Trends in Neurosciences

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Review

The calcium-dependent protease calpain in neuronal remodeling and neurodegeneration

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Calpains are evolutionarily conserved and widely expressed Ca^{2+}-activated cysteine proteases that act at neutral pH. The activity of calpains is tightly regulated, given that their abnormal activation can have deleterious effects leading to promiscuous cleavage of various targets. Genetic mutations in the genes encoding calpains are associated with human diseases, while abnormally elevated Ca^{2+} levels promote Ca^{2+}-dependent calpain activation in pathologies associated with ischemic insults and neurodegeneration. In this review, we discuss recent findings on the regulation of calpain activity and activation as revealed through pharmacological, genetic, and optogenetic approaches. Furthermore, we highlight studies elucidating the role of calpains in dendrite pruning and axon degeneration in the context of Ca^{2+} homeostasis. Finally, we discuss future directions for the study of calpains and potential therapeutic strategies for inhibiting calpain activity in neurodegenerative diseases.

Highlights

Calpains are evolutionally conserved and widely expressed cysteine proteases that act at neutral pH. Unlike all other proteases, calpains are activated by Ca^{2+}.

Under physiological conditions, cytoplasmic Ca^{2+} levels are typically in the μM range, which is much lower than the mM Ca^{2+} levels required for calpain activation in *in vitro* assays. Accordingly, how calpain is activated under physiological conditions has been a critical and long-standing question in the field.

A critical player in the activation of calpain appears to be Ttm50. Ttm50, a subunit of the TIM23 complex involved in the transport of proteins across the mitochondrial inner membrane, anchors calpain to Golgi/endoplasmic reticulum Ca^{2+} stores, while simultaneously increases the calcium sensitivity of calpain by directly interacting with calpain via its C-terminal FCP1 domain.

Calpains are activated by calcium transients in neuronal remodeling during development and by calcium overload in Wallerian degeneration and neurodegenerative diseases.

Given that axon loss is often an early sign of neurodegeneration, its prevention by inhibiting calpain activity may lead to treatments for neurodegenerative diseases.

Calpains are cysteine proteases activated by Ca^{2+}

Calpain was first discovered in 1964 during studies of proteolytic processes triggered by Ca^{2+} in the brain [1]. These proteolytic processes were mediated by a nonlysosome-associated intracellular cysteine protease with optimal activity at neutral pH. The characteristics of this protease activity, such as Ca^{2+} dependence and limited substrate proteolysis, suggest the role of calpain as a regulatory rather than a digestive protease [2]. The name 'calpain' was derived from its calcium-dependent activation and sequence homology to proteinase papain. So far, 15 genes encoding calpains have been identified in the human genome. Mammalian calpains 1, 2, 5, 7, 10, and 15 are ubiquitously expressed, whereas calpain 3 is restricted to skeletal muscle [3–5]. The most well-studied calpains include two conventional calpains micro (μ)-calpain and milli (m)-calpain, also known as calpains 1 and 2, respectively, which differ mainly in their *in vitro* Ca^{2+} requirements [6,7]. Hereafter, we use the calpain 1 and 2 nomenclature. Calpains, at least 1 and 2, exist as a heterodimer containing a shared small regulatory subunit (Capns1) (30 kDa) and a distinct, large catalytic subunit (80 kDa) (Figure 1).

The shared small subunit in calpains 1 and 2 is essential for the stabilization and activation of the large catalytic subunit of calpains. Conditional disruption of both calpains 1 and 2 in *Capns1*-knockout mouse brains alters dendrite morphology, impairs long-term potentiation (LTP), and promotes neuronal survival following injury [8]. However, each large calpain subunit alone is functional as active protease *in vitro* [6]. Furthermore, *Drosophila* calpains A and B, which, as discussed later, share features with the mammalian large calpain subunits, display effective enzymatic activity alone *in vitro* [9].

Calpastatin is a specific, endogenous calpain inhibitor that binds and inhibits both calpains 1 and 2 via its calpain-inhibitor domain when the proteases are activated by Ca^{2+} [10,11]. Thus far, the

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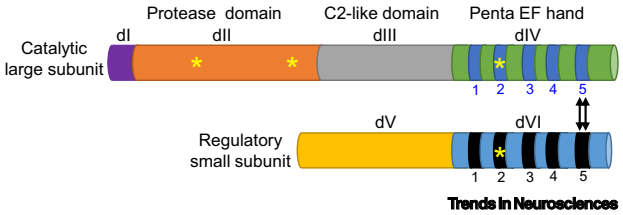

Figure 1. Schematic of the domain structure of the large and small subunits of calpain, with their respective Ca^{2+}-binding sites (asterisks). The most-studied conventional calpains comprise a large catalytic subunit containing four domains (dI–dIV) and a small regulatory subunit containing two domains (dV and dVI). The C-terminal calmodulin-like domain in both subunits contains a penta EF-hand motif, with Ca^{2+}-binding sites indicated by asterisks. The two subunits dimerize via the terminal EF-hand motif.

small regulatory subunit of calpain and calpastatin have been characterized in mammals, but not identified in invertebrates, such as fruit flies [12].

Mutations in genes encoding calpains result in diseases with distinct clinical manifestations, demonstrating the important roles of calpains in development and physiology. For example, a mutation in *CAPN1* has been associated with spastic paraplegia and spinocerebellar ataxia in humans and dogs [13–15], and a mutation in *CAPN3* is responsible for type 2A limb-girdle muscular dystrophy (LGMD2A) [4,16]. The biochemical and physiological functions of calpain have been reviewed elsewhere [2,5,17]. This review focuses on recently identified regulatory mechanisms of calpain activity and activation, primarily based on recent studies in *Drosophila*. Furthermore, we illustrate how calpain activation due to transient increase in Ca^{2+} influx is implicated in **neuronal remodeling** (see Glossary) and **axon degeneration** and summarize **neurodegenerative** diseases associated with elevated calpain activities. Of note, different calpains from different species may vary in their expression pattern and mode of activity regulation. However, the most well-studied calpains from mammals (i.e., calpains 1 and 2) and fruit flies (i.e., calpains A and B) share a similar Ttm50-mediated activation mechanism [9]. Lastly, we propose approaches for controlling calpain activity as potential strategies for the treatment of neurodegenerative diseases and other conditions in which calpains are hyperactivated.

Subcellular localization is critical for calpain activation

Given that calpain is activated by Ca^{2+}, and Ca^{2+} is not evenly distributed in cells, the subcellular localization of calpain is critical for its activation. Immunochemical studies have shown that calpain is widely distributed in cells; depending on the cell type, various mammalian calpains are diffused in the cytoplasm or are located in the endoplasmic reticulum (ER), Golgi, or plasma membrane [18,19]. The phosphoinositide PIP_2 is essential for the activation of calpain 2 and functions as a cofactor by promoting its anchorage to the plasma membrane [20]. However, phospholipids are typical activators of several enzymes and, therefore, cannot serve as specific activators of calpain [2]. A 2004 publication reported the localization of calpains 1 and 2 to the cytoplasmic surface of subcellular organelles, including the Golgi/ER in cultured cells [19]. A follow-up study addressed the mechanism by which calpain is localized to these organelles and the functional significance of the specific localization. It was found that *Drosophila* calpain A, which exhibits a similar sensitivity to Ca^{2+} as mammalian calpain 2 at milli-molar level, is localized primarily in Golgi/ER **Ca^{2+} stores** [9]. The localization of calpains in these Ca^{2+} stores places them in the vicinity of high Ca^{2+} microenvironments for efficient activation.

It was also found that the integral membrane protein Ttm50, a subunit of the **TIM23 complex** in the inner mitochondrial membrane involved in protein import into the mitochondrial matrix [21], mediates calpain localization at the Golgi/ER and, thus, facilitates calpain activation [9]. Ttm50 binds calpain A via its C-terminal domain [9]. Knockdown of Ttm50 disrupts the Ca^{2+}-dependent mobilization of calpain A to the Golgi and ER. Furthermore, the physical interaction between

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Ttm50 and calpain was enhanced by Ca^{2+}, and the localization of calpain A in Golgi was Ca^{2+} dependent [9]. Thus, Ca^{2+}-dependent calpain localization at the Golgi/ER via Ttm50 provides a possible explanation for the *in vivo* activation of calpains. Furthermore, it was demonstrated that full-length Ttm50 is located in the Golgi/ER, while Ttm50 is truncated by removing the N-terminal targeting sequence upon entering the inner mitochondrial membrane to assemble into the TIM23 complex [9]. Thus, the full-length but not the shorter mitochondrial, Ttm50 acts as a calpain anchor by localizing calpain to the Ca^{2+} stores Golgi/ER (Figure 2). How the two distinct functions of Ttm50 at different subcellular locations coordinate remains to be clarified (see Outstanding questions).

In addition to anchoring calpain at the Ca^{2+} stores of the Golgi/ER, Ttm50 binding increases calpain sensitivity to Ca^{2+} by approximately an order of magnitude [9]. Importantly, Tim50, the mammalian ortholog of *Drosophila* Ttm50, also increases the calcium sensitivity of calpains 1 and 2 *in vitro* and cultured cells [9], demonstrating an evolutionally conserved role of Ttm50 as a newly identified calpain activator. What is the mechanism by which Ttm50 proteins increase calpain sensitivity to Ca^{2+}? Ttm50 binding may result in calpain conformational change so that the Ca^{2+}-binding domains of calpain are exposed and accessible to Ca^{2+}. Alternatively, this may increase the binding affinity for Ca^{2+}. It is also possible that both mechanisms operate simultaneously. The main challenge is to rationalize the high levels of Ca^{2+} required for calpain activation (e.g., mM level of Ca^{2+} needed for calpain 2 activation) *in vitro* but low μM levels of cytoplasmic Ca^{2+} under physiological conditions. Although Ca^{2+} levels in specific cellular locations may be higher than the average in the overall cytoplasm, intracellular Ca^{2+} alone is probably unable to ensure efficient calpain activation in normal physiological and developmental processes. The identification of Ttm50 as a conserved anchor and activator of calpain explains the activation of calpains *in vivo* with a relatively low level of Ca^{2+} in physiological conditions.

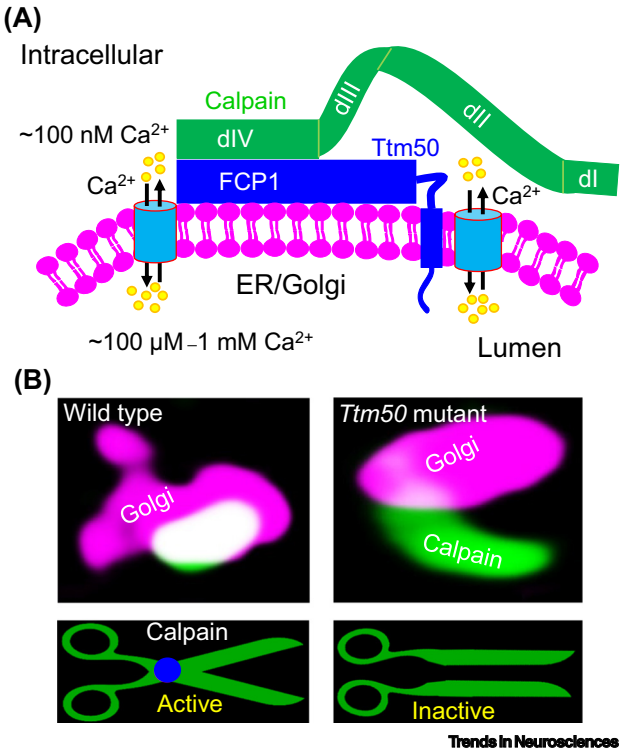

Figure 2. Ttm50 acts as a calpain anchor and activator. (A) A working model of calpain activation facilitated by Ttm50**.** Ttm50 acts as both an anchor and activator for calpain by localizing calpains at the membrane of the Ca^{2+} stores in the endoplasmic reticulum (ER) and Golgi. Ttm50 interacts with calpain via its C-terminal TFIIF-interacting CTD phosphatase 1 (FCP1) domain. The ER/Golgi localization facilitates calpain activation by positioning it in the vicinity of a relatively high concentration of Ca^{2+}. Whereas the Ca^{2+} concentration in the cytoplasm in resting state is ~100 nM, it is in the 100 μM–1 mM range in the ER/Golgi Ca^{2+} stores. (B) Representative confocal images of wild-type (left) and *Ttm50* mutant (right) *Drosophila* muscles co-stained with antibodies against calpain A (green) and the Golgi marker GM130 (magenta). Calpain colocalizes with the Golgi/ER in wild-type *Drosophila* muscles, but *Ttm50* mutation results in calpain mislocalization away from the Golgi/ER and leaves calpain inactive. Modified from [9].

Glossary

Axon degeneration: self-destruction process resulting in axon loss in different settings of development, injury, and disease, including Wallerian degeneration typically induced by axotomy.

Ca^{2+} homeostasis: intracellular Ca^{2+} levels in different cell types are maintained in an appropriate range depending on the status of the cell: resting state or activated. Among the primary routes of neuronal Ca^{2+} entry and egress are voltage-gated Ca^{2+} channels (VGCCs), potassium-dependent Na^{+}/Ca^{2+} exchangers (NCKs), and plasma membrane Ca^{2+} ATPases (PMCAs). To maintain Ca^{2+} homeostasis, the same amount of Ca^{2+} that enters the cytoplasm through VGCCs upon activation must be removed by NCKs and PMCAs in the resting state. Likewise, the same amount of Ca^{2+} that is released from Ca^{2+} stores by ryanodine receptors (RyR) and inositol 1,4,5-trisphosphate receptor (IP_3R) is taken back by the SERCA pump.

Ca^{2+} stores: intracellular organelles that store Ca^{2+}, such as the ER, Golgi, and mitochondria. The Ca^{2+} concentration in the stores is ~100 μM–1 mM, whereas the concentration in the cytoplasm in the resting state is ~100 nM.

Ca^{2+} transient: a transient increase in Ca^{2+} in the cytoplasm upon stimulation or excitation.

Dendrite pruning: a process by which neurons selectively remove exuberant or unnecessary dendrites without causing cell death. The process is crucial for the establishment of mature neural circuits during animal development.

Mitogen-activated protein kinases (MAPKs): proteins activated by a range of stimuli and mediating several physiological and pathological cellular functions. For example, activation of MAPK represents an early degenerative response to axon injury. MAPK signaling promotes the degradation of palmitoylated nicotinamide mononucleotide adenylyltransferase 2 (NMNAT2), while genetic or pharmacological inhibition of MAPK signaling results in upregulated NMNAT2 levels and axon protection.

Neurodegeneration: progressive loss of structure or function of neurons, including neuronal death. Prominent examples of neurodegenerative

Calpains function as a complex

Conventional calpains 1 and 2 are heterodimers, each comprising a large catalytic subunit with four domains (dI–dIV) and a small regulatory subunit with two domains of dV and dVI (Figure 1). The heterodimerization of the large and small subunits occurs through a unique interaction between their C-terminal domains [5,22] (Figure 1). However, the form in which the functional calpains exist for most calpains, including mammalian calpains 5–7 and 10–16, remains unknown. *Drosophila* calpain B was originally demonstrated to work as monomers based on *in vitro* studies of *Escherichia coli*-derived recombinant proteins [23], rather than as heterodimers, such as mammalian calpains 1 and 2 [5,22]. However, a recent study showed that, in *Drosophila,* different calpains act together *in vivo* to cleave its target GluRIIA at neuromuscular junction (NMJ) synapses [24]. Specifically, different *Drosophila* calpains are colocalized and interact physically *in vivo* and in cultured cells as detected by co-immunoprecipitation [24]. *Drosophila* calpain A [24] and calpain B [23] do not form homodimers, but instead form multimers comprising different calpains, including at least calpains A, B, and D. However the domain mediating calpain–calpain interactions remains to be defined. Of note, reducing the level of one subunit does not affect the protein level of another, but alters the autolysis specificity of the other and reduces the protease activity against targets, such as GluRIIA [24]. The finding of a calpain complex concurs with a previous study demonstrating that different calpains in *Drosophila* function synergistically downstream of **Ca^{2+} transients** to trigger **dendritic pruning** of sensory neurons during metamorphosis [25].

Similarly, mammalian calpains 8 and 9, expressed specifically in the gastrointestinal tract, interact physically, as detected by co-immunoprecipitation and gel filtration assays, to form a protein complex called 'gastric calpain' and participate in gastric mucosal defense [26]. Different from the calpain complex in *Drosophila*, the protein levels of calpains 8 and 9 are mutually dependent [26]. Thus, heterodimers or heteromultimers of different calpains without a small regulatory subunit, in addition to the conventional calpain heterodimers such as calpains 1 and 2 comprising a large and a small subunit, emerge as a new way of regulating the activity of calpains [24,26].

The Ca^{2+} sensitivity of single calpain subunits has been well established *in vitro* [6,9]. To better elucidate the activation of a single subunit versus calpain complex, it would be informative to determine and compare the Ca^{2+} sensitivity of the calpain complex with various compositions, including the recently identified Ttm50, with that of single subunits.

Intracellular Ca^{2+} requirement for calpain activation

Ca^{2+} is a highly versatile intracellular signal that impacts many different cellular processes [27,28]. The intracellular cytosolic Ca^{2+} concentration is typically ~100 nM, much lower than its extracellular concentration, which is on the order of mM at resting state. In response to various stimuli, Ca^{2+} enters cells via ionotropic receptors and voltage-gated Ca^{2+} channels in the plasma membrane and is released from intracellular stores, such as the ER, Golgi, and mitochondria (Figures 2 and 3). Together, these two processes cause a rapid increase in the intracellular Ca^{2+} concentration, often by 10–100 times [27,28]. Intracellular Ca^{2+} concentrations are generally assumed to increase by up to tens of μM. Thus, the μM-level Ca^{2+} concentrations required for calpain 1 activation might be reached under certain physiological conditions. However, intracellular Ca^{2+} concentrations cannot reach the mM level needed for calpain 2 activation, except under pathological conditions, such as stroke or tissue damage.

Ca^{2+} influx from the extracellular space can activate calpains [24,25], but is the Ca^{2+} released from Ca^{2+} stores also sufficient to activate calpains? To address this question, thapsigargin (Tg), an inhibitor of the sarco-ER Ca^{2+}-ATPase (SERCA) that pumps Ca^{2+} ions from the cytoplasm into

diseases include Alzheimer's disease, Parkinson's disease, amyotrophic lateral sclerosis, and multiple sclerosis. Such diseases are incurable, resulting in progressive degeneration of neurons.

Neuronal remodeling: used to describe multiple biological processes, including synapse elimination or strengthening, dendrite and axon elimination, and programmed cell death of specific neuronal populations. Remodeling involves specific elimination of existing connections, typically followed by strengthening of surviving synapses or neurite regrowth to form new connections.

Sterile alpha and TIR motif containing 1 (Sarm1): a protein with NAD^+ hydrolase activity both required and sufficient for axon degeneration. Sarm1 initiates a local axon destruction program involving rapid breakdown of NAD^+ after injury.

TIM23 complex: a protein complex with multiple subunits in the inner mitochondrial membrane involved in protein import into the mitochondrial matrix.

Wallerian degeneration: a specific form of genetically programmed axon degeneration in disease and injury. Active Wallerian degeneration requires Sarm1, while the NAD^+ synthetic enzyme NMNAT2 prevents degeneration. Some of the mechanisms identified in Wallerian degeneration may also operate during axon elimination in development.

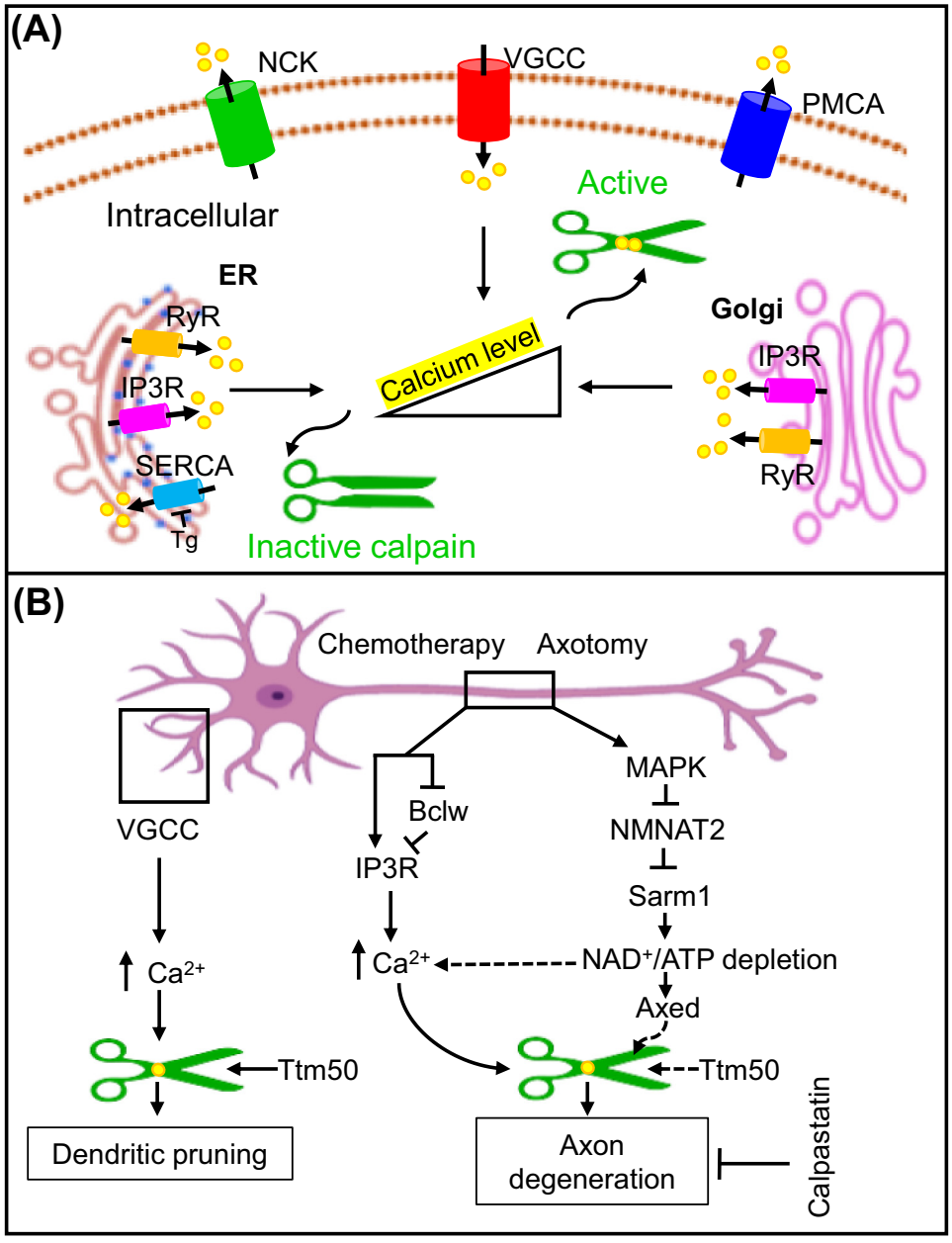

Figure 3. Ca^{2+} channels controlling Ca^{2+} transients and the calpain-involved pathways in neuronal remodeling. (A) Ca^{2+} channels known to affect calpain activity. Thapsigargin (Tg) is an inhibitor of the sarco-endoplasmic reticulum Ca^{2+}-ATPase (SERCA). (B) Dendritic and axonal pruning is regulated by Ca^{2+}-dependent and mitogen-activated protein kinase (MAPK)-mediated calpain activation. Voltage-gated Ca^{2+} channels (VGCCs) are critical for maintaining Ca^{2+} homeostasis to enhance Ca^{2+}-dependent calpain activation resulting in dendrite pruning. The ER inositol 1,4,5-trisphosphate receptor (IP_3R) Ca^{2+} channel is required for axon degeneration via calpain upon treatment with the chemotherapy drug paclitaxel. Axotomy activates the MAPK-sterile alpha and TIR motif containing 1 (Sarm1) signaling pathway, leading to calpain activation, the convergence point for execution of axonal degeneration. MAPK and Sarm1 are pro-degenerative, while nicotinamide mononucleotide adenylyltransferase 2 (NMNAT2) and NAD^+ are neuroprotective. The role of the mammalian Axed homolog in axon degeneration remains to be defined, as does the role of calpains in Wallerian degeneration in fruit flies. Arrows with dashed lines indicate that the regulatory mechanism is either unknown or unverified. Abbreviations: NCK, potassium-dependent Na^+/Ca^{2+} exchanger; PMCA, plasma membrane Ca^{2+} ATPase.

the ER lumen (Figure 3) was used and found not activate calpain [9]. Given that Tg-induced Ca^{2+} increases in the cytoplasm are mediated by Ca^{2+} leaking from the ER, the efficiency of the Tg-induced Ca^{2+} increase in the cytoplasm is believed to be lower than that of opening Ca^{2+} channels, such as ryanodine receptors (RyR) and inositol 1,4,5-trisphosphate receptors (IP_3R) in the ER/Golgi. When treated with ionomycin, a Ca^{2+} ionophore, in the presence of ambient Ca^{2+}, it is impossible to distinguish whether calpains are activated in response to Ca^{2+} entry through the plasma membrane, Ca^{2+} release from the ER/Golgi, or both. However, calpain can be activated by ionomycin in a Ca^{2+}-free medium [9], indicating that depletion of Ca^{2+} from the ER/Golgi is also sufficient to activate calpains.

In addition to ER/Golgi, mitochondria and lysosomes are also important Ca^{2+} stores [29,30]. However, the role of mitochondria- and lysosomes-mediated Ca^{2+} signaling in the regulation of calpain activity remains to be clarified.

Under resting conditions, the level of cytoplasmic Ca^{2+} is several orders of magnitude lower than that in intracellular Ca^{2+} stores or in the extracellular space [28]. The dynamics of Ca^{2+} is determined by the subcellular localization and the protein levels of Ca^{2+} channels and receptors permeable to Ca^{2+}. The opening of Ca^{2+}-permeable ion channels and receptors located in the plasma membrane, ER, or Golgi results in rapid Ca^{2+} influx to activate calpains. In neuronal cells, intracellular Ca^{2+} increases from several routes, including voltage-gated Ca^{2+} channels (VGCCs) [31] and NMDA receptors (NMDARs) [32,33]. Releasing Ca^{2+} from ER occurs as a result of IP_3 binding to IP_3Rs and activation of ryanodine receptors (RyRs) (Figure 3). In dendrites, Ca^{2+} transients depend on VGCCs along the dendritic shaft, while ionotropic glutamate receptor-mediated Ca^{2+} fluxes occur through NMDARs and AMPARs, which are predominantly localized in dendritic spines [34,35]. Specifically in neurons, membrane–membrane contacts controlling Ca^{2+} signaling is a complex unit between L-type VGCCs at the plasma membrane and RyRs in the ER [36]. The location and activity of Ca^{2+} channels and Ca^{2+}-permeable receptors affect the timing and frequency of Ca^{2+} transients and determine whether the changes in Ca^{2+} concentration occur in a global or localized fashion inside the cell [37,38]. Importantly, these spatiotemporal patterns of Ca^{2+} transients in neurons direct downstream processes, such as calpain activation involved in dendritic pruning and axon degeneration [25,39].

In addition to Ca^{2+} influx through the plasma membrane and Ca^{2+} release from Ca^{2+} stores, calpain activation also requires its recently identified interacting partner Ttm50 to localize at the ER/Golgi and sensitize to Ca^{2+}. In the absence of either a high level of Ca^{2+} or Ttm50, calpains cannot be effectively activated *in vivo* (Figure 2).

Calpain in neuronal remodeling

Through axon and dendrite pruning, remodeling of neuronal connectivity allows the formation of robust mature circuits. Multiple signaling pathways essential for axon and dendrite pruning and refinement have been identified in studies of both central and peripheral neurons [40–42]. During development, when the self-destruction processes in axons and dendrites are initiated, the ubiquitin proteasome system, caspases, and calpains are activated [41–44]. These pathways either act in parallel or intersect. For example, upon caspase activation leading to depletion of calpastatin, calpains with more promiscuous substrate specificity induce proteolysis of cellular proteins, including neurofilaments in axonal degeneration after injury and in development [45,46].

Calpain is activated by abnormally high Ca^{2+} levels associated with injury, ischemic insults, and neurodegenerative pathologies. Yet, the regulation of calpain activity in neuronal remodeling during normal development is largely unknown. Multiple processes, such as neuronal excitability,

muscle contraction, and stress, involve an increase in Ca^{2+} influx [27,28]. In various types of mammalian neuron, localized changes in the excitability of the dendritic branch resulting in an increase in local Ca^{2+} transits are observed [47,48]. In *Drosophila*, genetic and optogenetic manipulation of Ca^{2+} influx at NMJ synapses [24] and sensory neurons [25] revealed that, during normal development, calpains operate downstream of Ca^{2+} influx.

During neural development, highly branched dendritic arbors undergo selective pruning to create proper connectivity. How is localized Ca^{2+} precisely regulated to activate calpain in dendritic pruning during development? In an elaborate study, Emoto and colleagues used the genetically encoded Ca^{2+} indicator GCaMP3 and found compartmentalized Ca^{2+} transients induced by locally increased intrinsic excitability ~3 h before branch elimination in specific dendrite branches [25]. The Ca^{2+} waves were not synchronized between various classes of dendritic neuron, and dendrites were pruned in the same temporal order in which the transients of Ca^{2+} appeared. Dendrites were first pruned with the earliest Ca^{2+} transients, followed later by dendrites that initiated Ca^{2+} waves; the development of Ca^{2+} transients correlates with increased dendrite excitability mediated by VGCCs as the major Ca^{2+} regulator before pruning [25]. The authors further showed that Ca^{2+}-activated calpain is required for dendrite pruning, because loss of function in genes encoding a subunit of VGCC and calpain A or calpain B leads to defective pruning in sensory neurons [25]. Thus, the compartmentalized Ca^{2+} transients provide temporal and spatial triggers to induce dendrite pruning via Ca^{2+}-activated calpains. Future studies are needed to identify the upstream signals that trigger Ca^{2+} transients that activate calpains in dendrite and axon pruning (see Outstanding questions). It also remains to be determined whether the VGCC-mediated Ca^{2+} influx underlying dendrite pruning works in axon elimination.

At the *Drosophila* NMJ synapse, calpain downregulates GluRIIA triggered by Ca^{2+} influx through Ca^{2+} channels and/or from intracellular stores, such as the Golgi and ER [24]. Genetic and optogenetic analyses revealed that Ca^{2+}-induced activation of calpains is likely to occur under certain physiological conditions at NMJ synapses during development [24]. In the spinal cord of zebrafish, calpain activity induced by high-amplitude long-duration Ca^{2+} transients is involved in myelin sheath retraction, probably by localized degradation of cytoskeletal components [49].

It will be important to identify calpain targets in axonal and dendritic elimination, as well as in myelin dynamics, given the essential role of calpain in these processes (see Outstanding questions).

Calpain in axon degeneration

Axon degeneration is a specialized self-destructive process that eliminates unneeded or damaged axons in development, injury, and disease. Given that axon loss is involved in many neurodegenerative diseases, the molecular and cellular processes of axon degeneration have been intensively studied and reviewed elsewhere [40–42,48,50,51]. Here, we focus on recent studies that identified a crucial role of calpain in axon degeneration in different settings, including **Wallerian degeneration** typically caused by axotomy [46,50–52]. Ca^{2+} dysregulation is a key step that stimulates the involvement of calpain in axon degeneration following toxic and traumatic axon injury [46,50,51]. While Ca^{2+} influx via VGCCs has been shown to be critical for dendrite pruning [25], multiple studies have shown that Ca^{2+} released from intracellular stores can initiate the activation of calpain and axon degeneration after toxic or mechanical injury [51,52]. However, the regulation of this increase in cytoplasmic Ca^{2+} is not fully clear [46].

New insights into the mechanisms involved in calpain-dependent axonal degeneration have come from studies of peripheral neuropathy resulting from treatment with chemotherapeutic drugs, including paclitaxel, a commonly used chemotherapeutic drug for patients with breast,

ovarian, lung, and other cancers [39,53]. Paclitaxel activates calpain proteases by altering IP_3R1 phosphorylation and intracellular Ca^{2+} influx [39]. Concomitantly, paclitaxel selectively reduces axonal expression of the Bcl2 family member Bclw, an inhibitory binding partner of IP_3R1, thereby resulting in enhanced activation of calpain due to increased cytosolic Ca^{2+} [39] (Figure 3). Furthermore, RNAi knockdown of the Ca^{2+}-permeable IP_3R1 or treatment with Bclw BH4 mimetics prevents paclitaxel-induced degeneration, suggesting that the Bclw-IP3R1 pathway enables calpain-dependent axonal degeneration [39]. Apart from the Bclw-IP3R1 pathway, different pathways for Ca^{2+} influx or disruption of intracellular **Ca^{2+} homeostasis** have also been hypothesized to underlie calpain-involved axonal degeneration. Those pathways include direct Ca^{2+} influx through the damaged plasma membrane, the activation of VGCCs in the plasma membrane, or the activation of RyR and IP_3R or inhibition of SERCA in ER/Golgi (Figure 3). Depending on neuronal type as well as degenerative insult, one or more pathways could make a major contribution.

In addition to the Bclm-IP3R1 pathway [39], **sterile alpha and TIR motif containing 1** (Sarm1) signaling also leads to activation of calpain in axotomy-induced Wallerian degeneration (Figure 3) [54]. Specifically, loss of dSarm suppresses Wallerian degeneration for weeks after axotomy in *Drosophila* [55]. Relatedly, severed axons of mouse of *Sarm1*-null mutants exhibit remarkable long-term survival both *in vivo* and *in vitro*, indicating that Sarm1 pro-degenerative signaling is conserved in mammals [55]. Furthermore, Sarm1 initiates a local destruction program involving rapid breakdown of NAD^+ after injury. Formation of the Sarm1 TIR dimer triggers rapid breakdown of NAD^+ by NAD^+ hydrolase activity, whereas Sarm1-induced axon destruction could be counteracted by increased NAD^+ synthesis [56]. Tessier-Lavigne and colleagues originally showed that activated Sarm1 induces rapid **mitogen-activated protein kinase (**MAPK) activation, and that endogenous Sarm1 is necessary for MAPK activation after nerve injury *in vivo* and in *ex vivo* cultures of retinal ganglion cells, indicating a pro-degenerative Sarm1-MAPK pathway [54]. Similarly, the Sarm1-MAPK pathway has been reported in innate immunity signaling and in the control of odorant receptor expression in *Caenorhabditis elegans* [57,58] and in dendrite morphogenesis in mammals [59]. While the identity of signaling components of Sarm1-MAPK varies across organisms, a MAPK cascade has been identified to act downstream of Sarm1 in different processes [54,57–59].

However, a recent study in mouse dorsal root ganglia cultures and *Drosophila* suggested that MAPK signaling occurs upstream of SARM1 [60]. MAPK signaling speeds the turnover of nicotinamide mononucleotide adenylyltransferase 2 (NMNAT2), a crucial axon survival factor, therefore promoting axon degeneration [60]. MAPK signaling is necessary for injury-dependent NAD^+ depletion, but not for NAD^+ loss induced by activated Sarm1. MAPK signaling is also necessary for axon degeneration induced by axotomy, but not axon degeneration induced by activated Sarm1, thus placing MAPK signaling upstream of Sarm1 activation [60,61] (Figure 3). The exact regulation between Sarm1 and MAPK in Wallerian degeneration remains to be clarified. The pro-degenerative signaling of dSarm can be fully blocked by mutations in Axed, an axonal BTB and BLACK domain protein, demonstrating that Axed is a downstream effector of dSarm [62]. It is currently unknown how the MAPK-Sarm1 pathway mechanistically promotes calpain activity in local axons [40,54,63] (Figure 3).

By contrast, an increase in the levels of axonal calpastatin reduced degeneration induced by physical injury in cultured neurons, consistent with the protective effect observed in mice after calpastatin overexpression [46,64,65]. Tessier-Lavigne and colleagues found that rapid depletion of calpastatin releases its inhibitory effect on calpains, leading to the activation of calpain and axonal degeneration in cultured neurons [46]. Depletion of calpastatin, which occurs downstream of the caspase cascade and is followed by calpain activation, is a key process for the degeneration of nerve growth factor (NGF)-deprived sensory axons *in vitro*. Furthermore, calpastatin

critically regulates developmental pruning of retinal ganglion cell axons *in vivo* [46]. These findings revealed that caspases are a trigger of the degenerative signal instead of a direct executor of axon elimination. Calpastatin proteolysis by active caspase-3 releases inhibition of calpains, which are potent proteases with more promiscuous specificity of substrates, thus facilitating the pruning process by ensuring prompt clearance of undesirable axons during development. Notably, the activity of calpains depends on high Ca^{2+} concentrations, and endogenous calpastatin depletion alone is not sufficient to induce axon degeneration *in vitro* or *in vivo* [39,46]. Regardless, these findings highlight that cytosolic Ca^{2+} and calpastatin are key regulators of axonal survival and death in degenerative pathways.

In summary, while distinct pathways may be involved in axon degeneration in different settings, calpain activation by Ca^{2+} appears to be a shared mechanism underlying the processes in chemotherapeutic toxicity, traumatic injury, and normal development.

Calpain in neurodegenerative diseases

Diseases caused by mutations in different calpains had been systematically reviewed previously [2]. Here, we focus on the role of elevated levels or hyperactivated calpains in neurodegenerative diseases (Table 1). Calpain activation induced by Ca^{2+} influx due to disease or injury is a well-established observation in the pathology of the nervous system [46,66,67] (Table 1). During an excitotoxic event, a second uncontrolled rise in Ca^{2+} contributes to neuronal cell death, mediated by activation of the glutamate receptor [66,67]. Inhibition of calpain-mediated cleavage of one of the plasma membrane Na^{+}/Ca^{2+} exchanger isoforms, which is essential for Ca^{2+} homeostasis in neurons, can prevent secondary Ca^{2+} overload [66]. Calpain-cleaved mGluR1α maintains its ability to stimulate the increase in cytosolic Ca^{2+} even when it can no longer activate the neuroprotective PI_3K-Akt signaling pathways. A fusion peptide derived from the cleavage site of mGluR1α efficiently blocks NMDA-induced truncation of mGluR1α in primary neuronal cultures and prevents excitotoxicity both *in vitro* and *in vivo* [67].

Overactivation of calpains targeting specific substrates by abnormally high concentrations of Ca^{2+} has been associated with various neurodegenerative diseases, such as Alzheimer's (AD) and Parkinson's (PD) [2,68] (Table 1). For example, the activation ratio of calpain 1 is increased threefold in the prefrontal cortex in patients with AD compared with controls [69]. Calpain activation leads to the cleavage of p35 to p25, which contributes to the prolonged activation and mislocalization of cyclin-dependent kinase 5 (cdk5), resulting in neuronal death [70]. Deregulated cdk5 hyperphosphorylates tau, which also promotes neuronal death [70].

Table 1. Mouse models of neurodegenerative and brain disorders involving overactivation of calpains

Neurodegenerative disorders	Calpain targets	Refs
Brain ischemia and neurotoxicity	Na^{+}/Ca^{2+} exchanger and mGluR1α	[66,67]
Alzheimer's disease	p35 and APP	[64,69–73]
Parkinson's disease	Alpha-spectrin	[74]
Amyotrophic lateral sclerosis	TDP-43	[77]
Machado–Joseph disease	Ataxin-3	[78]
Huntington's disease	Huntingtin	[79]
Retinitis pigmentosa	Unknown	[80,81]

AD is associated with improper processing of the amyloid precursor protein (APP), which contributes to the extracellular accumulation of amyloid plaque aggregates. The disease is also associated with the generation of intracellular neurofibrillary tangles of tau protein [71]. Calpains are implicated in both these processes. Specifically, calpain may be directly responsible for APP proteolysis, which is the key mechanism behind amyloid plaque formation [72]. Consistently, calpain inhibition by overexpression of calpastatin improves memory and synaptic transmission in an AD mouse model [64]. Interestingly, specific calpain 1 inhibitors were designed and evaluated in preclinical settings as possible therapies for neurodegeneration in AD (Table 1) [73].

Calpain inhibition also reduces neurological and behavioral abnormalities in a mouse model of PD. *N*-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) evokes an increase in calpain-mediated proteolysis of alpha-spectrin in nigral dopamine neurons [74]. Inhibition of calpain activity by the calpain inhibitor MDL-28170 or overexpression of the endogenous calpain inhibitor calpastatin greatly attenuates MPTP-induced nigral dopamine neuron loss [74].

Amyotrophic lateral sclerosis (ALS) is the most common type of motor neuron disease, characterized by progressive loss of motor neurons resulting in muscle weakness and eventual paralysis [75,76]. Calpain-mediated cleavage in motor neurons at the C terminus of TDP-43, a nuclear protein involved in the regulation of RNA processing, has a crucial role in the pathogenesis of ALS in both patients and animal models [77]. Notably, reduction or elimination of calpain 1 in cells or mice resulted in decreased ataxin-3 cleavage without impacting its aggregation and partially rescued disease-related characteristics in Machado–Joseph disease models [78], similar to the rescue of nigral dopamine neuron loss in PD mouse models [74]. In addition, activated calpain was detected in the caudate nucleus of postmortem tissue from patients with Huntington's disease (HD) but not in age-matched controls [79]. Importantly, one of the major N-terminal huntingtin (Htt) proteolytic fragments found in HD brain appears to be derived from calpain cleavage [79]. Hyperactivation of calpains has also been observed in retinitis pigmentosa, a disease characterized by photoreceptor degeneration and vision loss, but the substrates targeted by calpain in the pathogenesis of this condition remain to be definitively characterized [80,81].

Concluding remarks

Calpains have a critical role in neuronal remodeling and neurodegeneration. Given that neuronal remodeling abnormalities contribute to neuropsychiatric conditions, such as autism and schizophrenia [41,82], understanding how neurites are remodeled should provide new insights into the pathophysiology of related mental illnesses. Although application of calpain inhibitors appears attractive as a potential therapeutic strategy, such use is challenging due to possible adverse effects of using nonspecific calpain inhibitors. A target to consider in this context is Ttm50, the conserved anchor and activator of calpain across evolution from fruit flies to mammals. Application of a small peptide to specifically block the calpain-Ttm50 interaction might attenuate calpain activity to prevent calpain-associated brain injury and neurodegeneration. It remains to be determined whether effective therapeutic strategies can be designed to inhibit the deleterious effects of calpain hyperactivation upon injury and degeneration (see Outstanding questions). In addition to the well-known regulators of calpains, such as Ca^{2+}, calpastatin, and Ttm50, what are the other regulatory mechanisms involved in the activation and inhibition of calpain? Recently, an *in vivo* reporter for calpain activity was developed [83], which would facilitate a better understanding of the regulators of calpain activity. Advances in this area will contribute to both a better understanding of the *in vivo* role of calpain and the development of potential therapeutic strategies for diseases associated with deregulated calpain activity. Prevention of axon loss appears to be among the leading venues to

Outstanding questions

What are the upstream signals that trigger Ca^{2+} transients leading to calpain activation during normal development and in disease states?

What are the direct substrates of calpains in axons and dendrites that undergo neuronal pruning during development and axon degeneration in disease and injury?

Ttm50 has two distinct functions: it is a subunit of the TIM23 complex involved in the transport of proteins across the mitochondrial inner membrane, and an anchor and activator of calpain. How are these two functions coordinated in the cell?

In addition to Ttm50, the recently identified anchor and activator of calpain, are there other regulators of calpain activity?

Inhibiting calpain activity may offer an approach to prevent the deleterious effects of calpain hyperactivation in neurodegeneration or upon injury. What would be the best therapeutic strategies for inhibiting calpain activity?

explore, given the central roles of calpain in axon degeneration and the broad relevance of axon loss as an early sign of various neurodegenerative diseases.

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Declaration of interests

The authors declare no competing interests in relation to this work

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