

Regulation of Axon Degeneration after Injury and in Development by the Endogenous Calpain Inhibitor Calpastatin

Jing Yang,^{1,2,4} Robby M. Weimer,^{2,4} Dara Kallop,² Olav Olsen,^{1,2} Zuhao Wu,¹ Nicolas Renier,¹ Kunihiro Uryu,³ and Marc Tessier-Lavigne^{1,2,*}

¹Laboratory of Brain Development and Repair, The Rockefeller University, New York, NY 10065, USA

²Research and Early Development, Genentech Inc., South San Francisco, CA 94080, USA

³Electron Microscopy Resource Center, The Rockefeller University, New York, NY 10065, USA

⁴These authors contributed equally to this work

*Correspondence: marctl@rockefeller.edu

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SUMMARY

Axon degeneration is widespread both in neurodegenerative disease and in normal neural development, but the molecular pathways regulating these degenerative processes and the extent to which they are distinct or overlapping remain incompletely understood. We report that calpastatin, an inhibitor of calcium-activated proteases of the calpain family, functions as a key endogenous regulator of axon degeneration. Calpastatin depletion was observed in degenerating axons after physical injury, and maintaining calpastatin inhibited degeneration of transected axons *in vitro* and in the optic nerve *in vivo*. Calpastatin depletion also occurred in a caspase-dependent manner in trophic factor-deprived sensory axons and was required for this *in vitro* model of developmental degeneration. *In vivo*, calpastatin regulated the normal pruning of retinal ganglion cell axons in their target field. These findings identify calpastatin as a key checkpoint for axonal survival after injury and during development, and demonstrate downstream convergence of these distinct pathways of axon degeneration.

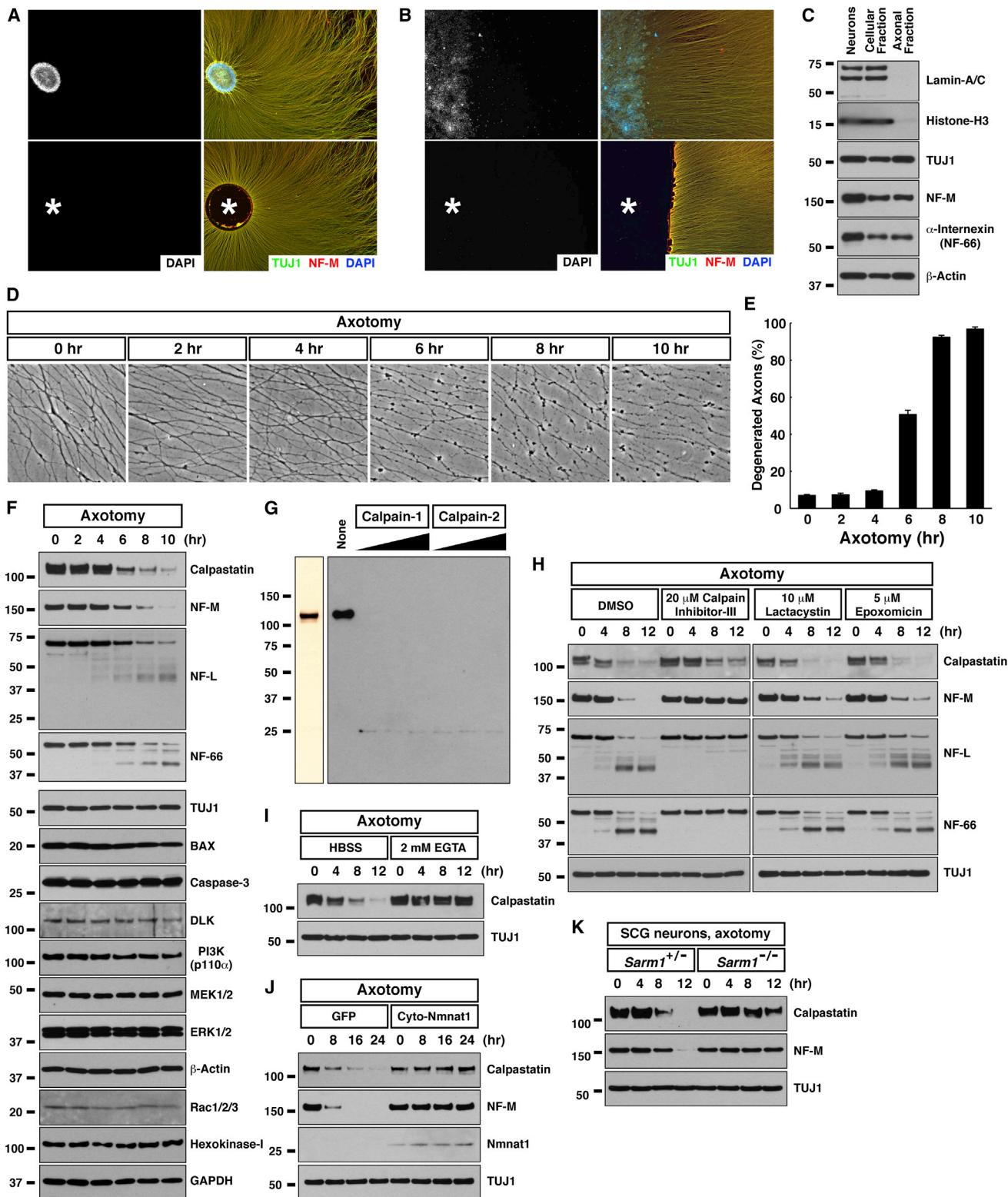
INTRODUCTION

Axon degeneration is characterized by elimination of axons or their branches, and results in loss of connectivity between neuronal cell bodies and their innervating targets. Axon degeneration has become increasingly appreciated as a critical feature of many neurological disorders including traumatic injury, Alzheimer's disease, Parkinson's disease, multiple sclerosis, and amyotrophic lateral sclerosis (Burke and O'Malley, 2012; Coleman, 2005; De Vos et al., 2008; Johnson et al., 2012; Wang et al., 2012). Damage to axons often precedes the onset of symptoms and neuronal cell death, further supporting the clinical relevance of axon degeneration in the progression of those dis-

orders. The functional preservation of axons is therefore thought to be an important therapeutic target for treatment of neurodegenerative disease.

An extensively studied model of axonal death is Wallerian degeneration. Following physical damage to a nerve, axon segments distal to the lesion site undergo a stereotyped form of self-destruction (Waller, 1850) characterized by loss of the action potential, destruction of the cytoskeleton, membrane fragmentation, and demyelination (Coleman, 2005; Coleman and Freeman, 2010). The discovery of the Wallerian Degeneration Slow (*Wld^s*) mouse (Lunn et al., 1989), and the subsequent characterization of the *Wld^s* fusion protein, whose gain-of-function delays Wallerian degeneration of distal axon segments for an extended period (Mack et al., 2001), demonstrated that the demise of injured axons is not triggered simply by loss of nutritive support from the neuronal cell body, but is instead regulated by an intrinsic mechanism in axons (Coleman, 2005; Coleman and Freeman, 2010). In support of this notion, we and our colleagues have recently shown that loss-of-function of the endogenous gene *Sarm1* also significantly delays Wallerian degeneration in mice, with a protective effect that is comparable to that observed in the *Wld^s* mutant (Osterloh et al., 2012). Despite these advances, the full molecular cascade underlying Wallerian degeneration remains to be elucidated.

Axon degeneration is not unique to neurodegenerative disorders, but is also widely observed as a normal feature of the development of nervous systems (Luo and O'Leary, 2005). The selective elimination of exuberant axons is crucial for the precise wiring of neural circuits. A classic example of axon degeneration in the developing nervous system is observed in the mammalian retinotectal system. During development, the axons of temporal retinal ganglion cells initially overshoot their future termination zone in the superior colliculus. Later, axon segments distal to the termination zone are stereotypically pruned through local degeneration (Feldheim and O'Leary, 2010). Studies in the past decade have revealed a variety of molecular signals responsible for eliciting axon pruning in different contexts, including activation of Semaphorin-Plexin or Eph-Ephrin signals. More recently, a critical role of the classic apoptotic pathway in developmental axon degeneration has begun to emerge. A signaling cascade involving the proapoptotic effector BAX and downstream

**Figure 1. Depletion of Axonal Calpastatin during Injury-Induced Degeneration**

(A and B) Enrichment of axonal proteins from neuronal cultures. Cultures of DRG explants (A) or dissociated DRG neurons (B) were set up, and their neuronal cell bodies were removed. Axons were visualized by immunostaining; asterisks denote the regions of neuronal cell bodies before the removal.

(C) Protein samples prepared from 5,000 dissociated DRG neurons, or their cell body fraction and axonal fraction, were subjected to immunoblot analysis.

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caspases, including caspase-9, caspase-3, and caspase-6, is essential for axonal death in *in vitro* models of pruning triggered by trophic-factor deprivation, and contributes to axon degeneration in the developing retinotectal system (Nikolaev et al., 2009; Simon et al., 2012; Vohra et al., 2010).

Interestingly, Wallerian degeneration appears independent of the apoptotic degeneration pathway (Simon et al., 2012; Vohra et al., 2010; Whitmore et al., 2003), and conversely, expression of *Wld^s* protein or genetic knockout of *Sarm1* is ineffective in delaying developmental axon degeneration in mouse or fruitfly (Hooper et al., 2006; Osterloh et al., 2012). Thus, whether divergent signaling pathways in pathological or developmental axon degeneration share particular regulatory step(s) remains unclear.

Calpains are Ca^{2+} -activated cysteine proteases implicated in a variety of physiological processes, whose aberrant activation can also result in proteolysis of vital cellular components (Goll et al., 2003; Liu et al., 2004). In mammals, the most well-characterized members of the calpain family are calpain-1 (μ -calpain) and calpain-2 (m -calpain), which are ubiquitously expressed in tissues, including the peripheral and central nervous systems (Croall and DeMartino, 1991; Goll et al., 2003). Calpain activity is critically controlled not only by intracellular Ca^{2+} , but also by the endogenous proteinaceous inhibitor calpastatin (Croall and DeMartino, 1991; Goll et al., 2003). Calpastatin binds and inhibits calpains via its calpain-inhibitor domains when the proteases are activated by Ca^{2+} (Hanna et al., 2008). Importantly, persistent activity of calpains can result in destruction of calpastatin, which has led to the notion that calpastatin is a “suicide inhibitor,” ensuring that calpains are kept in check in the case of transient fluctuations of intracellular Ca^{2+} and only reach their full proteolytic potential in the prolonged presence of the cation (Blomgren et al., 1999; Nakamura et al., 1989). Therefore, the cellular content and distribution of calpastatin, as well as its balance with calpain activity, are key determinants of how efficiently Ca^{2+} -activated calpains are inhibited (Goll et al., 2003; Hanna et al., 2008).

Previous studies have linked the overactivation of calpains to neurodegenerative disorders (Araújo and Carvalho, 2005; Camins et al., 2006; Liu et al., 2004). Calpains have also long been implicated in axon degeneration triggered by physical injury *in vitro* (George et al., 1995), and recently exogenous transgenic expression of calpastatin was used to provide definitive evidence for calpain involvement in sciatic and optic nerve degeneration after transection *in vivo* (Ma et al., 2013). Endogenous calpastatin has been proposed to play prosurvival roles

in adult neurons under degenerative conditions, including in models of ischemia-excitotoxicity (Bano et al., 2005; D’Orsi et al., 2012; Rao et al., 2008; Takano et al., 2005; Vosler et al., 2011) and MPTP/6-hydroxydopamine-induced parkinsonism (Crocker et al., 2003; Grant et al., 2009). However, whether endogenous calpastatin exerts any role in regulating survival specifically of axons in neurodegeneration models has not been determined. Moreover, involvement of calpastatin and calpains in other types of axon degeneration, e.g., in developmental pruning, remains unexplored.

We report that calpastatin is depleted rapidly in the injured axons of cultured neurons as well as in transected nerves, concomitant with full-scale activation of calpains and axonal death. Axons with a reduction of endogenous calpastatin undergo accelerated Wallerian degeneration, whereas, consistent with recent results (Ma et al., 2013), maintaining the protein’s level protects injured axons both in neuronal cultures and *in vivo*. Interestingly, the calpastatin-calpain system is also involved in axon degeneration during development. Occurring downstream of the caspase cascade, depletion of calpastatin, followed by calpain activation, is required for the death of nerve growth factor (NGF)-deprived sensory axons *in vitro*. Furthermore, calpastatin critically regulates developmental pruning of retinal ganglion cell axons *in vivo*. These results provide evidence that calpastatin functions as an endogenous determinant of axonal survival both after injury and during development, and that distinct pathways of axon degeneration converge on this checkpoint prior to dismantling the axons.

RESULTS

Enrichment of an Axonal Fraction from Neuronal Cultures

To study molecular mechanisms underlying axon degeneration, we optimized an approach allowing efficient enrichment of the axonal fraction for biochemical analysis, from either explant cultures or dissociated neuron cultures. With 4',6-diamidino-2-phenylindole (DAPI) staining, >99% of neuronal cell bodies could be reliably removed by this procedure (Figures 1A and 1B). To confirm separation of cell body contents from the axonal fraction, we compared the distribution of several cellular markers (Figure 1C). Cytoskeleton components (microtubules, neurofilaments, and actin filaments) were relatively evenly distributed among the cell body and axonal fractions, but the nuclear

(D–F) Depletion of axonal calpastatin during degeneration of injured axons. Representative phase-contrast images of axons at the indicated time points following axotomy are shown (D). The percentage of degenerated axons was quantified (E), mean \pm SEM; 1 μg of axonal protein harvested at the same time points were subjected to immunoblot analysis (F).

(G) Direct proteolysis of calpastatin by calpains. Recombinant mouse calpastatin was purified as described in the *Supplemental Experimental Procedures*, and protein purity was assessed by silver staining (left panel). Two hundred fifty nanograms calpastatin protein was incubated with increasing amounts of calpain-1 or calpain-2 in the presence of 5 mM Ca^{2+} at 37°C for 30 min, and then subjected to immunoblot analysis.

(H and I) Inhibition of calpains but not the proteasome preserved calpastatin in axotomized axons. DRG explants were treated with the small-molecule inhibitors for 1 hr prior to axotomy. One microgram of axonal protein harvested at the indicated time points were subjected to immunoblot analysis.

(J) *Wld^s/Nmnat1* functions upstream of calpastatin depletion. DRG neurons were transduced by lentivirus overexpressing GFP control or cyto-Nmnat1 as described in the *Supplemental Experimental Procedures*. One microgram of axonal protein harvested at the indicated time points postaxotomy were subjected to immunoblot analysis.

(K) Calpastatin depletion occurs downstream of *Sarm1*. 0.5 μg of axonal protein harvested from *Sarm1*^{+/-} or *Sarm1*^{-/-} SCG neurons at the indicated time points after axotomy was subjected to immunoblot analysis.

See also Figure S1.

proteins lamin-A/C and histone H3 were effectively excluded from the axonal fraction. This approach therefore enabled us to focus on molecular events occurring specifically in axons during degeneration. As well, while the *in vitro* experiments in the current study are largely based on mouse embryonic dorsal root ganglion (DRG) sensory neurons, this axon/neurite-enriching approach is also applicable to other neurons, including embryonic cortical neurons, embryonic motor neurons, and superior cervical ganglion neurons (data not shown), and thus represents a valuable tool for investigating axon degeneration in a variety of neuronal types.

Calpastatin Regulates the Survival of Physically Injured Axons

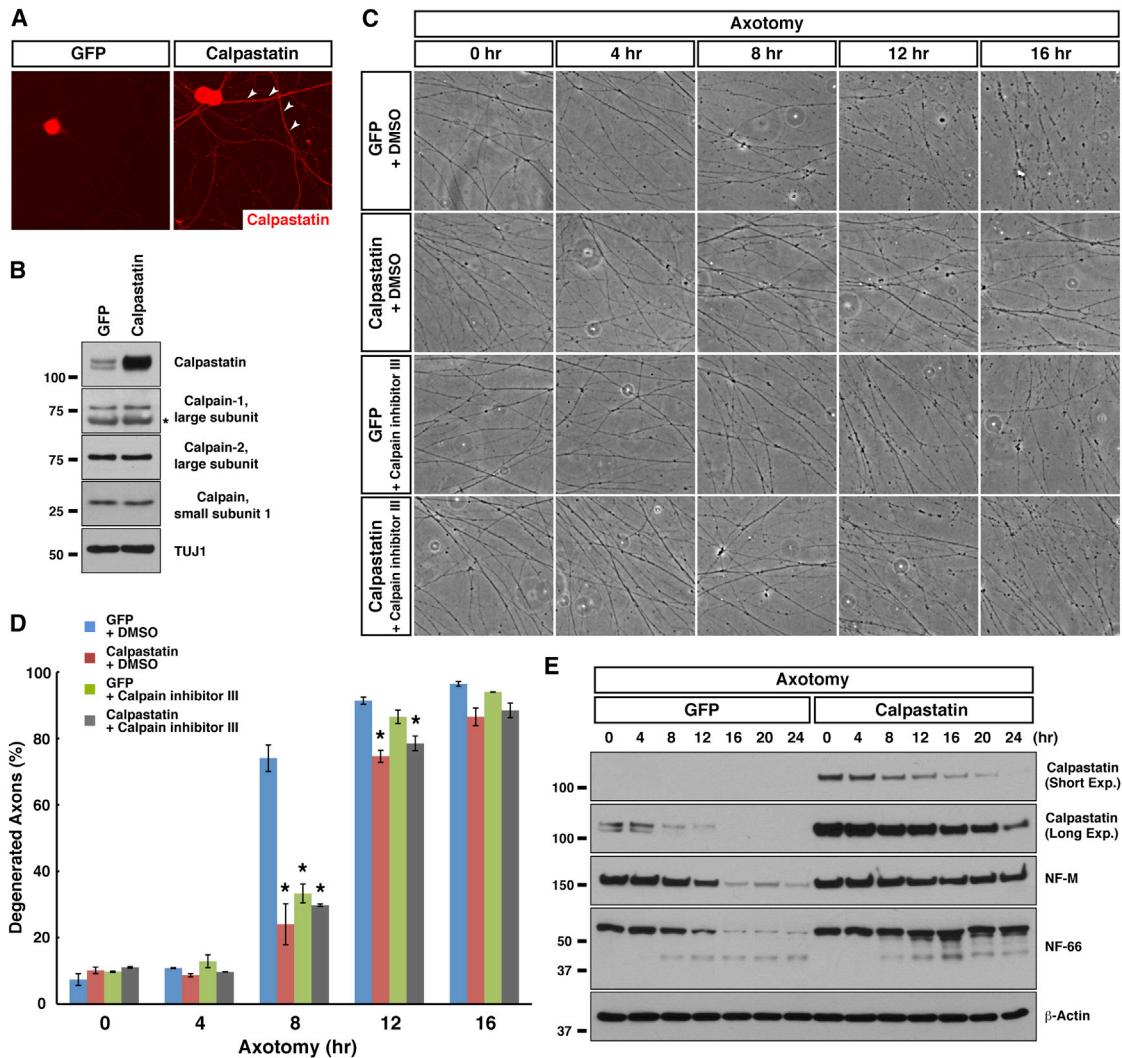
To explore critical regulators of axon degeneration, we first used a well-studied *in vitro* model of Wallerian degeneration (Figures 1D and 1E). Embryonic day 12.5 (E12.5) to E13.5 DRG explants were cultured with NGF. Upon separation from their cell bodies, axons remained intact for the first 4 hr, although all their growth cones collapsed (data not shown; Sasaki and Milbrandt, 2010). Signs of degeneration, including blebbing and fragmentation along axons, became evident around 6 hr postaxotomy, and the vast majority of axons degenerated at 8–10 hr postaxotomy (Figures 1D and 1E). We carried out biochemical analysis of axonal fractions prepared during this degenerative process to examine candidate proteins previously implicated in neuronal cell death. An abrupt decrease of the calpastatin level in axons was observed after axotomy at the 6 hr and later time points but not before (Figure 1F). Interestingly, this change of calpastatin level temporally correlated with the morphological degeneration of axons (Figure 1E), as well as the calpain-mediated degradation of neurofilaments, specifically neurofilament-M, neurofilament-L, and α -internexin/NF-66 (Figure 1F, and see below), consistent with a role for calpastatin in axon degeneration. Calpastatin appeared as doublet bands around 110 kDa, likely reflecting isoforms with different sites of transcription initiation (Goll et al., 2003).

Of note, levels of α -tubulin and β -tubulin protein monomers appeared unchanged during the degeneration of injured axons (Figure 1F; data not shown), providing a useful control for normalization of protein samples, even though polymerized axonal microtubules, visualized by TUJ1 immunostaining, were disassembled (Figures S1D and S2C available online; data not shown; Zhai et al., 2003). Moreover, the majority of the candidate proteins we examined also remained largely intact through the degenerative process, including proteins involved in the apoptotic pathway (BAX and caspase-3), Wallerian degeneration (DLK), trophic signaling (PI3K-p110 α , MEK1/2, and ERK1/2), cytoskeletal dynamics (β -actin and Rac1/2/3) and metabolism (Hexokinase-I and GAPDH; Figure 1F). Thus, the degeneration of injured axons does not involve nonspecific degradation of axosolic proteins, and the degradation of calpastatin and other select proteins is a specific response to injury.

Calpastatin functions as a “suicide inhibitor” for calpains to keep the proteases in check when they are transiently activated by intracellular calcium; however, when calcium levels increase persistently, calpains can eventually proteolyze calpastatin to overcome its inhibitory effect (Blomgren et al., 1999; Nakamura et al., 1989). Indeed, we observed in an *in vitro* biochemical

assay that calpastatin was readily degraded by both calpain-1 and calpain-2 (Figure 1G). Because calpain activity has been implicated in axon degeneration triggered by physical injury *in vitro* (George et al., 1995), the destruction of calpastatin that we observed could be due to activated calpains in axons. However, degradation of calpastatin has also been suggested to be mediated by the proteasome (Mellgren, 1997). To distinguish these possibilities, we inhibited the two types of proteases with small-molecule inhibitors. As a precaution, we compared three commonly used calpain inhibitors, ALLN, z-Val-Phe-CHO (calpain inhibitor-III), and MG-132, with two specific proteasome inhibitors, lactacystin and epoxomicin. ALLN, calpain inhibitor-III, and MG-132 showed comparable inhibitory activity toward calpain-1 and calpain-2, while lactacystin and epoxomicin had no effect (Figure S1A). However, treatment with ALLN, MG-132, lactacystin, or epoxomicin all resulted in the accumulation of polyubiquitinated proteins in axons, reflecting their inhibition of proteasome activity (Figure S1B). Therefore, among the three common calpain inhibitors, calpain inhibitor-III appeared relatively specific to calpains, whereas ALLN and MG-132 inhibited both calpains and the proteasome. When axotomized axons were treated with calpain inhibitor-III, it preserved the protein level of calpastatin, as well as inhibiting proteolysis of neurofilaments (Figure 1H). The observation that calpain inhibitor-III did not completely inhibit calpastatin depletion might be explained by the high binding affinity (low nanomolar range) of calpastatin toward calpains (Goll et al., 2003). The depletion of calpastatin after axotomy was also blocked in the presence of the Ca^{2+} -chelator EGTA (Figure 1I). In contrast, proteasome inhibition by lactacystin or epoxomicin had no effect on the calpastatin level (Figure 1H). Together, these results suggested that calpastatin is degraded in injured axons by calpains.

Previous studies have shown that the *Wld^s* mutant protein can significantly delay the degeneration of injured axons in mice (Mack et al., 2001). We compared the calpastatin level in axotomized axons overexpressing GFP (as a control) or a cytosolic version of Nmnat1(R125A/R127A; cyto-Nmnat1), which has been shown to be functionally equivalent to the protective *Wld^s* fusion protein (Sasaki et al., 2009). While calpastatin was rapidly degraded in control axons, depletion was completely blocked in the presence of cyto-Nmnat1 for at least 24 hr (Figure 1J), supporting the view that *Wld^s*/Nmnat1 inhibits an upstream step leading to calpastatin depletion. In addition, we examined depletion of calpastatin in *Sarm1*^{-/-} axons, which are also protected against Wallerian degeneration (Osterloh et al., 2012). Compared to the axons of *Sarm1*^{+/-} SCG neurons, the protein was significantly preserved in injured *Sarm1*^{-/-} axons (Figure 1K), suggesting that calpastatin depletion occurs downstream of the *Sarm1*-dependent degeneration signal. Interestingly, we observed that the previously documented depletion of axonal Nmnat2 or SCG10 after axotomy (Gilley and Coleman, 2010; Shin et al., 2012) was not prevented by cyto-Nmnat1 overexpression or *Sarm1*-knockout (Figure S1C; data not shown). Thus, there is not a necessary correlation between protection against Wallerian degeneration and maintenance of axonal proteins. The preservation of calpastatin therefore reflects a specific effect of *Wld^s*/Nmnat1 expression or *Sarm1* deletion, and fits with calpastatin being an integral component of the degeneration mechanism.

**Figure 2. Calpastatin Regulates Axonal Survival after Injury**

(A and B) Overexpression of calpastatin in axons. DRG neurons were subjected to lentiviral transduction, and exogenously-expressed calpastatin in axons was detected by immunostaining (A). One microgram of axonal protein harvested from GFP- or calpastatin-expressing neurons was subjected to immunoblot analysis (B). Asterisk denotes a nonspecific band cross-reacting with the calpain-1 large subunit antibody.

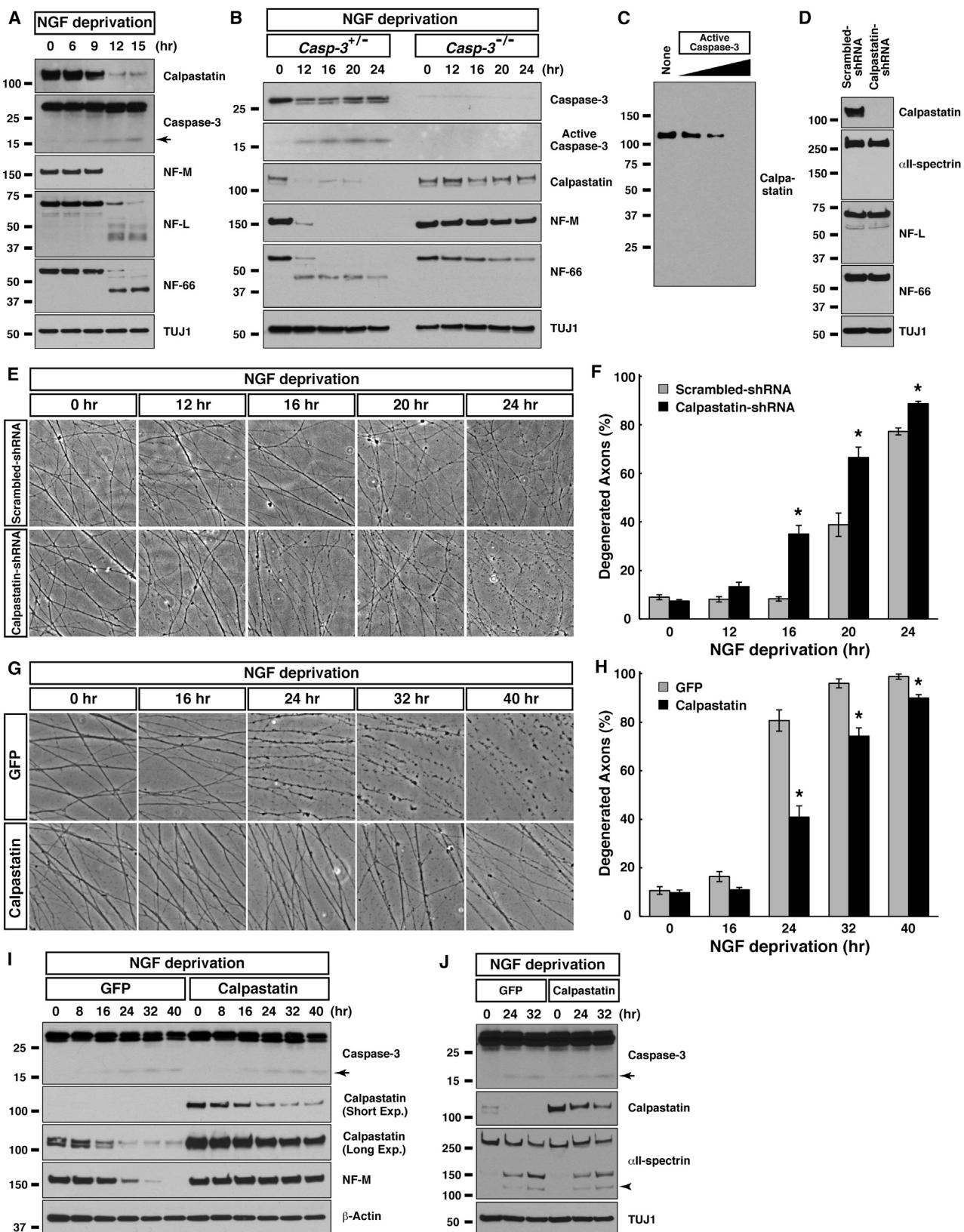
(C and D) Axonal calpastatin inhibited injury-induced degeneration. GFP- or calpastatin-expressing DRG neurons were treated with DMSO or 20 μ M calpain inhibitor-III for 1 hr prior to axotomy. Representative phase-contrast images of axotomized axons at the indicated time points are shown (C). The percentage of degenerated axons was quantified (D), mean \pm SEM, *p < 0.01.

(E) Calpastatin functions as a suicide-inhibitor. One microgram of axonal protein harvested from GFP- or calpastatin-expressing neurons at the indicated time points postaxotomy was subjected to immunoblot analysis.

As an aside, in the course of these experiments, we observed that, consistent with previous reports (MacInnis and Campenot, 2005; Zhai et al., 2003), inhibition of proteasome activity in the axons of SCG (sympathetic) neurons led to a significant delay of degeneration upon axotomy (Figures S1D and S1E). However, treatment of axotomized DRG (sensory) axons with lactacystin or epoxomicin showed no effect on neurofilament proteolysis (Figure 1H) or axon degeneration (Figure S2C) following axotomy. Similarly, the proteasome inhibitors failed to block the injury-induced degeneration of embryonic motor neuron axons, or either pre- or postcrossing spinal commissural axons (data not shown), suggesting that the previously reported involvement of

the proteasome in Wallerian degeneration is not universal but rather neuronal-type dependent.

Given the temporal correlation between the depletion of axonal calpastatin and the degeneration of physically injured axons, it appeared plausible that calpastatin could function as a checkpoint for axonal survival. We therefore tested whether an increase of the calpastatin level can enhance its capacity to protect injured axons against degeneration. DRG neurons were transduced with lentivirus expressing calpastatin, which resulted in a significant increase of the protein's level in both cell bodies and axons compared to neurons transduced with a control lentivirus expressing GFP (Figure 2A). The transduction rate under



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our culture conditions was over 95% across experiments (data not shown). Immunoblot analysis of axonal fractions showed that expression of exogenous calpastatin was over 5-fold greater than the endogenous level (Figure 2B). Despite this increase, the axons exhibited indistinguishable morphology compared to GFP-expressing axons (Figure 2C). In addition, there was no detectable change in the axonal level of calpain-1, calpain-2, or their common small subunit (Figure 2B). Following axotomy, the majority of control GFP-expressing axons degenerated by 8 hr (Figures 2C and 2D). Indeed, the increase in calpastatin significantly delayed the degeneration, i.e., over 70% of axotomized axons remained intact after 8 hr, compared to less than 25% in controls (Figures 2C and 2D). Moreover, although most of the calpastatin-overexpressing axons showed blebbing signs 16 hr postaxotomy, their morphological continuity was still maintained, which contrasted sharply with the complete fragmentation of control axons. Consistent with this, the integrity of neurofilaments in calpastatin-overexpressing axons was also significantly protected over a more extended time course (Figure 2E). Prior studies have demonstrated an exquisite inhibitory specificity to calpastatin (Goll et al., 2003), but to confirm that this observed protection of axons by calpastatin relied on its inhibition of calpains, we examined the effect of calpain inhibitor-III on calpastatin-overexpressing axons. Treatment of control axons with calpain inhibitor-III delayed degeneration following injury, though to a weaker extent than with calpastatin overexpression. Importantly, there was no additive protection when calpain inhibitor-III was added to calpastatin-overexpressing axons (Figures 2C and 2D), supporting the model that calpastatin functions as a critical regulator of axonal survival following injury through its inhibition of calpains.

It is interesting to note that, although exogenously expressed calpastatin preserved axonal survival, the protein itself was continuously proteolyzed with the same time course as the endogenous protein (Figure 2E). This is consistent with the model that as a suicide inhibitor, calpastatin suppresses full-scale activity of calpains downstream of the signal leading to initial activation of the proteases. The destruction of exogenously expressed calpastatin might explain the residual cleavage of α -internexin / NF-66 seen in calpastatin-overexpressing

axons at time points when axons were still largely intact; alternatively, the small fraction of neurons not transduced by the lentiviral construct might also account for this.

Calpastatin Regulates Degeneration of Trophic-Factor Deprived Axons

Axon degeneration occurs not only in pathological conditions, but also as part of normal neural development. Therefore, we explored the possibility that the calpastatin-calpain system might be involved in developmental axon degeneration. As an in vitro model of developmental pruning, axons of embryonic DRG neurons undergo caspase-dependent degeneration upon acute deprivation of NGF (Nikolaev et al., 2009; Vohra et al., 2010). Interestingly, rapid depletion of axonal calpastatin was observed with the same time course as that of axon degeneration (Figure 3A). Moreover, calpastatin degradation temporally correlated with activation, 9–12 hr after NGF-deprivation, of caspase-3 (Figure 3A), a key effector of axon degeneration in this in vitro paradigm (Simon et al., 2012). To test whether depletion of calpastatin was downstream of caspase-3 activation, axonal calpastatin was examined in caspase-3^{-/-} DRG neurons, which are completely resistant to degeneration upon NGF withdrawal in this assay (Simon et al., 2012). Strikingly, while the protein was largely degraded around 12 hr in NGF-deprived caspase-3^{+/+} axons, its level remained unchanged in caspase-3^{-/-} axons for at least 24 hr, the latest time examined (Figure 3B). Of note, the starting level of axonal calpastatin was not altered in caspase-3^{-/-} axons compared to caspase-3^{+/+} axons.

Previous studies have revealed that calpastatin can be a substrate of active caspase-3 (Kato et al., 2000; Neumar et al., 2003; Pörn-Ares et al., 1998; Wang et al., 1998a). Consistent with this, we found in the biochemical assay that calpastatin protein can be directly proteolyzed by active caspase-3 (Figure 3C). On the other hand, in a cell-free assay for neurofilament fragmentation, efficient cleavage of axonal neurofilament-M, neurofilament-L, or α -internexin/NF-66 was not observed with active caspase-3, but was observed with calpain-1 or calpain-2, which generated proteolytic patterns resembling those seen in the degenerating axons after axotomy (Figure S2A). Further, the cleavage of these neurofilament components also occurred in NGF-deprived

Figure 3. Calpastatin Regulates Caspase-Dependent Axon Degeneration during Trophic-Factor Deprivation

- (A) Depletion of axonal calpastatin correlates with caspase-3 activation during NGF deprivation. DRG explants were treated with a final concentration of 50 μ g/ml NGF-neutralizing antibody. One microgram of axonal protein harvested at the indicated time points was subjected to immunoblot analysis. Arrow denotes the 17-kDa large fragment of cleaved caspase-3.
- (B) Caspase-3-dependent calpastatin depletion in NGF-deprived axons; 0.5 μ g of axonal protein harvested from caspase-3^{+/+} or caspase-3^{-/-} DRG explants at the indicated time points was subjected to immunoblot analysis.
- (C) Direct proteolysis of calpastatin protein by active caspase-3; 250 ng calpastatin protein was incubated with increasing amounts of active caspase-3 at 37°C for 30 min, and then subjected to immunoblot analysis.
- (D) Endogenous calpastatin was not required for axonal survival under physiological conditions; 1 μ g of axonal protein harvested from DRG neurons targeted by scrambled-shRNA or calpastatin-shRNA was subjected to immunoblot analysis.
- (E and F) Knockdown of endogenous calpastatin accelerates axon degeneration upon NGF deprivation. Representative phase-contrast images of the axons at the indicated time points are shown (E). The percentage of degenerated axons was quantified (F), mean \pm SEM, *p < 0.01.
- (G and H) Increase of axonal calpastatin inhibits the degeneration of NGF-deprived axons. Representative phase-contrast images of GFP- or calpastatin-expressing axons at the indicated time points are shown (G). The percentage of degenerated axons was quantified (H), mean \pm SEM, *p < 0.01.
- (I and J) Calpastatin functions downstream of caspase-3 to regulate the axonal survival; 1 μ g of axonal protein harvested from GFP- or calpastatin-expressing DRG neurons at the indicated time points following NGF deprivation was subjected to immunoblot analysis. Arrows denote the 17-kDa fragment of cleaved caspase-3, and arrowhead denotes the 115-kDa fragment of α II-spectrin released by caspase-3 cleavage.

See also Figure S2.

wild-type or caspase-3^{+/-} but not caspase-3^{-/-} axons (Figures 3A and 3B). Together, these genetic and biochemical data support a model in which active caspase-3 in NGF-deprived axons directly cleaves calpastatin, which releases the suppression of calpains, leading to neurofilament degradation and axon degeneration.

To determine the role of endogenous calpastatin in regulating the degeneration of NGF-deprived axons, we transduced DRG neurons with shRNAs against calpastatin to reduce the endogenous protein to an undetectable level in axons (Figure 3D). Intriguingly, there was no spontaneous axon degeneration with this significant reduction of calpastatin level, as assessed by the phase-contrast images (Figure 3E) or by the integrity of axonal neurofilament components or nonerythroid α -spectrin (α II-spectrin; Figure 3D), a known calpain and caspase substrate (see below). This observation fits with the fact that mice with genetic ablation of *calpastatin* are viable, and do not show detectable signs of spontaneous neurodegeneration in the central nervous system (Takano et al., 2005), suggesting that calpastatin is not required for the general viability of axons under normal physiological conditions. However, when the calpastatin-depleted axons were subjected to NGF deprivation, they underwent accelerated degeneration, i.e., whereas axons targeted by control scrambled-shRNA were intact at 16 hr following NGF deprivation, a significant fraction of axons targeted by calpastatin-shRNA had already degenerated at this time point. This acceleration of the degenerative process was also evident at later time points (Figures 3E and 3F). Conversely, we tested whether increasing the calpastatin level in axons could prolong their survival during NGF deprivation. We employed the same strategy to transduce DRG neurons with the lentivirus expressing calpastatin. In contrast to control GFP-overexpressing axons, which degenerated around 24 hr after NGF withdrawal, most calpastatin-overexpressing axons remained intact at this time point. Moreover, a portion of calpastatin-overexpressing axons persisted until 40 hr postdeprivation, a late time point at which control axons had completely fragmented (Figures 3G and 3H). Consistent with the preservation of morphological integrity, the destruction of neurofilaments in calpastatin-overexpressing axons was also blocked (Figure 3I).

Importantly, activation of caspase-3 was unaffected in calpastatin-overexpressing axons during NGF deprivation when compared to control (Figure 3I), further supporting that calpastatin functions downstream of caspase-3. To rule out the possibility that in the presence of exogenously expressed calpastatin, the cleaved caspase-3 might somehow stay inactive, we examined cleavage of axonal α II-spectrin. Calpain- and caspase-mediated degradation of α II-spectrin both give rise to fragments around 150 kDa detected by a monoclonal antibody (clone AA6), but further cleavage of the 150-kDa fragment by caspase-3 results in a degradation product of 115 kDa (Jänicke et al., 1998; Wang et al., 1998b). Consistent with the involvement of caspase-3 in axon degeneration resulting from NGF deprivation but not physical injury (Simon et al., 2012), the appearance of the 115-kDa α II-spectrin fragment was evident only during the degeneration of NGF-deprived but not axotomized axons (Figure S2B). Importantly, this caspase-3-specific cleavage of α II-spectrin was indistinguishable between control

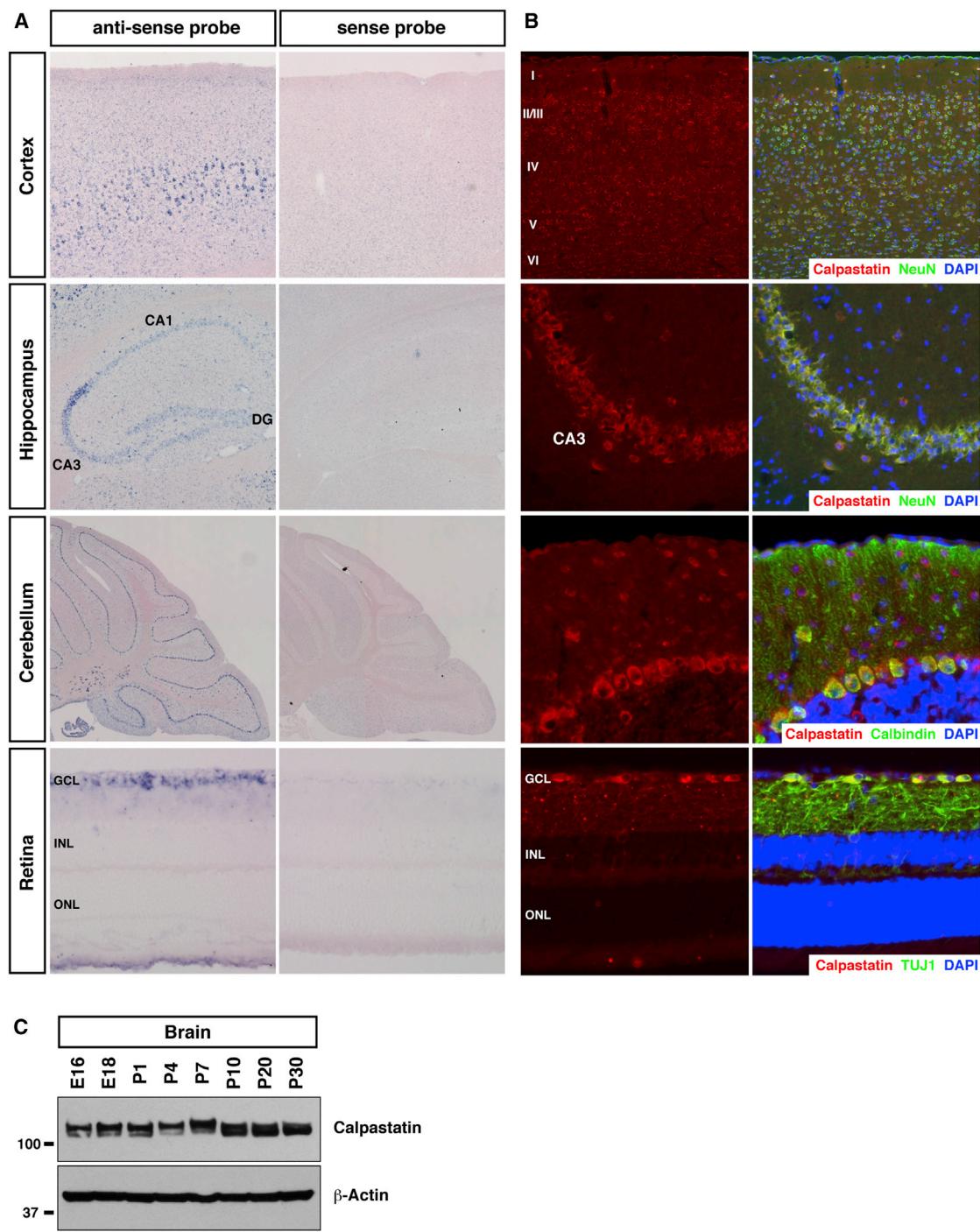
GFP- and calpastatin-overexpressing axons upon NGF deprivation (Figure 3J), suggesting that maintaining the calpastatin level can preserve axonal survival even when caspase-3 is functionally activated. Together, the results demonstrate that calpastatin regulates axon degeneration downstream of the NGF deprivation-induced caspase cascade.

Calpastatin Is Broadly Expressed in the Nervous Systems

To begin to examine the function of calpastatin in regulating axon degeneration *in vivo*, we studied the expression pattern of calpastatin in the nervous systems. We first carried out *in situ* hybridization (ISH) to probe expression of *calpastatin* mRNA in the mouse central nervous system. *Calpastatin* mRNA is broadly detected throughout the adult brain, including cortex and hippocampus, and is highly enriched in Purkinje cells in the cerebellum and in retinal ganglion cells (Figure 4A). Next, we performed immunohistochemistry (IHC) to determine the expression of calpastatin protein, in combination with several neuronal markers (NeuN, calbindin, or TUJ1; Figure 4B). Matching our ISH result, calpastatin protein is expressed in neuronal populations in the cortex, hippocampus (e.g., pyramidal neurons), cerebellum (e.g., Purkinje neurons), and retina (e.g., retinal ganglion cells). In addition, axonal calpastatin was revealed by IHC in the optic nerve and sciatic nerve (Figure 5A and Figure S4A). Finally, we examined expression of calpastatin in the developing brain. ISH showed that *calpastatin* mRNA is expressed across the developing brain at postnatal day 1 (Figure S3A), with higher levels in several regions including the cortex, the pontine formation, the facial motor nucleus, and the inferior olive. Consistent with this, calpastatin protein is expressed in the brain through developmental stages from E16 to P30 as assessed by immunoblot (Figure 4C). Interestingly, we observed a shift of calpastatin during development from a larger to a small isoform, which are thought to derive from transcripts with different initiation sites (Goll et al., 2003). Together, these results demonstrate that calpastatin is broadly expressed in the central and peripheral nervous systems, both during development and in adulthood, supporting a role for calpastatin in regulating axonal survival in different contexts.

Calpastatin Regulates Wallerian Degeneration *In Vivo*

To test whether endogenous calpastatin plays a regulatory role in Wallerian degeneration *in vivo*, we examined the protein level in axons following nerve transection. Resembling our observations *in vitro*, there was a dramatic decrease of the calpastatin level in RGC axons following optic nerve injury (Figures 5A and 5B), which correlated temporally with the degeneration process (Figures 5D and 5E). Depletion of axonal calpastatin also occurred following sciatic nerve transection (Figures S4A and S4B). Interestingly, we noticed that there was a significant increase of calpastatin-expressing nonneuronal cells in the sciatic nerve after transection, which may represent infiltrating macrophages (Figure S4A and data not shown). In fact, presumably because of these nonneuronal cells, there was actually an increase of calpastatin in the total protein prepared from sciatic nerves undergoing Wallerian degeneration (Figure S4C). This result is in line with a previous study showing that there was no

**Figure 4. Calpastatin Is Broadly Expressed in the Nervous System**

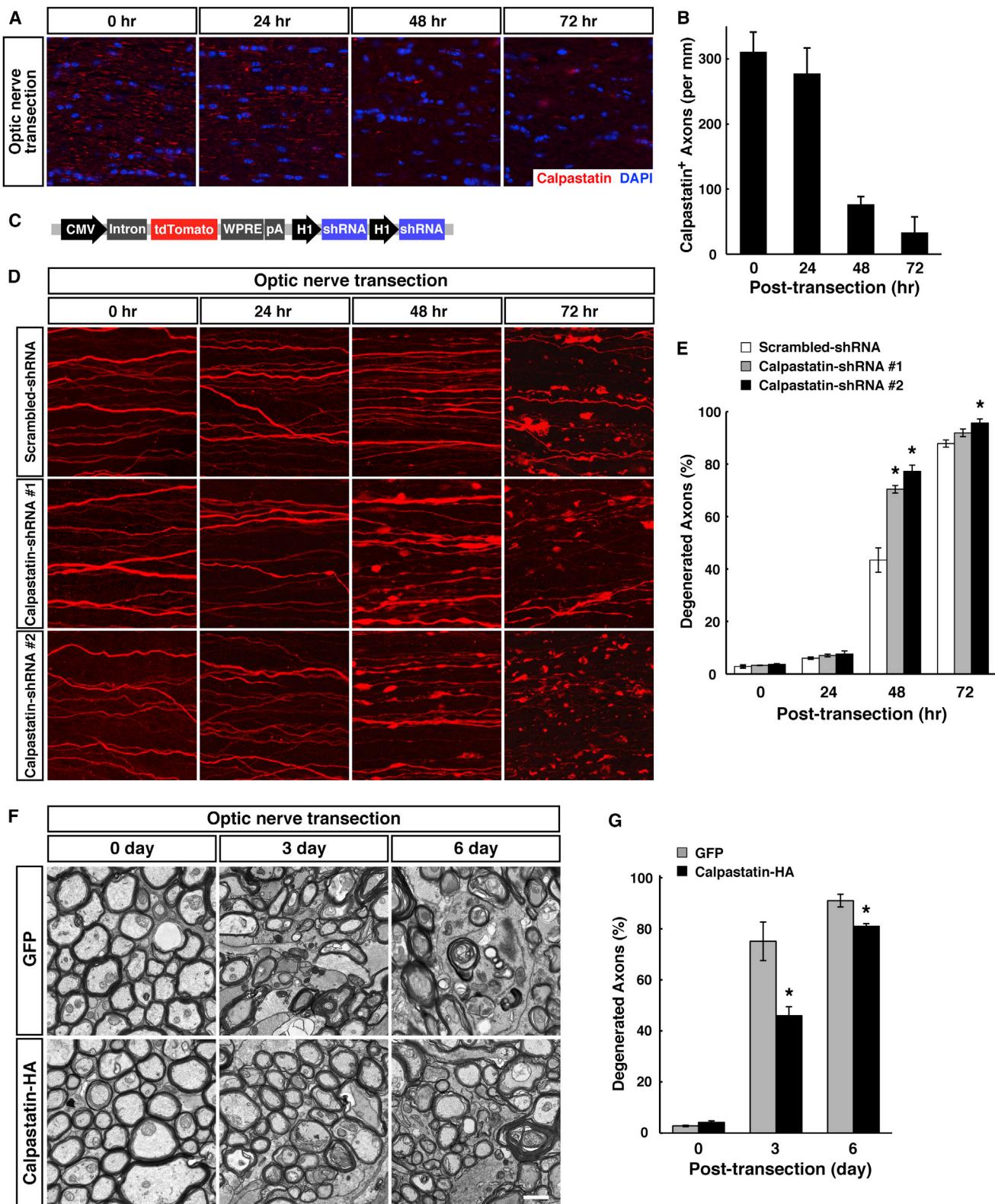
(A and B) Calpastatin is broadly expressed in the nervous system. In situ hybridization (A) and immunohistochemistry (B) were carried out on sagittal sections of brain and retina from 8-week-old mice. DG, dentate gyrus; GCL, ganglion cell layer; INL, inner nuclear layer; ONL, outer nuclear layer.

(C) Expression of calpastatin in the developing brain; 20 μ g of total protein prepared from mouse brains at the indicated developmental stages were subjected to immunoblot analysis.

See also Figure S3.

decrease of total calpastatin in sciatic nerve at various time points after injury (Glass et al., 2002), but emphasizes the importance of examining the protein level specifically in axons.

To determine the function of endogenous calpastatin in Wallerian degeneration, we constructed adeno-associated virus (AAV2) expressing shRNAs against calpastatin together with a

**Figure 5. Calpastatin Regulates Wallerian Degeneration In Vivo**

(A and B) Depletion of axonal calpastatin in the transected optic nerve. Optic nerve transection was performed, and axonal calpastatin in the nerves was examined by immunohistochemistry at the indicated time points after injury (A). The number of calpastatin-positive axons was quantified (B), n = 3 for each time point, mean \pm SEM (legend continued on next page)

TdTomato fluorescent marker (Figure 5C and Figure S4D). We sparsely transduced RGCs via intravitreal injection of these AAV2 vectors, which enabled us to directly image TdTomato-labeled RGC axons in the optic nerve (see [Experimental Procedures](#)). Following optic nerve transection, RGC axons targeted by control scrambled-shRNA remained intact at 24 hr, and a fraction of axons then started to degenerate at 48 hr, with the majority fragmented at 72 hr after injury. Consistent with our *in vitro* observations, shRNA-knockdown of endogenous calpastatin did not result in spontaneous degeneration of RGC axons (Figure 5D), again indicating that calpastatin is dispensable for general viability of axons. However, RGC axons targeted by calpastatin-shRNAs showed a significant acceleration of Wallerian degeneration after optic nerve transection, as the number of degenerated axons at 48 hr was almost double that seen with control scrambled-shRNA, and the degeneration process was almost complete by 72 hr (Figures 5D and 5E).

Conversely, we examined the effect of calpastatin overexpression on the degeneration of injured RGC axons. Intravitreal delivery of AAV2 vector expressing mouse calpastatin-HA transduced 70 to 80 percent of total RGCs in the retina, as estimated by colocalization of HA-staining either with TUJ1-positive RGCs in retinal cryosections (Figure S4E, upper panels) or with Brn3-positive cells in the whole-mount retina preparation (Figure S4E, lower panels). GFP-expressing AAV2 achieved a similar transduction rate of RGCs (data not shown). Without injury, GFP- and calpastatin-HA-expressing RGC axons appeared to possess normal myelination and axonal cytoskeleton, as assessed by electron microscopy (Figure 5F). Three days after optic nerve transection, most GFP-expressing axons had undergone demyelination and destruction of their cytoskeleton (Figures 5F and 5G), consistent with that observed with our TdTomato-labeling approach (Figure 5E). After 6 days, a complete breakdown of axonal structures was observed across the entire nerves (Figures 5F and 5G). In contrast, there was a significant protection of calpastatin-expressing RGC axons against Wallerian degeneration: over 50% of the axons retained a tightly packed myelin-sheath and intact cytoskeleton 3 days after injury, and some axons still appeared intact at 6 days post-transection (Figures 5F and 5G). In parallel, we carried out immunoblot analysis of the protein extracts from transected nerves, and expression of calpastatin-HA significantly protected neurofilament components after injury (Figure S4F). We also observed a depletion of exogenously expressed calpastatin-HA in transected nerves (Figure S4F), resembling our *in vitro* results (Figure 2E). Together, these results support the idea that the calpastatin cell autonomously regulates Wallerian degeneration *in vivo*.

(C–E) Knockdown of endogenous calpastatin in RGC axons accelerated the degeneration following transection. Intravitreal injection of viral vectors was carried out as described in the [Experimental Procedures](#). Design of AAV2-shRNA vectors for labeling of RGC axons is shown in (C). Representative maximum-intensity-projection images of TdTomato-labeled RGC axons at the indicated time points posttransection are shown (D). The percentage of degenerated axons was quantified (E), $n = 4$ per condition for each time point, mean \pm SEM, * $p < 0.01$.

(F and G) Increase of calpastatin level in RGC axons inhibited Wallerian degeneration. RGCs were transduced by AAV2 vectors expressing GFP or hemagglutinin (HA)-tagged calpastatin. Representative electron-microscope images of RGC axons at the indicated time points posttransection are shown (F). The percentage of degenerated axons was quantified (G), $n = 4$ per condition for each time point, mean \pm SEM, * $p < 0.01$.

See also [Figure S4](#).

Calpastatin Functions Downstream of Caspases in Developmental Pruning

Finally, we explored the function of calpastatin in developmental degeneration of axons *in vivo*. The stereotyped pruning of developing RGC axons in the superior colliculus was chosen as the model system because it has been shown to occur via local degeneration ([Feldheim and O'Leary, 2010](#); [Hooper et al., 2006](#)). Moreover, our recent studies have demonstrated that this degeneration process involves the same caspase cascade that operates in the degeneration of NGF-deprived sensory axons ([Simon et al., 2012](#)). We employed an *in utero* electroporation approach to target RGCs at E13 and then examine their labeled axons in the superior colliculus at indicated postnatal days (see [Experimental Procedures](#)); this approach yields equivalent results to the more classic Dil-labeling method ([Simon et al., 2012](#)). First, we determined the function of endogenous calpastatin in the pruning by electroporating control scrambled-shRNA or calpastatin-shRNA plasmids into the RGCs of wild-type mice. Because RGC axons are normally pruned back between postnatal day 1 to day 7 (P1–P7; [Feldheim and O'Leary, 2010](#); [Simon et al., 2012](#)), the mice were examined at P3 when the pruning is in process. Strikingly, while there was still a large number of axons present in the extreme posterior edge of the colliculus (posterior superior colliculus [PSC]; Figure 6A) in control scrambled-shRNA condition, the vast majority of axons targeted by calpastatin-shRNA had already been pruned back (Figures 6B and 6C). Thus, the endogenous calpastatin cell autonomously regulates developmental axon degeneration *in vivo*. Next, we tested whether overexpression of calpastatin could inhibit pruning of RGC axons by examining GFP- or calpastatin-electroporated wild-type mice at P6, when the normal pruning process should have been largely completed. We observed 3-fold more axons in the PSC of calpastatin-electroporated animals compared to GFP-electroporated controls (Figures 6D and 6E), showing that increasing the calpastatin level delays axon pruning *in vivo*. Of note, expression of GFP or calpastatin did not affect the initial overshooting of RGC axons past the future termination zone on the superior colliculus at P1 (Figure S5A).

Our results with the *in vitro* model of NGF deprivation have shown that caspase-3 activation functions upstream of calpastatin in axon degeneration. In fact, a similar delay of RGC axons pruning has also been reported in *caspase-3*^{-/-} mice ([Simon et al., 2012](#)). We therefore set out to determine the genetic interaction of *caspase-3* and *calpastatin* *in vivo*. Neither shRNA-knockdown of calpastatin (Figures 6F and 6G) nor overexpression of calpastatin (Figures 6H and 6I) modified the delayed pruning of RGC axons in *caspase-3*^{-/-} animals, suggesting that calpastatin and caspase-3 function in the same

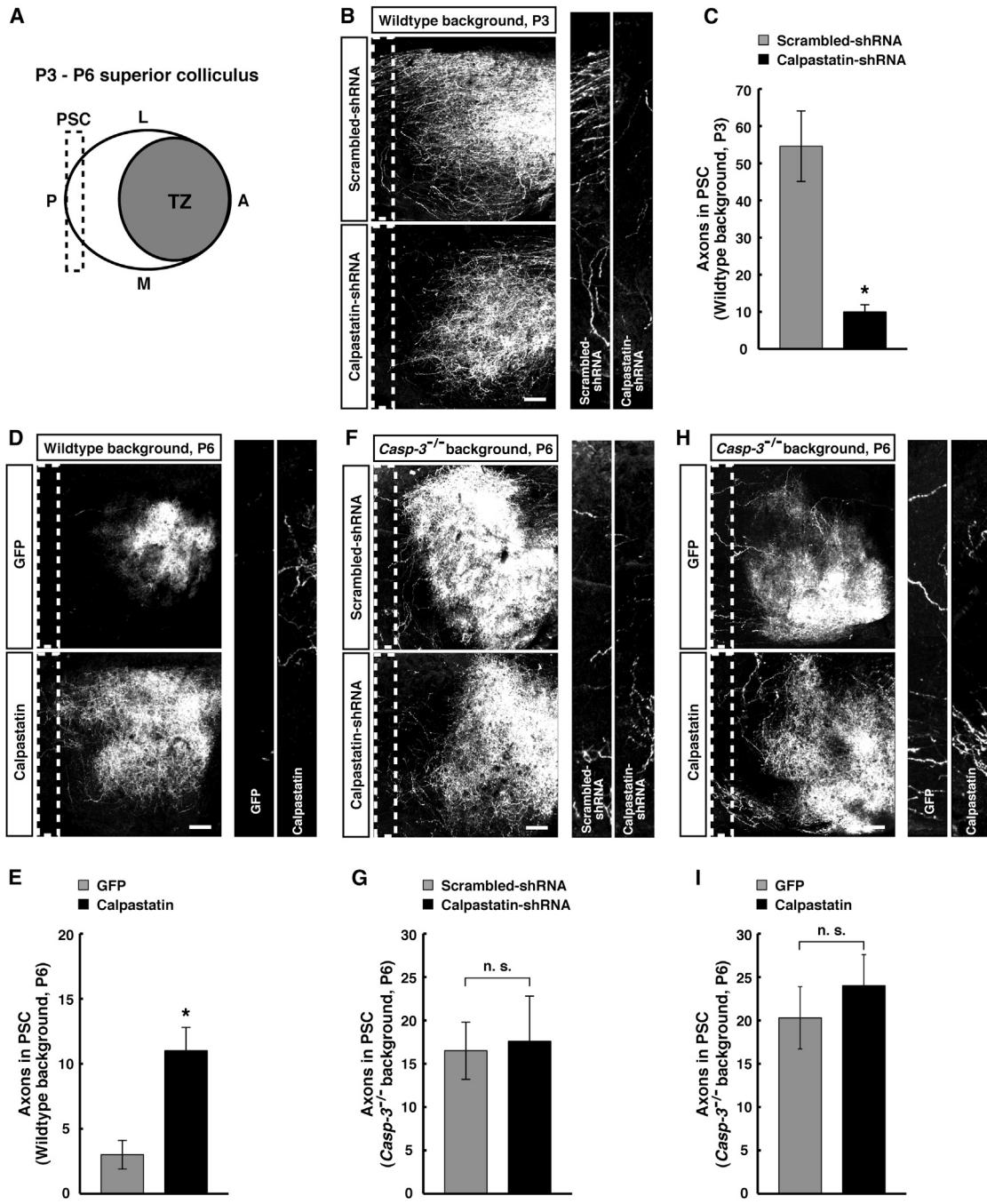


Figure 6. Calpastatin Regulates the Developmental Pruning of RGC Axons Downstream of Caspase-3

In utero electroporation of RGCs in E13.5 mouse embryos, and the imaging of superior colliculus at the indicated postnatal days were carried out as described in the [Experimental Procedures](#).

(A) Diagram of mouse superior colliculus (SC). A, anterior; P, posterior; M, medial; L, lateral; and TZ, termination zone. The PSC is defined as the region within 100 μ m of the posterior edge of the SC.

(B and C) Knockdown of endogenous calpastatin accelerated the pruning of RGC axons. Representative maximum-intensity-projection images of DsRed-positive axons within the SC of scrambled-shRNA or calpastatin-shRNA-electroporated mice at postnatal day 3 (P3) are shown (B). The number of DsRed-positive axons in the PSC regions was quantified (C), n = 7 for scrambled-shRNA and n = 4 for calpastatin-shRNA.

(D and E) Increasing the calpastatin level delayed the pruning of RGC axons. Representative maximum-intensity-projection images of DsRed-positive axons within the SC of GFP- or calpastatin-electroporated mice at P6 are shown (D). The number of DsRed-positive axons in the PSC was quantified (E), n = 5 for GFP and n = 13 for calpastatin.

(legend continued on next page)

genetic pathway in the developmental degeneration of RGC axons. These results further support that calpastatin functions downstream of caspase-3 to regulate developmental axon degeneration *in vivo*.

DISCUSSION

In this study, we provide evidence identifying endogenous calpastatin as a key regulator of axonal survival in both injury-induced and developmental degeneration. Our results suggest that destruction of endogenous calpastatin in injured axons releases its inhibitory action on calpains, leading to full-scale calpain activation and the demise of axons. Conversely, increasing the axonal level of calpastatin via viral delivery inhibited degeneration in neuronal cultures as well as *in vivo*, consistent with the protection recently reported in calpastatin transgenic mice *in vivo* (Ma et al., 2013). Unexpectedly, this calpastatin-calpain regulatory module also functions in models of developmental axonal death involving the classic apoptotic pathway, with depletion of endogenous calpastatin downstream of caspase activation required for degeneration. The fact that axons can be rescued by maintaining the calpastatin level without affecting upstream caspase activation implies that caspases are a trigger of the degenerative signal rather than direct executioners of axons. This might reflect the relatively stringent specificity of caspases, which renders the proteases inefficient at degrading axonal components such as neurofilaments (Posmantur et al., 1998; Figure S2A), an event required to dismantle axons. Instead, proteolysis of calpastatin by active caspase-3 releases inhibition of calpains, which are potent proteases with more promiscuous substrate specificity (Goll et al., 2003), ensuring a timely elimination of unwanted axons during development. This possibility is supported by our finding that the pruning of RGC axons in the superior colliculus is critically regulated by the calpastatin level (Figure 6). Because we observed that calpastatin is broadly expressed in the developing brain, it might function in regulating other instances of developmental axon degeneration as well.

We also found that the previously reported involvement of the proteasome in Wallerian degeneration (MacInnis and Campenot, 2005; Zhai et al., 2003) is neuronal-type dependent. In fact, among the four neuronal types tested, only the axotomized axons of SCG neurons could be protected by specific inhibitors of the proteasome. What the unique component(s) in SCG axons is that confers this proteasome-dependence remains to be elucidated. Intriguingly, while inhibition of the proteasome had little effect on physically injured axons of DRG neurons, it significantly blocked axonal death upon NGF deprivation by delaying caspase-3 activation (Figures S2C and S2D). Together with previous

studies in *Drosophila* (Watts et al., 2003), these results suggest a more conserved role of the proteasome in the regulation of developmental pruning, with a hierarchy of protease actions, i.e., the proteasome most upstream, caspases further downstream, and calpains further yet.

The activity of calpains depends on high concentrations of Ca²⁺, and depletion of endogenous calpastatin alone is not sufficient to cause axon degeneration *in vitro* or *in vivo* (Figures 3D and 5D). We therefore infer that, besides proteolyzing calpastatin, caspases need to activate additional downstream pathway(s) to increase cytosolic Ca²⁺. Different mechanisms for Ca²⁺ influx or loss of intracellular Ca²⁺ homeostasis have been proposed to underlie axon degeneration. Those mechanisms—including direct Ca²⁺ influx through the damaged plasma membrane (Schlaepfer, 1974), activation of voltage-gated Ca²⁺ channels (George et al., 1995), reverse operation of Na⁺/Ca²⁺ exchangers (LoPachin and Lehning, 1997), or opening of the mitochondrial permeability transition pore (Barrantes et al., 2011)—are not mutually exclusive. Depending on neuronal type as well as degenerative insult, one or more mechanism(s) could make a prominent contribution. In fact, we found that degeneration of embryonic DRG axons induced by either injury or NGF deprivation could both be inhibited by amiloride (Figure S2E), suggesting a role for Ca²⁺ influx via amiloride-sensitive ion channel(s) at least in these *in vitro* models. Regardless of the source of Ca²⁺ that activates calpains, our results highlight that calpastatin is a key checkpoint for axonal survival onto which multiple degenerative pathways converge (Figure S5B).

It will be of interest to determine whether calpastatin can exert a protective function in axons during the progression of neurodegenerative disorders besides traumatic injury, especially those with documented axonal dysfunction or damage. In fact, a significant reduction of calpastatin has been reported along dendrites of pyramidal neurons in the cortices of Alzheimer's disease patients (Rao et al., 2008). Whether the protein undergoes a similar change in the axonal/neurite compartments in other disease conditions requires further detailed examination. Meanwhile, sustaining the level of calpastatin via viral vectors, or delivering its derivative peptides, might be of therapeutic benefit in treating such neurodegenerative disorders. The approach of blocking the detrimental activity of calpains using their natural inhibitor could complement approaches based on conventional small-molecule inhibitors that, in the case of calpains, so far lack great specificity.

EXPERIMENTAL PROCEDURES

Antibodies, AAV2 preparation, and other information can be found in the [Supplemental Experimental Procedures](#).

(F and G) Knockdown of endogenous calpastatin did not alter the delayed pruning of RGC axons in caspase-3^{-/-} mice. Representative maximum-intensity-projection images of DsRed-positive axons within the SC of scrambled-shRNA or calpastatin-shRNA-electroporated caspase-3^{-/-} mice at P6 are shown (F). The number of DsRed-positive axons in the PSC was quantified (E), n = 11 for scrambled-shRNA and n = 7 for calpastatin-shRNA.
 (H and I) Increasing the calpastatin level had no additive effect on the delayed pruning of RGC axons in caspase-3^{-/-} mice. Representative maximum-intensity-projection images of DsRed-positive axons within the SC of GFP- or calpastatin-electroporated caspase-3^{-/-} mice at P6 are shown (H). The number of DsRed-positive axons in the PSC regions was quantified (I), n = 4 for GFP and n = 5 for calpastatin. Dashed boxes in (B), (D), (F), and (H) indicate the PSC regions shown with higher magnification. All the values are presented as mean ± SEM, *p < 0.01, n.s., not significant. Scale bar, 100 μm.
 See also Figure S5.

Intravitreal Injection and Optic Nerve Transection

All surgical procedures in mice were performed in compliance with the protocols approved by the IACUC of Genentech and the Rockefeller University. Intravitreal injection of AAV2 expressing calpastatin-HA or shRNAs against calpastatin was performed on 8- to 10-week-old mice. The animals were anesthetized, and a customized 33G 30°-beveled needle (Hamilton) attached to the Hamilton syringe was passed through the sclera into the vitreous. For overexpression of calpastatin-HA or GFP, 1×10^7 transduction units of AAV2 vectors were delivered, which transduced over 70% of total RGCs in the retina. For shRNA-knockdown, 5×10^3 transduction units of AAV2-shRNA vectors were delivered, which transduced approximately 500 to 1,000 RGCs in the retina. Three to four weeks after the viral injection, the animals were anesthetized, and the left optic nerves were completely transected via enucleation.

At indicated time points after transection, the nerve samples were processed either for immunohistochemistry or biochemical analysis as described in the *Supplemental Experimental Procedures*. To visualize TdTomato-labeled RGC axons, mice were transcardially perfused with ice-cold 4% paraformaldehyde/10% (w/v) sucrose/PBS. The optic nerves were postfixed in 4% paraformaldehyde / PBS overnight. After washing three times in PBS, the nerves were transferred into 30% sucrose/PBS at 4°C overnight. The segment of nerves about 2 mm proximal to the optic chiasm were imaged by confocal microscope, and 100–150 axons in each nerve sample were examined, with any sign of blebbing or fragmentation scored as degeneration. The p value was calculated by an unpaired two-tailed t test.

In Utero Electroporation of Retinal Ganglion Cells

In utero electroporation of RGCs was carried out as previously described (Garcia-Frigola et al., 2007; Simon et al., 2012). C57B/6 timed-pregnant females were anesthetized at E13. The uterine horns were exposed by an incision through the abdominal dermis and body wall muscle. The right eye of each embryo was injected through the uterine wall using a fine glass capillary needle connected to a pressure injector (Picospritzer, Parker), and 0.5 μl of 1 μg/μl plasmid mixture (CAGGS promoter-driven calpastatin-IRES-GFP or IRES-GFP control, mixed with DsRed plasmid at 1:1 molar ratio) was delivered, followed by the electroporation by application of a series of five square electrical pulses (36 V, 50 ms, 450 ms interval) through a pair of 3-mm-diameter paddle electrodes (Tweezertrode, BTX). The uterine horns were then placed back into the abdominal cavity, the incision was closed by suture, and the females were treated with an analgesic prior to recovery from anesthesia. In the whole-mount retina preparations from the electroporated pups, GFP/DsRed cotransfected RGC populations located primarily in the central region of the retina, and the cotransfection efficiency was observed to be >90% across the experiments.

To examine the developmental pruning of RGC axons in the superior colliculus, the electroporated pups were transcardially perfused with ice-cold 4% paraformaldehyde/10% (w/v) sucrose/PBS at indicated developmental ages. After postfixation overnight in 4% paraformaldehyde/PBS, each brain sample was immersed in PBS and stereotactically positioned under an objective lens (10× NA 0.6, Olympus). A volume of ~1.0 × 1.0 × 0.4 mm of the left superior colliculus was imaged en bloc by two-photon laser scanning microscopy with 1,020 nm pulsed laser (Mai Tai DeepSee laser, Spectra Physics) and 620/60 nm bandpass filter (Chroma), at a resolution 1.1 μm/pixel (Ultima IV, Prairie Technologies). The extent of axon pruning was determined by counting the number of DsRed-expressing axons within 100 μm of the posterior edge of the superior colliculus. The p value was calculated by an unpaired two-tailed t test.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures and five figures and can be found with this article online at <http://dx.doi.org/10.1016/j.neuron.2013.08.034>.

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