), and ascribing some of these changes to calpains would be an intriguing next step. As AAD is temporally and spatially dissimilar to Wallerian degeneration, the importance of calpains in two different injury paradigms suggests that calpain-mediated degeneration may be a generalizable mechanism of axonal pathology.

In summary, calpains are important contributors to Wallerian degeneration, particularly neurofilament proteolysis. The causal mechanisms underlying axolemmal pathology, microtubule disruption, and "dark degeneration" still require further exploration. Axonal degeneration is likely multifactorial, and, in addition to pathologic calpain activity, may include Ca^{2+} dependent non-calpain mechanisms, the UPS, or autophagy (Beirowski et al., 2010; Knöferle et al., 2010; Yang et al., 2007; Zhai et al., 2003).

B. Axonal swelling, neurofilament compaction, and secondary axotomy after traumatic axonal injury

B.1. Pathophysiology of traumatic axonal injury—In the mammalian CNS, immediate severing or transection of axons at the moment of trauma, known as primary axotomy, is an irreversible process and is thought to only occur at the most severe mechanical strains (Maxwell et al., 1997; Povlishock and Katz, 2005). Instead, after blunt TBI, injured axons do not typically demonstrate primary axotomy, but evidence suggests that a biochemical cascade is triggered within axons, ultimately disrupting their continuity (secondary axotomy; Jafari et al., 1997; Maxwell and Graham, 1997; Povlishock et al., 1997; Povlishock and Katz, 2005; Saatman et al., 2003). In addition, blunt TBI disrupts axonal transport, leading to focal accumulation of vesicles and organelles, or axonal swellings (Büki and Povlishock, 2006). These swellings, also called spheroids or varicosities, are almost universally observed in CNS neurodegenerative diseases (Beirowski et al., 2010; Coleman, 2005). As secondary axotomy and axonal swellings are potentially preventable or reversible, they are a major focus of TBI researchers.

Support for secondary axotomy after mechanical strain comes from *in vivo* investigations. Within 1 h of fluid percussion injury in cats, intra-axonal accumulations of an anterograde

tracer, horseradish peroxidase (HRP) conjugated to wheat germ agglutinin, occurs in labeled efferent fibers (Erb and Povlishock, 1988; Povlishock et al., 1983). Accumulations first show continuity with proximal and distal intra-axonal peroxidase profiles (axonal swellings). Over several hours, more of these accumulations appear to be disconnected from their distal profiles (axonal bulbs). A similar progression is observed after injury in rats (Kelley et al., 2006; Singleton et al., 2002). It is possible that continued anterograde transport of HRP may occur distal to a site of complete anterograde transport disruption, resulting in its disappearance from the distal segment and the illusion of axonal disconnection (Erb and Povlishock, 1988); however, secondary axotomy is also supported by ultrastructural analysis (Erb and Povlishock, 1988; Povlishock et al., 1983). Delayed axonal disconnection appears to be temporally and spatially linked to axonal swellings. It is important to note that it is the presence of axonal swellings that focuses the investigator on that segment of axon, and it is not known if secondary axotomy occurs at non-swollen sites.

An attractive hypothesis is that transport disruption is due to microtubule loss or depolymerization (Maxwell, 1996; McCracken et al., 1999; Saatman et al., 2003). Within 5 min after moderate to severe fluid percussion injury in cats, axons with altered axolemmal permeability in the ventral pontomedullary region display microtubule loss (Pettus and Povlishock, 1996). Fifteen min after optic nerve stretch in the guinea pig, there is a dramatic loss of microtubules (Maxwell and Graham, 1997). Importantly, internodal axonal swellings containing significantly reduced numbers of microtubules develops within 2 h of injury. In addition, mechanical strain has been proposed to physically rupture microtubules, leading to transport disruption (Tang-Schomer et al., 2012, 2010). Despite the strong circumstantial evidence, a direct link between microtubule loss and/or rupture to axonal swellings and transport disruption has not been established.

In addition to microtubule loss, neurofilament compaction has been suggested to impair axoplasmic transport (Smith and Meaney, 2000). When neurofilament subunits polymerize to form filaments, the central rod domains are normally masked by the phosphorylated sidearms. Binding of the antibody RMO-14, which is a commonly used histologic marker of TAI, to the rod domain of NFM suggests that proteolysis or dephosphorylation of the sidearm has occurred (Buki et al., 2003; Maxwell et al., 1997; Povlishock et al., 1997). Because the sidearms contribute to interneurofilament spacing, RMO-14 immunostaining likely indicates neurofilament compaction (Povlishock et al., 1997; Stone et al., 2001), and has been observed after human TBI (Stone et al., 2001) and in animal models of TAI (Buki et al., 2003; Stone et al., 2001). As RMO-14 immunoreactive axons in the pyramidal tract do not stain with β-APP nor have evidence of organelle pooling after impact acceleration head injury in rats (Stone et al., 2001), neurofilament compaction as a causal mechanism for transport disruption has been questioned (Ma et al., 2012b). In the setting of blunt TBI, the functional consequence of proteolysis or dephosphorylation of neurofilament sidearms remains unknown.

B.2. Role of calpains in traumatic axonal injury—Axonal calpain activity is elevated within minutes after experimental TAI (Büki et al., 1999; Saatman et al., 2003). Calpain proteolysis of αII-spectrin, measured by Ab38 immunolabeling, occurs in axons in the pyramidal tracts and medial lemnisci within 15 min of impact acceleration head injury in rats (Büki et al., 1999). After optic nerve stretch in the rodent, an *in vivo* model that isolates mechanical strain primarily to axons, Ab38 signal is detectable 20-30 min post-injury (Ma et al., 2012a; Saatman et al., 2003). As tubulin and MAPs are known calpain substrates (Billger et al., 1988; Fischer et al., 1991; Johnson et al., 1989), early calpain activity following injury makes calpains attractive candidates for the mechanisms underlying TAI pathology.

Calpains have been implicated in neurofilament compaction and impaired axonal transport after TAI. As early as 15 min after impact acceleration head injury, Ab38 immunoreactivity consistently colocalizes with RMO-14, suggesting that neurofilament compaction is temporally and spatially linked to pathologic calpain activity (Büki et al., 1999). Calpain inhibitor studies have provided further support for the causal role of calpains in TAI pathology (Table 2; Fig. 2). ALLN reduces neurite beading 60-90 min after fluid shear stress injury to dissociated forebrain neurons (Kilinc et al., 2009). Mechanical strain is not limited to axons, however, as there is increased calpain activity diffusely in the soma at the earliest time point (5 min post-injury). A single 30 mg/kg intravenous (IV) dose of MDL-28170 30 min prior to impact acceleration head injury in rats reduces β-APP and RMO-14 staining in brainstem fiber tracts 2 h post-injury (Buki et al., 2003). MDL-28170 also decreases β-APP labeling in the corpus callosum up to 7 d after fluid percussion injury (Ai et al., 2007). ALLM administered via continuous arterial infusion throughout the entire survival period reduces axonal swellings and/or bulbs in the corpus callosum and other white matter tracts 24 h after cortical impact injury (Posmantur et al., 1997), although quantification is not reported. Care must be taken when interpreting findings from TBI models in which the mechanical forces may not exclusively act upon axons (e.g., *in vitro* fluid shear stress, fluid percussion injury, impact acceleration head injury, and cortical impact). It is difficult to tease apart primary versus secondary injury to axons. Theoretically, a drug, when given systemically, may directly protect non-axonal structures, which in turn reduce the secondary injury seen within axons. In addition, neither of the inhibitors used in the *in vivo* studies (MDL-28170 and ALLM) is completely specific for calpains (Markgraf et al., 1998; Posmantur et al., 1997). Even though these studies suggest that MDL-28170 or ALLM may be beneficial after human TBI, their limitations make establishing a mechanistic link between intra-axonal calpain activity and TAI pathology tenuous.

To overcome these potential limitations, calpastatin overexpression was used in conjunction with the rat optic nerve stretch model (Ma et al., 2012b). Calpastatin overexpression within retinal ganglion cell axons partially preserves retrograde transport of Fluoro-gold™. In contrast, intensive therapy with MDL-28170 is ineffective in the same injury paradigm, possibly due to the short duration of treatment or inadequate intra-axonal concentration (Ma et al., 2012a).

The contribution of calpains specifically to microtubule loss or secondary axotomy has not been experimentally investigated. Nonetheless, the evidence in total suggests that calpains are responsible for key aspects of TAI, such as neurofilament compaction, and anterograde and retrograde transport disruption.

B.3. Commonalities and differences between Wallerian degeneration and TAI

—In the previous sections, I limited my discussion of axonal degeneration to axonal transection models and emphasized transport disruption and neurofilament compaction in TAI models. This distinction is somewhat artificial but does reflect the different focus of nerve biologists and traumatologists. Evidence of transport disruption and degeneration is almost universally present after axonal injury irrespective of injury model. For example, *in vivo* rat optic nerve crush or transection results in the appearance of swellings close to the lesion site by 6-10 h post-injury, followed by their appearance ~5 and ~15 mm distal to the lesion site at 24 and 48 h post-injury, respectively (Beirowski et al., 2010). Even though swellings result from both axonal transection and TAI, there are key spatiotemporal differences. The centrifugal progression and delayed appearance of axonal swellings during Wallerian degeneration sharply contrast with experimental TAI models, where swellings first appear in presumably nonaxotomized axons within 30-60 min (Erb and Povlishock, 1988; Povlishock et al., 1983; Singleton et al., 2002). These spatiotemporal differences may

be due to variations in the localization, degree, or duration of calpain activity. Concurrent or sequential non-calpain mechanisms may also contribute to these differences.

There are likely key mechanistic differences between Wallerian degeneration and TAI. Apoptotic effectors, in particular caspases, have been implicated in TAI (Büki and Povlishock, 2006; Büki et al., 2000; Chen et al., 2004; Stone et al., 2002) but they are unlikely to mediate degeneration of the transected axon (Finn et al., 2000; Schoenmann et al., 2010; Whitmore et al., 2003). Inhibition of the UPS protects transected neurites and axons *in vitro* and *in vivo* (Zhai et al., 2003) but accelerated progression to secondary axotomy after *in vitro* neurite stretch injury (Staal et al., 2009). The reasons behind these potentially important differences have been unexplored.

C. Disruption of the morphology and function of specialized axonal domains

C.1. Calpain substrates in the axolemma and subaxolemmal cytoskeleton—

Several transmembrane proteins, specifically Nav1.2 and the Na^+-Ca^{2+} exchanger-3 (NCX3; Fig. 1), are calpain substrates under various pathological conditions. Their proteolysis may further contribute to injury cascades (Iwata et al., 2004; Pannaccione et al., 2012). In addition to directly proteolyzing transmembrane proteins, calpains may cleave the underlying subaxolemmal cytoskeleton, thereby disrupting the localization and/or function of various transmembrane proteins.

Calpain proteolyzes the Nav1.2 α-subunit in calcium ionophore-treated cortical cultures, in an *in vitro* TBI model, and after controlled cortical impact in mice (Schoch et al., 2013; von Reyn et al., 2009). Nav1.2 α-subunit contains the tetrodotoxin (TTX) binding site, selectivity filter, channel pore, and structural elements responsible for activation and inactivation. Inactivation refers to the process by which an open voltage-gated Na^+ channel closes even in the presence of maintained depolarization. Calpain proteolysis occurs at two predicted sites: within the intracellular I-II loop and II-III loop (von Reyn et al., 2009). There is possibly a third cleavage site but this is uncertain. As demonstrated *in vitro*, the channel fragments may remain in the plasma membrane but the functionality of the remaining cleaved channel is not known (von Reyn et al., 2009). However, it has been proposed that proteolysis of the α-subunit prevents Nav1.2 inactivation, resulting in Na^+ influx and indirectly to persistent Ca^{2+} influx (Iwata et al., 2004). Particularly relevant to this review, stretching of isolated axons (MAP2-negative neurites) in cortical cultures results in proteolysis of the Nav1.2 α-subunit, which is blocked by generalized protease inhibition (Iwata et al., 2004). While recognizing the limitations of protein size estimation by western blot, the sizes of Nav1.2 α-subunit fragments generated by calpain activity (von Reyn et al., 2009) appear to differ from those resulting from isolated axonal stretch, suggesting that calpains may not solely be responsible for the proteolysis of the α-subunit in this model. Nonetheless, a proposed feed-forward pathway linking axonal stretch to sustained elevations in intra-axonal [Ca^{2+}] via proteolysis of the Nav1.2 α-subunit (Iwata et al., 2004), is intriguing.

In normal physiology, plasmalemmal NCX, of which there are three known isoforms (NCX1, NCX2, and NCX3), couples Na^+ influx to Ca^{2+} extrusion (forward mode). NCX1 immunoreactivity is detectable in myelinated axons in the sciatic nerve, optic nerve, and spinal cord, but not in the corpus callosum (Steffensen et al., 1997). All three isoforms are detectable in a small subset of axons and terminals in the rat hippocampus and cortex (Minelli et al., 2007). Of the three, NCX3 is a calpain substrate (Bano et al., 2005). Calpain proteolysis of NCX3 after focal brain ischemia and in glutamate-treated cerebellar cultures (Bano et al., 2005) or AMPA (α-amino-3-hydroxy-5-methylisoxazole-4-propionate) receptor-stimulated hippocampal neuronal cultures (Araújo et al., 2007) results in 58-60 kDa

fragments. After brain ischemia, NCX1 is proteolyzed by an unidentified protease, but NCX2 is not proteolyzed (Bano et al., 2005).

The function of the calpain-proteolyzed NCX3 is controversial. Early data suggests that the NCX3 is inactivated by calpains, and that its inactivation contributes to Ca^{2+} dysregulation and neuronal death (Bano et al., 2005). However, using patch-clamp technique in whole-cell configuration, Pannaccione et al. (2012) demonstrated a strong causal association between calpain proteolysis of NCX3 and increased NCX currents in the reverse mode of operation (couples Ca^{2+} influx to Na^{+} extrusion), suggesting that cleavage generates a hyperfunctional form of the antiporter. Axoplasmic [Na+] are acutely elevated after injury, driving the NCX to work in reverse mode (see section VI), so a hyperfunctional NCX3 may further exacerbate the axoplasmic [Ca^{2+}] rise.

Of the spectrin subunits, αII, βII, and βIV-spectrin are calpain substrates (Fig. 1; Glantz et al., 2007; Harris and Morrow, 1990; Roberts-Lewis et al., 1994; Schafer et al., 2009). Commonly used as a sensitive and specific way to assay for calpain activity (Czogalla and Sikorski, 2005; Roberts-Lewis et al., 1994), αII-spectrin proteolysis is observed after nerve transection (Glass et al., 2002), TAI (Ma et al., 2012a; Saatman et al., 2003), and anoxic insult to nerves (Jiang and Stys, 2000). Two commonly used antibodies (i.e., Ab37 and Ab38) specifically recognize the calpain cleaved form of αII-spectrin by binding to the newly formed carboxyl-terminus of the ~150-kDa amino-terminal fragment (Roberts-Lewis et al., 1994). The preferred site of calpain proteolysis is between residues Tyr^{1176} and Gly^{1177}, immediately adjacent to the calmodulin-binding domain of αII-spectrin (Glantz et al., 2007). Proteolyzed αII-spectrin significantly retains its actin binding and crosslinking activity *in vitro*, but these activities are disrupted in the presence of calmodulin (Harris and Morrow, 1990). Bovine βII-spectrin is proteolyzed by calpains into 165- and 125-kDa fragments in the presence of calmodulin. Concurrent proteolysis of both αII- and βII-spectrin causes fragmentation of the spectrin heterodimer and permanently impairs spectrin binding to actin regardless of calmodulin. Furthermore, calpain digest of αII/βII-spectrin inhibits its *in vitro* association with ankyrin-independent membrane binding sites (Hu and Bennett, 1991). Additional calpain cleavage sites in αII- and βII-spectrin have been identified (Glantz et al., 2007). For βIV-spectrin, calpain proteolysis yields a ~45 kDa fragment (Schafer et al., 2009). Together with cleavage of ankG (reportedly by calpains to generate ~72 and 95 kDa fragments), βIV-spectrin proteolysis likely disrupts AIS morphology. In addition, spectrin proteolysis may contribute to increased axolemmal permeability, thereby contributing to further Ca^{2+} influx in a feed-forward spiral (Czeiter et al., 2009).

Whether or not actin is a calpain substrate is controversial. Even though some investigators did not observe actin proteolysis by calpains (Goll et al., 1991; Siman and Noszek, 1988), there is evidence suggesting that actin may be a calpain substrate (Fig. 1). *In vitro* digest of purified actin isolated from bovine lens (43 kDA; isoform not specified) with m-calpain generates ~40 and 42 kDa fragments (Yoshida et al., 1984). Constitutive apoptosis in human neutrophils results in proteolysis of β-actin between residues Val^{43} and Met^{44}, resulting in a ~38 kDa carboxyl-terminal fragment; proteolysis is inhibited by ALLN application (Brown et al., 1997). In cultured apoptotic neurons, generation of ~40 and 41 kDa actin fragments is reduced by ALLN or ALLM (Villa et al., 1998). Amino-terminal sequencing of the ~40 kDa carboxyl-terminal fragment determined that proteolysis occurs after the Arg residue corresponding to residue 37 in β-actin (Villa et al., 1998) and γ–actin (based on the sequence in Khaitlina (2001)). Directly microsequencing the ~41 kDa fragment from its amino-terminal end is not possible, but by using Lys-C digestion, high-performance liquid chromatography, and sequencing, the investigators confirmed that it is an actin fragment; the 2 peptides sequenced are common to α, β, and γ–actin. All 6 actin isoforms contain the two

proteolytic sites (based on the sequences in Khaitlina (2001)). The last two studies suggest but do not definitively establish that calpains directly cleave actin. Apart from their broad specificity (Goll et al., 2003), ALLM and ALLN reduce apoptosis (Brown et al., 1997; Villa et al., 1998); multiple pathologic cascades may be active in a dying cell, one of which may be a yet unidentified protease[1] that directly cleaves actin. The consequence of actin proteolysis is not known. The deoxyribonuclease I (DNase I) binding loop (residues 39-51) may be important for polymerization and stability of F-actin (Khaitlina and Strzelecka-Golaszewska, 2002). Although its relevance to the axon is unclear, disruption of this binding site has been proposed to interfere with actin binding to, and inhibition of, DNase 1, which degrades deoxyribonucleic acid (DNA) during apoptosis (Villa et al., 1998). It is not known whether actin acutely undergoes proteolysis in primarily axonal injury models, or whether actin proteolysis contributes to the disruption of the subaxolemmal cytoskeleton.

Of the calpain substrates in the axolemma (Nav1.2 and NCX) and subaxolemmal cytoskeleton (actin, spectrin, and ankyrin), there is reasonably strong evidence for calpain proteolysis only of αII-spectrin (Ma et al., 2012a; Saatman et al., 2003), βIV-spectrin (Schafer et al., 2009), and Nav1.2 (Schoch et al., 2013) in axons in mature animals. It is challenging to isolate the effects of intra-axonal calpains from calpain activity in the neuronal soma and dendrites. This task is made easier by the availability of antibodies for immunohistochemistry that selectively recognize the calpain-cleaved fragment (αII-spectrin; Roberts-Lewis et al., 1994), or the almost exclusive localization to axons of proteins (isoforms or splice variants) such as βIV-spectrin and Nav.1.2 (Berghs et al., 2000; Komada and Soriano, 2002; Westenbroek et al., 1989). An important next step is to identify the functional consequences of calpain proteolysis of these substrates within the axon.

C.2. Evidence of calpain involvement in the disruption of specialized domains—As calpain substrates include the subaxolemmal cytoskeleton and transmembrane proteins, pathologic calpain activity can have far-ranging effects. I will now discuss the evidence supporting intra-axonal calpains as key mediators of the morphological disruption of specialized domains—AIS, node, and terminal (Table 3; Fig. 2). In addition, their contribution to axonal and synaptic dysfunction will be examined.

The AIS consists of a unique cytoskeleton comprised of βIV-spectrin and ankG (Schafer et al., 2009), which are required for Na^+ channel clustering (Komada and Soriano, 2002; Zhou et al., 1998). Six h after middle cerebral artery occlusion (MCAO), the number of cortical neurons with intact immunostaining for Na^+ channel, ankG, and βIV-spectrin at the AIS is significantly reduced (Schafer et al., 2009). Calpain proteolysis of βIV-spectrin and ankG occurs early (3 h post-occlusion), and this has been proposed to disrupt AIS morphology. Treatment with MDL-28170 preserves the βIV-spectrin staining at the AIS 24 h post-MCAO. Interestingly, for unclear reasons, MCAO does not disrupt the organization of nodes, which share a similar molecular composition to the AIS (Schafer et al., 2009). In primary cortical neurons exposed to oxygen glucose deprivation (OGD), MDL-28170 and calpastatin overexpression preserve βIV-spectrin immunostaining of the AIS (Schafer et al., 2009). Photolytic uncaging of Ca^{2+} at the AIS in live neurons was performed *in vitro* using a cell permeant acetoxymethyl ester derivative of the caged compound *o*-nitrophenyl EGTA. This results in a decrease in immunofluorescence intensity of the CAM neurofascin-186 at the AIS, implicating local calpain activity. The evidence in total suggests that calpains localized to the AIS are responsible for AIS disassembly.

[1]The caspase inhibitor Z-VAD-FMK (carbobenzoxy-Val-Ala-Asp-fluoromethyl ketone; 100 μm) inhibits nuclear condensation, but not actin proteolysis, in human neutrophil cultures undergoing constitutive apoptosis (Brown et al., 1997).

Using a model of anti-GD1a antibody directed complement activation, the role of calpains in the disruption of nodal morphology and function was examined (McGonigal et al., 2010). The ganglioside GD1a is present in the node and motor nerve terminal, sites that correspond to those predicted to be affected in motor axonal forms of Gullain-Barré syndrome. In this model, GD1a is enriched in axons through using the GD3 synthase knockout mice; their axons are particularly vulnerable to anti-GD1a antibody mediated complement activation. A consequence of complement activation is the formation of bi-directional non-specific ion and water pores (membrane attack complex pores) on the plasma membrane. In intramuscular axons in *ex vivo* triangularis sterni muscle preparations treated with anti-GD1a antibody and complement, immunoreactivity for Nav1.6, ankG, and Caspr is decreased at nodes and paranodes, and this loss is blunted by AK295. However, AK295 does not ameliorate the loss of perineural currents at the node and motor nerve terminal, which indicates an inability to generate Na^+ and K^+ currents. The same laboratory also measured compound action potentials (CAPs) in experimentally desheathed phrenic nerve trunks exposed to anti-GD1a antibody and complement (McGonigal et al., 2010). Despite modest protection of nodal and paranodal morphology, injury-induced decrement of CAP amplitude is not reduced by AK295. CAP amplitude correlates with number of axons generating action potentials. The failure of the axolemma to maintain ionic homeostasis, when punctured by membrane attack complexes, was suggested to be the critical factor in mediating axonal conduction block (McGonigal et al., 2010).

Similarly, pharmacologic calpain inhibitors do not provide electrophysiological protection (CAP) to optic nerves exposed to *ex vivo* anoxia-reoxygenation or OGD (Jiang and Stys, 2000; Stys and Jiang, 2002). As removing extracellular Ca^{2+} is completely protective of CAP, other Ca^{2+}–dependent pathways, such as phospholipases, phosphatases, or kinases, were suggested as potential factors in mediating electrophysiological deterioration. The disruption of action potential propagation may be due to multiple interdependent axonal factors, e.g., loss or mislocalization of nodal proteins, dysregulated activity of ion channels, and perturbation of ionic homeostasis, as well as extra-axonal factors. Inhibiting intra-axonal calpains may be inadequate for preserving such a complex process as action potential propagation but is likely a necessary component of any effective therapeutic strategy to maintain nodal function after injury.

In addition to contributing to nodal disassembly, calpains have been causally linked to the degeneration of the axon terminal in *ex vivo* and *in vivo* injury models. Extensor digitorum longus (EDL) muscle preparations from transgenic mice whose motor neurons express yellow fluorescent protein (YFP) were exposed to simulated ischemia/reperfusion (Talbot et al., 2012). Pre-treatment with calpain inhibitor VI [N-(4-fluorophenylsulphonyl)-L-valyl-L-leucinal] preserves neuromuscular junction (NMJ) innervation, assessed by YFP fluorescence. Morphological, including ultrastructural, protection of NMJs by calpain inhibitors was demonstrated in several additional injury models (O'Hanlon et al., 2003). NMJs in hemidiaphragm preparations were treated with anti-GQ1b ganglioside antibodies and complement, or the spider neurotoxin, α-latrotoxin—both form non-selective pores within the target membrane. Both calpeptin (carbobenzyloxy-Leu-normleucinal) and calpain inhibitor V (Mu-Val-HPh-CH_2F) reduce the loss of NFH immunostaining and "cytoskeletal bundles" in the motor nerve terminal. The *in vivo* role of calpains in the NMJ was characterized using sciatic nerve transection in transgenic mice overexpressing calpastatin (Ma et al., 2013). Degeneration of the presynaptic terminal and synaptic dysfunction are observed early after nerve transection. Calpastatin overexpression preserves the morphology of preterminal axons and terminals (neurofilament and synaptic vesicle immunostaining) (Ma et al., 2013). However, there is no difference in compound muscle action potential (CMAP) amplitudes after nerve transection between wild-type and transgenic animals. As early impairment of CMAP likely represents synaptic dysfunction rather than failure of

action potential conduction, CMAP amplitudes are expected to correlate with number of functioning synapses. In all of these injury models, calpains mediate key aspects of synaptic terminal degeneration; the cytoskeleton seems particularly vulnerable. Like nodes, the electrophysiologic function of synaptic terminals cannot be protected solely by calpain inhibition.

In summary, calpains are causally linked to the morphological degeneration of the AIS, nodes, and synaptic terminals. However, no study to date has demonstrated any improvement in electrophysiological outcome measures specifically of injured axons using calpain inhibition. In contrast, improvement of electrophysiological outcome measures (CAP amplitude and latency, tail sensory nerve action potential amplitude, long-term potentiation) with pharmacologic calpain inhibitors has been reported in animal models of TBI, taxol-induced sensory neuropathy, and Alzheimer's disease (Ai et al., 2007; Trinchese et al., 2008; Wang et al., 2004). Calpain activity in the neuronal somata, dendrites, glial cells, or other structures may be one of many important pathologic mechanisms. Given the complexity of these injury models, the systemic administration of calpain inhibitors, and the lack of improvement in axonal-specific electrophysiological outcomes by calpains, it is unlikely that the electrophysiological protection seen in these animal models can be attributed solely to inhibition of intra-axonal calpains. Calpain inhibition is probably a component of any therapeutic strategy that fully protects axonal and synaptic function after injury.

VI. Mechanisms of Ca^{2+} entry into axoplasm

As calpains require elevated [Ca^{2+}] for activity, the sources and mechanisms of Ca^{2+} entry into the axoplasm are particularly relevant to this review (Fig. 4). Most studies implicate the source of elevated [Ca^{2+}] to be extracellular, while others point to intracellular Ca^{2+} stores. Because of the challenges of manipulating and measuring [Ca^{2+}] *in vivo*, most of the studies have been performed *in vitro* or *ex vivo*, which limits generalizability to a living adult animal. In the following, I will discuss the routes of Ca^{2+} entry into the axoplasm in several injury models: neurite/axon transection, neurite/axon stretch, *in vitro* and *in vivo* TBI, and ischemia/anoxia. Studies that directly measure cation concentrations will be emphasized here.

As a whole, neurite/axon transection studies provide limited information about the sources of axoplasmic Ca^{2+}. In one study, axoplasmic [Ca^{2+}] was measured in pharmacologically treated-transected axons in the time frame coinciding with AAD, and not Wallerian degeneration (Knöferle et al., 2010). Topical application of a cocktail of amiloride (T-type Cav blocker), amlodipine (L-/N-type Cav blocker), and NBQX (1,2,3,4-tetrahydro-6-nitro-2,3-dioxo-benzo[f]quinoxaline-7-sulfonamide disodium salt hydrate; AMPA receptor blocker) prevents *in vivo* AAD after axon transection in the optic nerve (Knöferle et al., 2010). Without these antagonists, axoplasmic [Ca^{2+}] rapidly rises within 30 sec, although normal resting [Ca^{2+}] is quickly restored over the next 60 sec. The authors suggested that the pharmacologic inhibitors also blunt the early rise in [Ca^{2+}] (Knöferle et al., 2010) but results of any statistical analysis were not provided. Manipulating Ca^{2+} influx while directly measuring axoplasmic [Ca^{2+}] during Wallerian degeneration has not been reported. George et al. (1995) assessed neurite degeneration, which is dependent on elevated intra-axonal [Ca^{2+}]. Chelating extracellular Ca^{2+} or applying L-type Cav channel inhibitors reduces degeneration of transected DRG neurites, assessed 4 days post-injury. Pharmacological inhibition of N-type Ca^{2+} channels or TTX-sensitive Na^{+} channels is ineffective. Given the caveat that TTX-resistant Na^{+} channels are present on 10-15% of immature DRG cell bodies (Kostyuk et al., 1981), it is not clear if extracellular Na^{+} significantly contributes to axoplasmic [Ca^{2+}] elevation. Extracellular Ca^{2+} influx, likely through L-type channels,

appears to be a major contributor to the axoplasmic [Ca^{2+}] elevation in Wallerian degeneration and possibly AAD.

Studies using severe axonal stretch models strongly implicate extracellular Ca^{2+} and Na^{+} in the elevation of axoplasmic [Ca^{2+}]. In a model where neurites from N-Tera2cl/D2 neurons are stretched 75% beyond their initial lengths, an immediate rise in intra-axonal [Ca^{2+}] is observed but can be reduced if extracellular Ca^{2+} or Na^{+} is removed or if neurons are pretreated with inhibitors to Nav channels (TTX), P/Q- and N-type Cav channels (ω–conotoxin MVIIC), or NCX (bepridil) (Wolf et al., 2001). In another study, stretching axons of rat primary cortical neurons to 70-75% beyond their initial lengths results in an immediate rise in axoplasmic [Ca^{2+}], followed by a decrease toward resting [Ca^{2+}] at 2 min post-stretch, and a subsequent secondary rise in [Ca^{2+}] over the next 60 min (Iwata et al., 2004). Preinjury treatment with TTX completely abolishes the Ca^{2+} elevation, measured at 2, 20, and 60 min post-injury, whereas treatment starting at 5 min post-injury reduces the secondary [Ca^{2+}] rise only at 60 min compared with injured nontreated cultures. Pre- or post-injury treatment with a protease inhibitor cocktail, which inhibits serine and cysteine proteases, metalloproteases, trypsin, and others, only reduces the 60 min [Ca^{2+}] elevation. Based on these results, Iwata et al. (2004) proposed a model by which Na^{+} entry through mechanically sensitive Na^{+} channels triggers an increase in intra-axonal Ca^{2+} via opening of Cav channels and reversal of the NCX. Furthermore, the authors suggested that proteolysis of the Nav1.2 α-subunit results in loss of channel inactivation and additional Na^{+} influx, which further drives Ca^{2+} influx. These two studies together implicate axolemmal ion channels and exchangers, specifically TTX-sensitive Na^{+} channel, Cav channel, and NCX, as the predominant mechanisms underlying axoplasmic [Ca^{2+}] elevation.

In contrast, the increase in axoplasmic [Ca^{2+}] following mild neurite stretch injury originates principally from intracellular stores (Staal et al., 2010). The investigators deformed neurite bundles from rat cortical neurons using a fluid pulse, generating a transient 1-6% increase in original neurite length. Stretch injury induces a biphasic increase in intra-axonal [Ca^{2+}], with the larger, initial peak occurring within 1-3 sec of injury. The immediate rise in intra-axonal [Ca^{2+}] after stretch is not altered when Ca^{2+} is removed from the bath solution, suggesting that the early [Ca^{2+}] rise is due to Ca^{2+} release from intracellular stores (Staal et al., 2010); however, the relevant intracellular compartments have not been identified. The smaller and slightly delayed peak is reduced in Ca^{2+}-free media, suggesting that part of the [Ca^{2+}] elevation in this injury model may be dependent on Ca^{2+} influx. Axoplasmic [Ca^{2+}] elevation is primarily due to Ca^{2+} release from intracellular stores after mild axonal stretch injury, and influx of extracellular Ca^{2+} and Na^{+} after more severe axonal stretch.

In vitro and *in vivo* TBI models have been utilized to address the phenomenon of mechanoporation, or the mechanically induced creation of physical defects in the plasma membrane. Mechanoporation of the axolemma will be the focus here. For this section and Fig. 4, the *in vitro* TBI model, where the entire neuron is exposed to mechanical strain, is distinguished from *in vitro* models where strain is isolated to neurites. The [Ca^{2+}] rise in neurites and influx of extracellularly applied membrane-impermeable 480 Da Lucifer yellow ammonium salt within minutes of applying fluid shear stress to chick forebrain neurons is reduced by post-injury application of poloxamer 188, whose mechanism of action is thought to be sealing damaged cell membranes (Kilinc et al., 2009, 2008). In this model, the entire neuron, as evidence by increased calpain activity in the soma and neurites (Kilinc et al., 2009), is injured so the source of axoplasmic Ca^{2+} and 480 Da tracer could partially be due to disruption of the plasmalemma surrounding the soma, as well as axolemmal mechanoporation. In two *in vitro* models, where mechanical strain is isolated to neurites, axolemmal permeability to a 570 Da fluorescent tracer (Alexa 488 hydrazide) is not

observed early after injury (<1 h; Smith et al., 1999; Staal et al., 2007). The reason for this discrepancy is unclear.

Further support for the existence of axolemmal mechanoporation comes from *in vivo* TBI studies. First, ultrastructural evidence of mechanoporation, using electron microscopy, is observed after lateral acceleration of the head in non-human primates (Maxwell et al., 1993). Second, axolemmal permeability to the ~44 kDa HRP after TBI has been reported (Pettus et al., 1994). After infusing HRP in the intrathecal space, moderate fluid percussion injury (2.0-2.8 atm) results in axolemmal permeability to HRP as early as 5 min after injury. It is intriguing that treatment with MDL-28170 prior to impact acceleration head injury results in decreased permeability to HRP 2 h post-injury (Table 2; Czeiter et al., 2009). Post-injury calpain activity, possibly via spectrin proteolysis, may further worsen axolemmal permeability in a feed forward fashion (Czeiter et al., 2009).

Mechanisms of Ca^{2+} and Na^{+} entry into the axoplasm have been more thoroughly studied in anoxic and ischemic axons[2] than in other injury models. The interested reader is referred to more comprehensive reviews (Stys, 2005, 2004). During ischemia, axoplasmic Ca^{2+} rises from a presumed baseline of ~100 nM to the tens of micromolar (Stys, 2005). In *ex vivo* nerves exposed to simulated ischemia, elevation of axoplasmic [Ca^{2+}] predominantly results from influx of extracellular Ca^{2+} with a smaller contribution from internal stores (Nikolaeva et al., 2005). Recently, glutamate receptors have been implicated (Ouardouz et al., 2009a, 2009b).

Ca^{2+} influx from the extracellular environment predominantly occurs via reversal of the NCX, driven by axonal Na^{+} accumulation through TTX-sensitive Na^{+} channels, in anoxic optic nerves (Stys and Lopachin, 1998) and chemically ischemic dorsal roots (Petrescu et al., 2007). Depolarization also stimulates the NCX to operate in reverse mode (Stys, 2004). These two studies show no or inconsistent effects on axoplasmic [Ca^{2+}] or [Ca][3] with L-type Cav channel inhibitors (Petrescu et al., 2007; Stys and Lopachin, 1998). Another study provides some support for a role for L-type Cav channels in Ca^{2+} influx in anoxic optic nerves by demonstrating that the inhibitor diltiazem ameliorates the anoxia-induced decrease in extracellular [Ca^{2+}] (Brown et al., 2001). Axoplasmic Ca^{2+} was not directly measured, however. Besides extracellular Ca^{2+} influx, Ca^{2+} release from internal stores is also important. When optic nerves are bathed in Ca^{2+}-free artificial cerebrospinal fluid, the Ca^{2+} contribution from internal stores during simulated ischemia originates from the endoplasmic reticulum and mitochondria, and its release is dependent on influx of extracellular Na^{+} (Nikolaeva et al., 2005). Even in the absence of axonal depolarization, Na^{+} accumulation may drive the mitochondrial NCX to export Ca^{2+} into the axoplasm, positively modulate ryanodine receptors, and promote IP_3 generation by phospholipase C. The studies together support that in anoxic and ischemic injury, axoplasmic [Ca^{2+}] elevation is predominantly due to TTX-sensitive Na^{+} channels and plasmalemmal NCX (operating in reverse mode), with a smaller contribution from internal Ca^{2+} stores.

The importance of excitotoxicity during anoxic and ischemic injury has recently gained more momentum. Glutamate, the main excitatory neurotransmitter in the mammalian CNS (Stys, 2005), may directly contribute to the rise of axoplasmic [Ca^{2+}]. During anoxic injury to dorsal column slices, glutamate is released from the axoplasm and oligodendrocytes likely via reversal of Na^{+}-dependent glutamate transport (Li et al., 1999). Glutamate is also

[2]These *ex vivo* studies often use white matter tissue taken acutely from the adult animal. In contrast, for example, axonal stretch studies typically use neuronal cultures. When comparing these investigations, it is important to recognize that the presence and density of channels and receptors may be developmentally regulated (Brown et al., 2001), or differ between white matter regions in the same animal (Tekkök et al., 2007).

[3]Electron probe X-ray microanalysis measures total (free plus bound) concentrations of elements (Stys and Lopachin, 1998).

released from optic nerves undergoing OGD (Tekkök et al., 2007). Overactivation of kainite and AMPA glutamate receptors, which are present on glia, is believed to exacerbate white matter injury by directly disrupting glia (Ouardouz et al., 2006; Stys, 2005). However, these receptors have recently been detected on myelinated axons in dorsal columns (Ouardouz et al., 2009a, 2009b). Even though the specific mechanisms of Ca^{2+} entry into the axoplasm vary depending on subunit composition of the glutamate receptors, they may include Ca^{2+} influx through the glutamate receptor, downstream activation of L-type Ca^{2+} channels (via influx of Na^{+} and localized depolarization), and release from IP_3-and ryanodine-dependent intracellular stores. The specific contribution of glutamate receptors *localized on axons* to axoplasmic [Ca^{+}] elevation and axonal pathology from anoxia and ischemia still remains to be characterized.

Neuromuscular junctions have also been studied during simulated ischemia. Motor nerve terminals are more sensitive to ischemia than axons and muscle fibers (Mäkitie and Teräväinen, 1977; Tömböl et al., 2002). EDL preparations were harvested from transgenic mice expressing YFP in motor neurons and their axons (Talbot et al., 2012). Chelating bath Ca^{2+} or adding pharmacologic inhibitors for plasma membrane NCX, P/Q-type Cav channels, L-type Cav channels, or Na^{+},K^{+},$2Cl^{-}$ co-transporter reduces the degeneration of EDL terminals, as assessed by YFP fluorescence, 120 min post-OGD. Even though intra-terminal [Ca^{2+}] was not measured in the EDL preparation, elevated intracellular [Ca^{2+}] within levator auris longus NMJs during OGD was observed. Given that NMJ degeneration is likely associated with increased intracellular [Ca^{2+}], this study suggests that many of the mechanisms of Ca^{2+} entry into the axon terminal are shared with the axon fiber.

Taken together, these studies highlight the heterogeneity of mechanisms for [Ca^{2+}] elevation. The sources and mechanisms of Ca^{2+} entry into the axoplasm and terminal depend on the injury and model system. Directly preventing Ca^{2+} elevation in human diseases may require different and/or multiple therapeutic interventions. As Ca^{2+}-dependent non-calpain pathways may be important in Wallerian degeneration (Zhai et al., 2003), TAI (Staal et al., 2010), and ischemia (Stys, 2005), blunting the axoplasmic Ca^{2+} rise may confer additional benefits to calpain inhibition alone. Nonetheless, increased activity of axoplasmic calpains, which is downstream of elevated [Ca^{2+}], is deleterious across many injury paradigms and remains a therapeutic target for many human diseases.

VII. Concluding Remarks

There has been growing awareness of the contribution of axonal pathology to the morbidity and mortality of many human diseases, including CNS and PNS trauma, cerebral ischemia, neurodegenerative disorders, and peripheral neuropathies. Axonal dysfunction and degeneration in these seemingly disparate disorders likely share common molecular injury mechanisms, one of which is dysregulated intra-axonal calpain activity. This review has attempted to bring together research findings from various disciplines to facilitate a more unified understanding of the pathologic roles played by calpains in axons and to highlight gaps in our current knowledge. Significant progress has been made over the past decade, yet much work still remains. The identification of the intra-axonal calpain contribution to morphological, electrophysiological, and functional abnormalities, particularly *in vivo*, is lacking in many injury models. In particular, our understanding of the downstream consequences of calpain proteolysis of specific substrates in axons remains vague. As we move forward, elucidating these effects in axons may be critically important as the outcome of substrate proteolysis may depend on their subcellular localization. Despite these shortcomings, it is clear that inhibition of intra-axonal calpains is likely a necessary component of any effective therapeutic strategy for a wide spectrum of human diseases.

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ABBREVIATIONS

AAD	acute axonal degeneration
AIS	axon initial segment
AMPA	α-amino-3-hydroxy-5-methylisoxazole-4-propionate
ankG	ankyrinG
β-APP	β-amyloid precursor protein
CAM	cell adhesion molecule
CAP	compound action potential
Caspr	contactin-associated protein
Cav	voltage-gated Ca^{2+} channel
CMAP	compound muscle action potential
CNS	central nervous system
CSS1	common small subunit 1
CSS2	common small subunit 2
DIC	differential interference contrast
DNA	deoxyribonucleic acid
DNase I	deoxyribonuclease I
DRG	dorsal root ganglia
dSarm	sterile α/Armadillo/Toll-Interleukin receptor homology domain protein
EDL	extensor digitorum longus
EGTA	Ethylene glycol-bis(2-aminoethylether)-*N*,*N*,*N'*,*N'*-tetraacetic acid
GDC	granular disintegration of the axonal cytoskeleton
GFP	green fluorescent protein
HRP	horseradish peroxidase
IA	intra-arterial
IP	intraperitoneal
IV	intravenous
Kv	voltage-gated K^+ channel
MAP	microtubule-associated protein
MCAO	middle cerebral artery occlusion

Nav	voltage-gated Na^+ channel
NCX	Na^+-Ca^{2+} exchanger
NFH	neurofilament heavy
NFL	neurofilament light
NFM	neurofilament medium
Nmat-1	nicotinamide mononucleotide adenyl transferase 1
NMJ	neuromuscular junction
NrCAM	neuronal cell adhesion molecule
OGD	oxygen glucose deprivation
PNS	peripheral nervous system
SCG	sympathetic superior ganglia
SCI	spinal cord injury
TAI	traumatic axonal injury
TBI	traumatic brain injury
TTX	tetrodotoxin
Ube4b	ubiquitination factor E4B
UPS	ubiquitin-proteasome system
YFP	yellow fluorescent protein
ZNRF1	zinc and ring finger 1

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HIGHLIGHTS

1. Axonal injury and degeneration significantly contribute to outcome in many diseases.
2. Transection, stretch, ischemia, and complement activate intra-axonal calpains.
3. Calpain activity disrupts axonal transport, cytoskeleton, and specialized domains.
4. Calpain substrates include the Na^+ channel, Na^+-Ca^{2+} exchanger, and neurofilament.
5. Inhibition of intra-axonal calpains is likely beneficial for many human diseases.

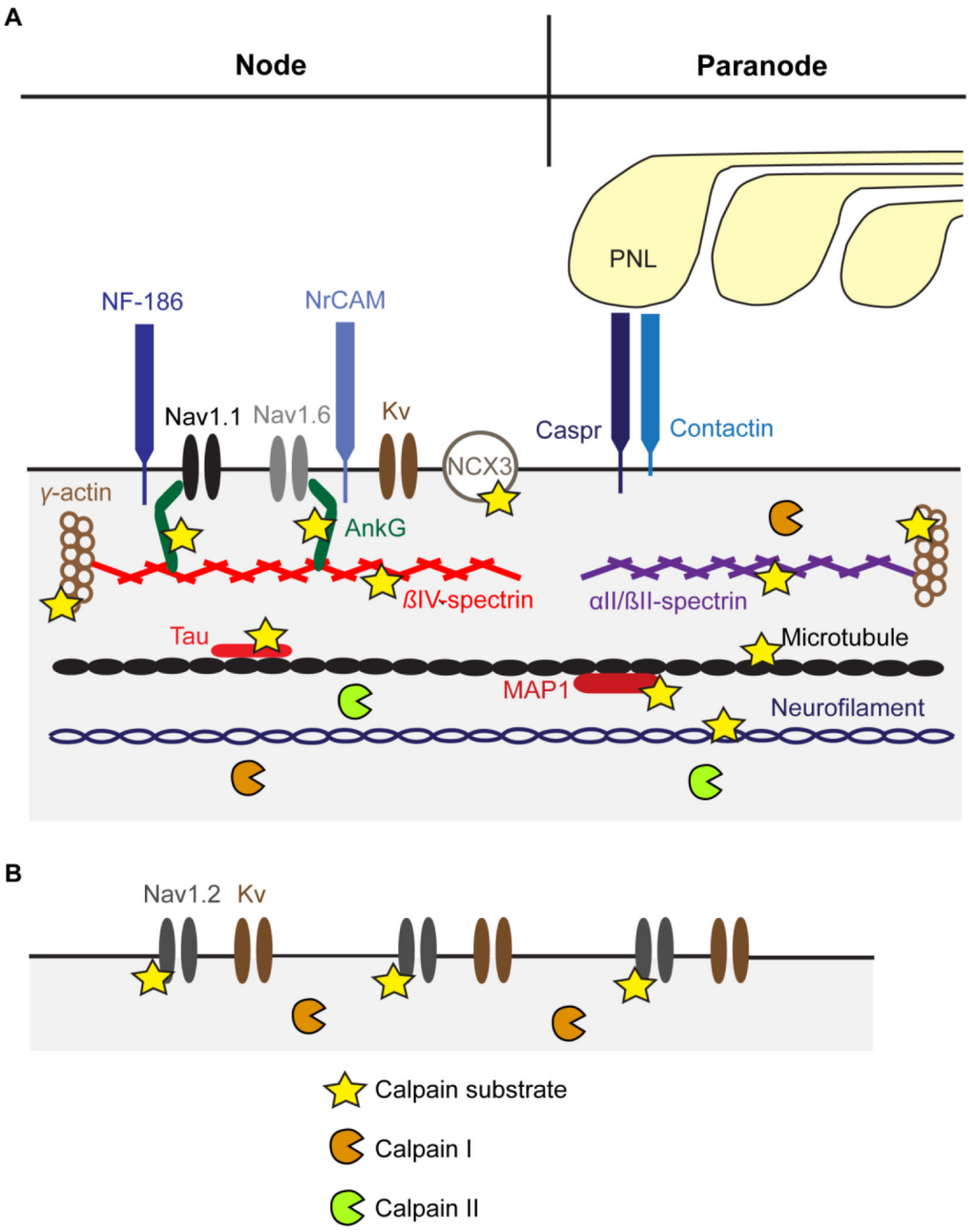

Fig. 1.

Selected calpain substrates and localization of calpain I and II in axons. Schematic of a representative myelinated (A) and unmyelinated axon (B). The nodal and paranodal regions of the myelinated axons are shown. The axon initial segment, which also contains Nav1.2, shares a similar molecular composition to the node. Calpain proteolysis of substrates in the axolemma and cytoskeleton (subaxolemmal and central) may contribute to the structural and functional disruption seen in injured axons. Whether or not calpain II is present in unmyelinated axons has not been reported. Represented locations of proteins are not exhaustive nor do they necessarily reflect their distribution in axons. AnkG, ankyrinG; Caspr, contactin-associated protein; Kv, voltage-gated K^+ channel; MAP1, microtubule-

associated protein 1; Nav, voltage-gated Na^+ channel; NCX3, Na^+-Ca^{2+} exchanger-3; neurofascin-186, NF-186; NrCAM, neuronal cell adhesion molecule; PNL, paranodal cytoplasmic loop

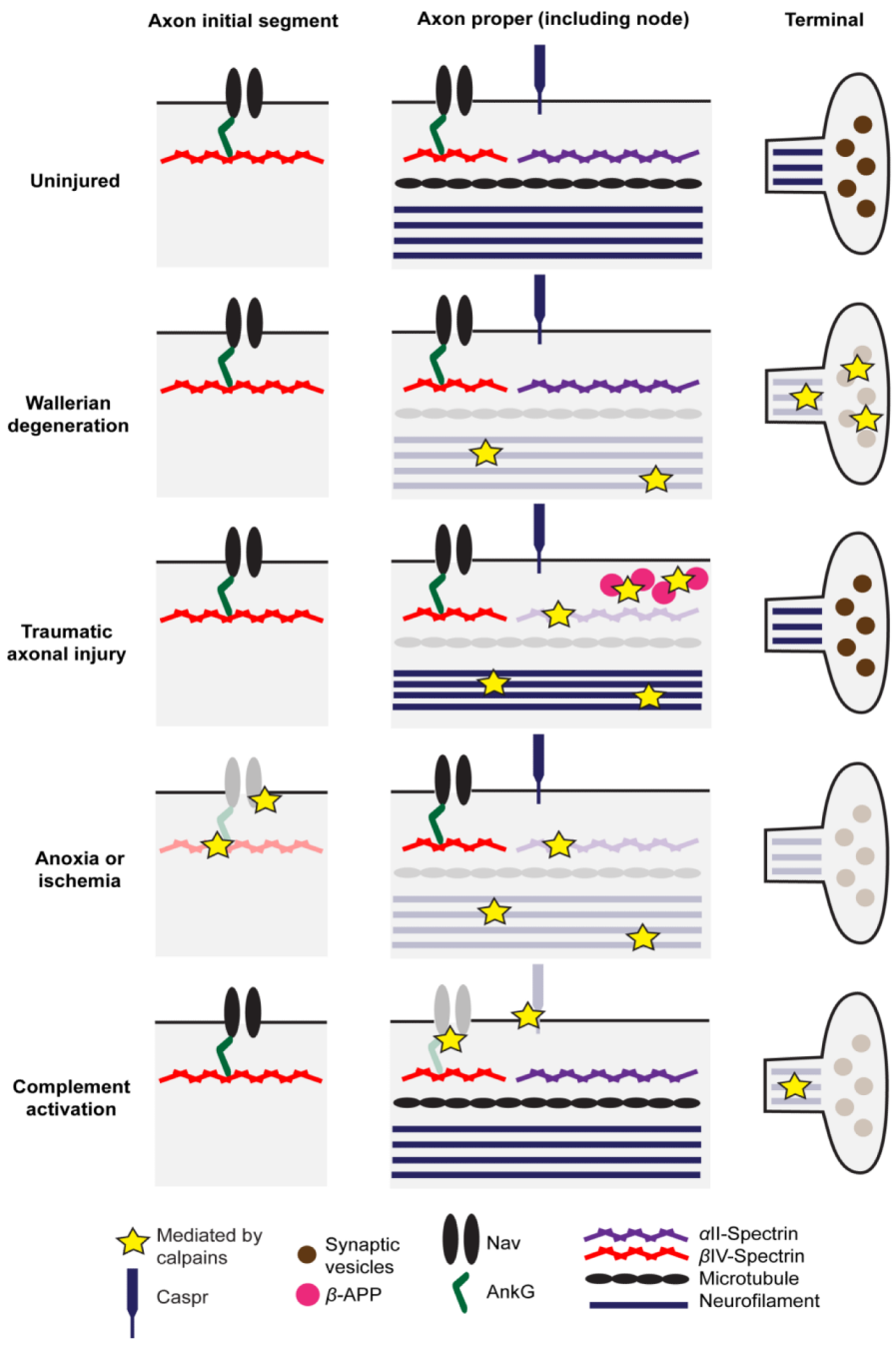

Fig. 2.

Axonal and synaptic pathology mediated by calpains in various injury models. In Wallerian degeneration, there is loss of microtubules and neurofilament in the axon proper, and loss of pre-terminal neurofilaments and synaptic vesicles. Prominent pathology after traumatic axonal injury includes *α*II-spectrin proteolysis, *β*-APP accumulation, microtubule loss, and neurofilament compaction. Based on these studies (Jafari et al., 1997, 1998; Maxwell and Graham, 1997), the number of neurofilaments early after axonal stretch injury appears to be extremely variable. An anoxic or ischemic insult is followed by AIS disassembly, *α*II-spectrin proteolysis, microtubule loss (Waxman et al., 1992), and neurofilament breakdown. There is also loss of preterminal neurofilaments and synaptic vesicles (Baxter et al., 2008;

Tömböl et al., 2002). Finally, complement activation results in nodal and synaptic disruption. Calpains contribute to many, but not all, of these abnormalities. Color fading represents loss or proteolysis of the protein. Note that not all of the above proteins nor the mechanisms underlying the various abnormalities have been studied in each injury model. AnkG, ankyrinG; β-APP, β-amyloid precursor protein; Caspr, contactin-associated protein; Nav, voltage-gated Na^+ channel

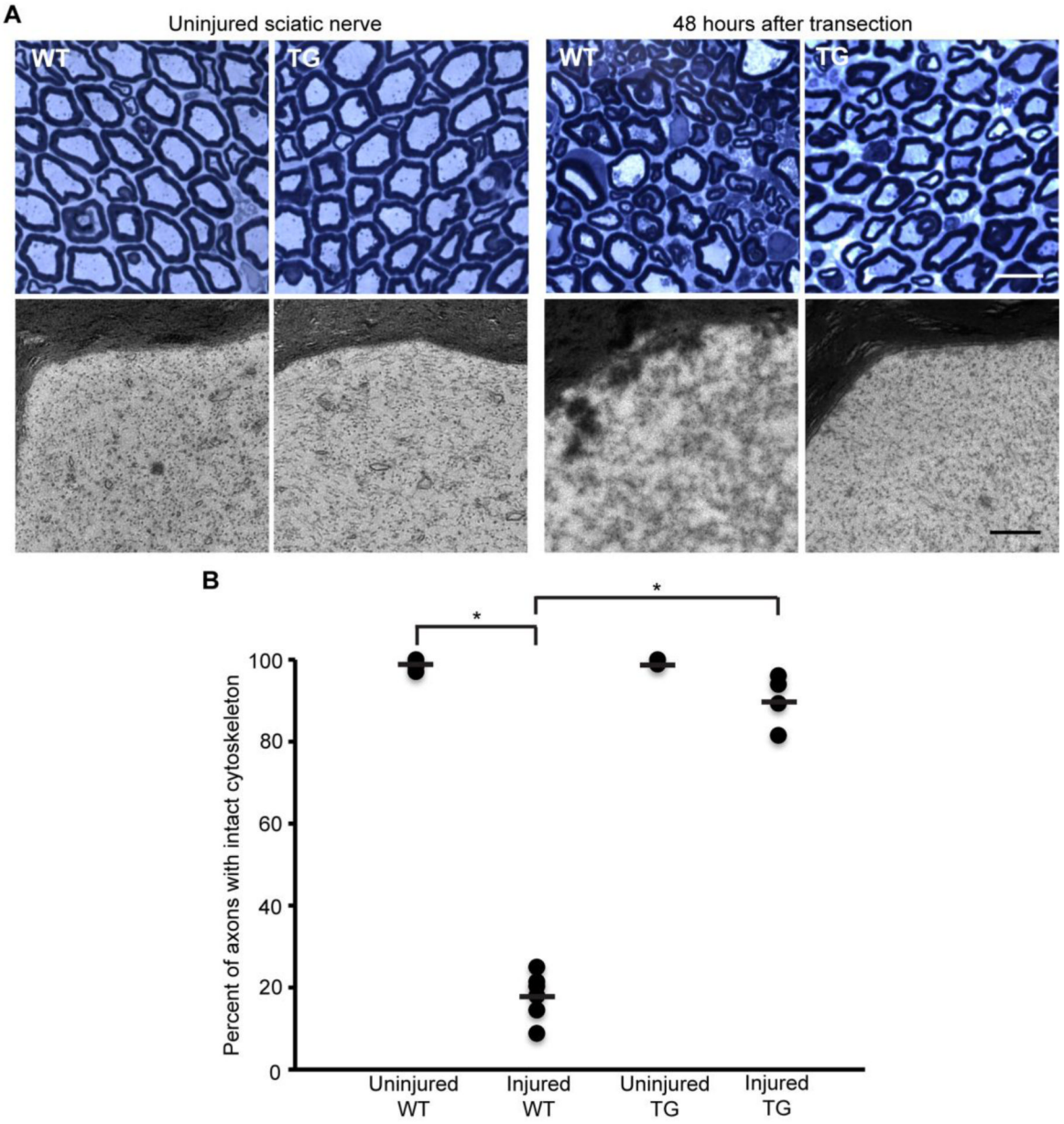

Fig. 3.

Axonal cytoskeleton 48 h after sciatic nerve transection in wild-type and calpastatin-overexpressing transgenic mice. (A) Representative light and electron microscopic images of sciatic nerves from wild-type (WT) and transgenic (TG) mice. Scale bar=10 μm (light microscopy), 0.5 μm (electron microscopy). (B) Percentage of axons scored with intact cytoskeleton 48 h after sciatic nerve transection (n=7 WT and 4 TG). Each circle represents an individual nerve, while horizontal bars represent means. *p<0.0001. Protection of the axonal cytoskeleton in sciatic nerves of TG mice is nearly complete at 48 h post-transection. Reprinted from Ma et al. (2013), with permission from Elsevier.

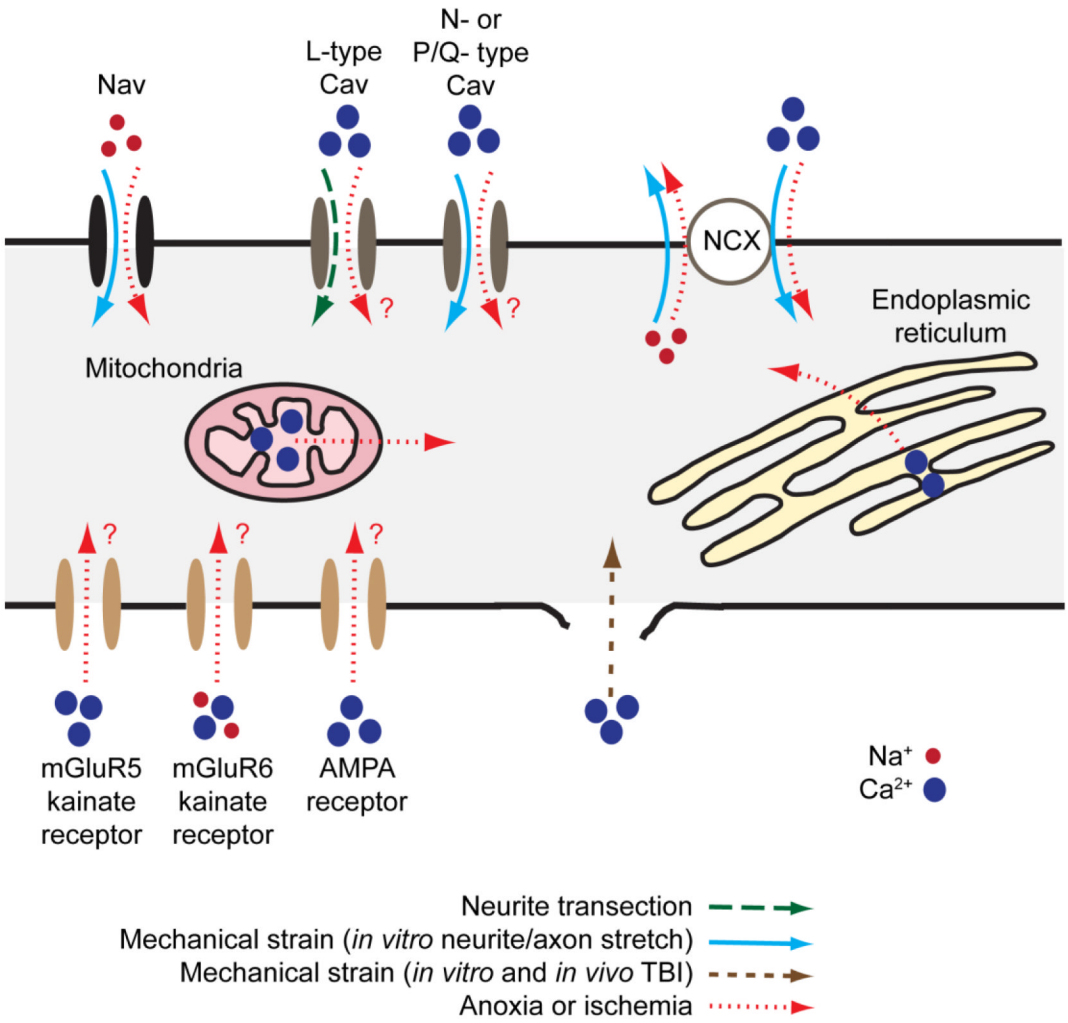

Fig. 4.

Mechanism of Ca^{2+}entry into the axoplasm after transection, mechanical strain, or anoxic/ischemic insult. Mechanisms by which axoplasmic [Ca^{2+}] become elevated likely vary according to injury model (designated by arrow type). Note that several of the mechanisms have not been adequately studied in various injury models.

Based on experiments demonstrating protection of compound action potential with channel antagonists, N-type Ca^{2+} channels may contribute to Ca^{2+} influx during anoxic injury (Fern et al., 1995; Imaizumi et al., 1999)

AMPA, α-amino-3-hydroxy-5-methylisoxazole-4-propionate; Cav, voltage-gated Ca^{2+} channel; Nav, voltage-gated Na^{+} channel; NCX, Na^{+}-Ca^{2+} exchanger; TBI, traumatic brain injury.

Table 1
Effect of calpain inhibitors in neurite/axon transection models

Model	Inhibitor(s)	Effect of Calpain Inhibitor(s)	Reference
Neurite transection; DRG explant culture	**1** ALLM **2** ALLN **3** EST	Reduces neurite degeneration (phase contrast microscopy) 96 h post-transection. IC_{50} for all 3 = ~10-20 μM.	George et al., 1995
Neurite transection; DRG explant culture	AK295 (50 μM)	Reduces neurite degeneration (MAP1B immunolabeling) 72 h post-transection.	Wang et al., 2000
Neurite transection; DRG explant culture	ALLN (200 μM)	Preserves NFH immunolabeling of neurites 24 h post-transection. No effect on axolemmal degeneration (DIC microscopy or immunolabeling for cytoplasmic enzyme PGP 9.5 or cell-surface antigen Thy-1.1).	Finn et al., 2000
Neurite transection; SCG explant culture	ALLN (50 μM)	Delays neurite degeneration (phase contrast microscopy). Decreases loss of intact NFL and NFM (western blot). No effect on "fragmentation" of microtubules (βIII-tubulin immunolabeling).	Zhai et al., 2003
Neurite transection (saponin); dissociated cortical neuronal culture	ALLN (10 μM)	No effect on axon fragmentation (βIII-tubulin immunolabeling) 4, 8 and 24 h post-transection.	Kilinc et al., 2011
Optic nerve transection *in vivo*	Calpastatin overexpression	Reduces NFL proteolysis (western blot) 5 days post-transection. No ultrastructural protection of axonal cytoskeleton (electron microscopy).	Ma et al., 2013
Optic nerve crush *in vivo*	Mu-F-hF-FMK (topical)	Reduces axonal degeneration, myelin vacuolization and delamination, and reactive astrogliosis (electron microscopy) 4 days post-crush.	Couto et al., 2004
Sciatic nerve transection *in vivo*	Calpastatin overexpression	Reduces NFL proteolysis and NFH loss (western blot) 48 h post-transection. Protects axonal cytoskeleton (electron microscopy) 2 and 5 days post-transection. Preserves NMJ (neurofilament and synaptic vesicle immunolabeling) 18 h post-transection. No effect on CMAP amplitudes 14 h post-transection.	Ma et al., 2013
Sciatic nerve transection *in vivo*	Leupeptin IP	No effect on axonal degeneration (light microscopy) or loss of intact NFL (western blot) 3 days post-transection.	Glass et al., 2002
Transection of GFP-labeled DRG axons in spinal cord *in vivo*	Mixture of EST and MDL-28170	Completely prevents acute axonal degeneration (fragmentation of GFP) between 5 and 30 min post-transection.	Kerschensteiner et al., 2005

CMAP, compound muscle action potential; DIC, differential interference contrast; DRG, dorsal root ganglia; EST, (2S,3S)-*trans*-epoxysuccinyl-L-leucylamido-3-methylbutane ethyl ester; GFP, green fluorescent protein; IP, intraperitoneal; MAP1B, microtubule-associated protein 1B, NFH, neurofilament heavy; NFL, neurofilament light; NFM, neurofilament medium; NMJ, neuromuscular junction; SCG, sympathetic superior ganglia

Table 2
Effect of calpain inhibitors on axonal pathology in TBI and TAI models

Model	Inhibitor(s)	Effect of Calpain Inhibitor(s)	Reference
Fluid shear stress; dissociated forebrain neurons	ALLN (3 μM)	Reduces neurite beading (phase contrast microscopy) 60-90 min post-injury.	Kilinc et al., 2009
Impact acceleration head injury	MDL-28170 (30 mg/kg) IV 30 min before injury	Reduces β-APP and RMO-14 immunostaining of axons in the corticospinal tract and medial longitudinal fasciculus at 2 h post-injury.	Buki et al., 2003
Fluid percussion injury	MDL-28170 IV (various dosing strategies)	Inhibitor effect varies depending on dosing strategy and post-injury survival duration. Inhibitor reduces β-APP immunostaining in the corpus callosum. Although its effect on CAP is less consistent, inhibitor partially normalizes CAP amplitude and latency of first and second major peaks (corresponding to myelinated and unmyelinated axons, respectively). Amplitude correlates with number of axons generating action potentials, while latency represents velocity of action potential propagation.	Ai et al., 2007
Controlled cortical impact	ALLM IA bolus and infusion (total dose ~2.7 μmoles)	Reduces axonal swellings, bulbs, and "breaks" in white matter tracts (NFL immunostaining) 24 h post-injury.	Posmantur et al., 1997
Optic nerve stretch *in vivo*	MDL-28170 IV bolus and infusion (total dose 67.5 mg/kg)	No improvement in retrograde transport of Fluoro-gold 4 days post-injury.	Ma et al., 2012a
Optic nerve stretch *in vivo*	Calpastatin overexpression	Improves retrograde transport of Fluoro-gold 4 days post-injury.	Ma et al., 2012b
Impact acceleration head injury	MDL-28170 (30 mg/kg) IV 30 min before injury	Reduces the length, but not the thickness, of HRP-labeling of axons in the corticospinal tract 2 h post-injury. No effect observed in the medial longitudinal fasciculus.	Czeiter et al., 2009

β-APP, β-amyloid precursor protein; CAP, compound action potential; HRP, horseradish peroxidase; NFL, neurofilament light; IA, intra-arterial; IV, intravenous

Table 3

Effect of calpain inhibitors on axonal pathology from anoxia, ischemia, complement activation, and α-latrotoxin

Model	Inhibitor(s)	Effect of Calpain Inhibitor(s)	Reference
OGD; cortical neuronal culture	**1** MDL-28170 **2** Calpastatin overexpression	Preserves βIV-spectrin labeled AIS 24 h post-OGD. IC_{50} for MDL-28170 = ~10 μM.	Schafer et al., 2009
MCAO (90 min) *in vivo*	MDL-28170 IV bolus and infusion (total dose 40 mg/kg)	Preserves βIV-spectrin and Nav labeled AIS in infarcted cortex 24 h post-MCAO.	Schafer et al., 2009
Anoxia± reoxygenation; optic nerves *ex vivo*	**1** MDL-28170 (50-100 μM) **2** ALLN (30-100 μM)	Reduces αII-spectrin proteolysis (western blot), presumably in axons. No improvement in CAP (area under the CAP).	Jiang and Stys, 2000
Anoxia or OGD; optic nerves *ex vivo*	**1** MDL-28170 (50 μM) **2** ALLN (10 μM)	Reduces NFM and NFH proteolysis, and NFH dephosphorylation (western blot). No improvement in CAP (area under the CAP).	Stys and Jiang, 2002
OGD; extensor digitorum longus preparation from mice that express YFP in motor neurons	Calpain inhibitor VI (100 μM)	Reduces NMJ denervation (loss of YFP fluorescence) 120 min post-OGD.	Talbot et al., 2012
Anti-GD1a antibody with complement; intramuscular axons in *ex vivo* triangularis sterni muscle	AK295 (100 μM)	Preserves immunostaining for Nav1.6, ankG, and Caspr at nodes and paranodes, and immunostaining for NF at NMJs. No improvement in perineural currents at nodes or terminals.	McGonigal et al., 2010
Anti-GD1a antibody with complement; desheathed phrenic nerves *ex vivo*	AK295 (100 μM)	Preserves immunostaining for Nav1.6 and Caspr, but not ankG, at nodes and paranodes. No improvement in CAP amplitude.	McGonigal et al., 2010
α-latrotoxin; NMJs in hemidiaphragm preparations	Calpeptin (50 μg/ml or 138 μM)	Preserves NFH immunostaining and "cytoskeletal bundles" in NMJs. Synaptic vesicle depletion is less pronounced with calpain inhibitor.	O'Hanlon et al., 2003
Anti-GQ1b antibody with complement; NMJs in hemidiaphragm preparations	**1** Calpeptin (50 μg/ml or 138 μM) **2** Calpain inhibitor V (50 μg/ml or 123 μM)	Preserves NFH immunostaining and "cytoskeletal bundles" in NMJs. Does not reduce mitochondrial damage. No effect on synaptic vesicle depletion.	O'Hanlon et al., 2003

AIS, axon initial segment; ankG, ankyrin G; CAP, compound action potential; Caspr, contactin-associated protein; MCAO, middle cerebral artery occlusion; Nav, voltage-gated Na^+ channel; NF, neurofilament; NFH, neurofilament heavy; NFM, neurofilament medium; NMJ, neuromuscular junction; OGD, oxygen glucose deprivation