

Chapter 2

Calcium and Calpain Activation

Morin Ryu and Toru Nakazawa

Abstract Even with clinical treatment to reduce intraocular pressure (IOP), visual field defects still progress in many patients. Recent research has shown that the optic nerve head (ONH) is a key location in this process, where mechanical, ischemic, and inflammatory stresses are associated with axonal transport defects and retinal ganglion cell (RGC) apoptosis.

Glutamate, an excitatory neurotransmitter, causes excitotoxic damage in RGCs via *N*-methyl-D-aspartate receptors (NMDARs) in certain pathologic conditions, particularly ischemia. Activated NMDARs cause Ca^{2+} dysregulation, which activates the Ca^{2+} -dependent cascade and results in RGC degeneration, calpain activation, the degeneration of neuronal processes, and, finally, apoptosis.

Axonal transport damage induced by optic nerve crush (ONC) is commonly used as a model in glaucoma research. This model is known to induce deficits in neurotrophic factors in the central nervous system and ATP, to impair the mitochondria, to create oxidative stress and Ca^{2+} dysregulation, and to cause calpain activation.

Additionally, hyperglycemia-induced dysfunction and pathology occurs earlier in the RGCs than in the retinal vessels. Previous reports also identified Ca^{2+} dysregulation and calpain activation in this process.

Although various triggers induce RGC degeneration and apoptosis via various mechanisms, it is believed that they all result in Ca^{2+} dysregulation and calpain activation. Targeting this point of convergence thus holds promise as a neuroprotective therapy, especially for patients who do not respond to IOP-lowering treatment.

Keywords Calcium channel blocker • Calcium dysregulation • Calpain inhibitor • Neuroprotection • Oxidative stress

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2.1 Ca^{2+} and RGC Degeneration

Intracellular Ca^{2+} homeostasis plays a pivotal role in many biological processes, including fertilization, gene expression, and apoptosis [1, 2]. The products of cell metabolism accumulate with age and together with oxidative stress gradually impair Ca^{2+} homeostasis, making neurons more vulnerable to stresses that can lead to neurodegeneration [3, 4]. Ca^{2+} dyshomeostasis in these chronic pathologies comprises mitochondrial and endoplasmic reticulum (ER) dysfunction, Ca^{2+} buffering impairment, and glutamate excitotoxicity.

RGC degeneration in glaucoma has similarities with processes in other neurological diseases [5, 6] such as Parkinson’s disease and Alzheimer’s disease. These similarities include the accumulation of amyloid precursor protein [7], the dependence on neurotrophic factors [8], and the involvement of various Ca^{2+} -mediated cascades [6].

Intracellular Ca^{2+} dysregulation triggers many pathogenic mechanisms in the retina that affect RGC survival, including glutamatergic excitotoxicity via *N*-methyl-D-aspartate (NMDA) receptors [9], optic nerve blockade-induced RGC axonal damage (as occurs in the optic nerve crush (ONC) model) [10], and hyperglycemia-induced RGC degeneration [11]. All these mechanisms begin with a Ca^{2+} influx from extra- and intracellular storage in the ER and mitochondria into the cytoplasm, followed by the activation of Ca^{2+} -dependent cascades, ultimately resulting in RGC degeneration and apoptosis. These Ca^{2+} -dependent cascades include calpain, the caspase pathway, and oxidative stress.

Despite the varying etiology of glaucoma and other ocular neural degenerative diseases, there are many important common elements, including compartmentalized programs of degeneration targeting axons, dendrites, and cell bodies [12–14]. These programs, which are linked to several specific neuronal cascades, all converge in the dysregulation of Ca^{2+} [14] (Fig. 2.1).

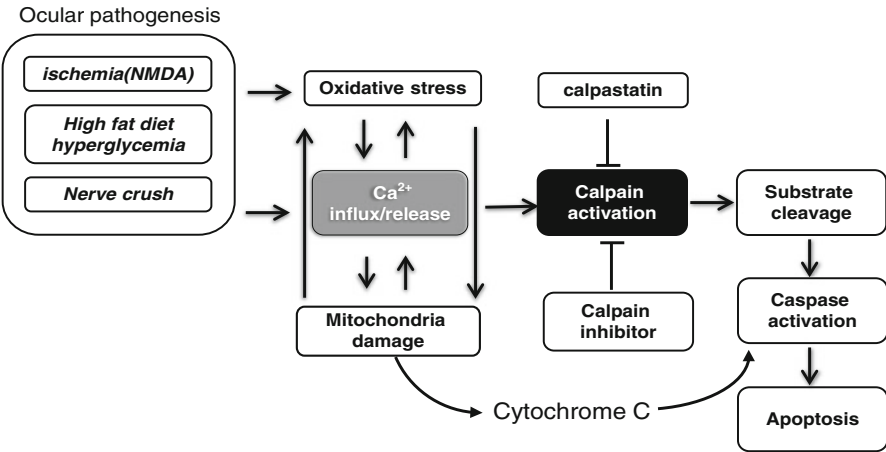


Fig. 2.1 Calcium dysregulation and calpain activation in RGCs

2.2 Calpain Activation

Calpains are a family of cytoplasmic, Ca^{2+} -activated, cysteine proteases. They are ubiquitous in the cells of all living creatures, from human beings to microorganisms. The enzymatic activity of calpains is regulated by Ca^{2+} and the endogenous inhibitory protein calpastatin [15].

In normal cells, Ca^{2+} concentration is lower than 0.05 mM. Under these conditions, calpains act as a biomodulator for Ca^{2+} -dependent events, such as signal transduction, cell proliferation, cell cycle progression, differentiation, and apoptosis [16]. The most abundant members of the calpain family are calpains 1 and 2. They differ in their sensitivity to Ca^{2+} , with their half maximal Ca^{2+} requirements in vitro being approximately 3–50 mM and 400–800 mM, respectively [16]. The level of Ca^{2+} required to activate calpains in vivo, however, has not been determined.

Uncontrolled and prolonged calpain-mediated proteolysis has been associated with a number of neurodegenerative conditions, including Parkinson's disease, Alzheimer's disease [17, 18], stroke [19], and trauma [20].

In the RGCs, calpain activation has been demonstrated in many pathologies, such as in the optic nerve crush model [10], the hyperglycemia model [11], and the NMDA-induced excitotoxic injury [21]. Calpains have various substrates including their endogenous inhibitor calpastatin [22], caspase 3, poly (ADP-ribose) polymerase [23], fodrin [24], p53 [25], p35 [26], and calcineurin [27]. Cleavage of calpastatin, fodrin, p35, or calcineurin is associated with the induction of apoptosis.

2.3 Pathologies Causing Ca^{2+} Dysregulation and Calpain Activation

2.3.1 *Oxidative Stress and Ca^{2+} Dysregulation*

The central nervous system (CNS) becomes increasingly susceptible to oxidative stress with age. DNA, proteins, and lipids are all affected [28, 29]. Markers of oxidative stress, such as lipid peroxidation [30], protein carbonyl [31], and advanced glycation end products (AGEs) [32], are also detectable in glaucoma models and in the tissues of glaucoma patients [33].

Furthermore, NF-E2-related factor 2 (Nrf2), a transcription factor that plays a pivotal role in endogenous protection against oxidative stress, is implicated in glaucoma models [34]. Nrf2, which is activated by oxidative stress and electrophiles, controls various detoxifying enzymes and antioxidants. In normal conditions, the cytosolic regulatory protein Kelch-like ECH associated protein 1 (Keap1) represses Nrf2 and anchors it in the cytoplasm. When cells are exposed to oxidative or electrophilic stress, Nrf2 is released from Keap1 and translocates to the nucleus,

where it enhances the transcription of antioxidant genes [35]. The authors identified the expression of Nrf2 and its translocation in the RGCs with an ONC model. Furthermore, CDDO-Im, a potent activator of Nrf2, has a neuroprotective effect.

Oxidative stress impairs intracellular Ca^{2+} homeostasis, and the resulting Ca^{2+} overload causes additional oxidative stress, creating a positive feedback loop. Ca^{2+} dysregulation induces oxidative stress through several mechanisms, including an increased metabolic rate [36] and the activation of reactive oxygen species (ROS)-producing enzymes, such as nitric oxide synthase and nicotinamide adenine dinucleotide phosphate oxidase [37, 38]. ROS can directly damage proteins, lipids, and nucleic acids, thereby impairing mitochondrial respiration and depolarizing the mitochondrial membrane. This decreases the ability to buffer Ca^{2+} [39, 40]. Furthermore, it is likely that ROS not only increase the release of Ca^{2+} from the ER [41], but also directly damage the plasma membrane proteins (Ca^{2+} ATPase) responsible for maintaining Ca^{2+} homeostasis [42, 43].

2.3.2 Glutamate Toxicity

Elevated intracellular Ca^{2+} can cause the influx of Ca^{2+} from the extracellular environment or its release from the ER, where the Ca^{2+} concentration is 1,000 times higher than in the cytoplasm. Essentially, Ca^{2+} enters cells through two Ca^{2+} channels in the plasma membrane: receptor-operated channels (ROCs) and voltage-operated Ca^{2+} channels (VOCCs) [44]. These ROCs include *N*-methyl-D-aspartate receptors (NMDARs) and α -amino-3-hydroxy-5-methylisoxazole-4-propionate acid receptors (AMPA receptors). Ca^{2+} influx through the ROCs is activated by the direct binding of a specific agonist (e.g., glutamate).

RGC apoptosis in glaucoma has been attributed to glutamate-mediated toxicity [45]. Under hypoxic conditions, retinal cells release glutamate [46], exposure to which is toxic at high levels. Low but chronic levels of elevated glutamate have also been found to be toxic to ganglion cells [47].

Glutamate is the principal excitatory neurotransmitter in the CNS, which includes the retina. In response to a variety of insults, the balance of glutamate shifts to a net accumulation of extracellular glutamate, which induces the overactivation of glutamatergic ion channels in the neurons. Due to their high permeability to Ca^{2+} , neurons are injured by the activation of NMDARs, and treatment with NMDAR antagonists can reduce the damage associated with glutamatergic excitotoxic injury [48].

Nakazawa et al. showed that after NMDAR activation induced an influx of Ca^{2+} , the protein phosphatase 2A (PP2A) was activated to dephosphorylate Akt. Phosphorylated Akt is apoptosis suppressive and thus prevents RGC death. On the other hand, brain-derived neurotrophic factor (BDNF) stimulates the TrkB/ShcC/PI3K signaling pathway and results in an increase in phosphorylated Akt [9].

2.3.3 Hyperglycemia-Induced RGC Degeneration

Diabetic retinopathy (DR) remains one of the major causes of blindness [49]. DR is diagnosed based on vascular pathology findings in the retina, but in animal models, hyperglycemia has been observed to lead to neurological effects after 2 weeks [50]. In patients without retinopathy, the thickness of the circumpapillary retinal fiber layer has been observed to decrease with the progress of DR [51].

Hyperglycemia-related calcium dysregulation may be caused by a decrease in releasable Ca^{2+} stored in the ER [52], Ca^{2+} influx via impaired Ca^{2+} channels in the plasma membrane, and Ca^{2+} release from the ER and mitochondria [53]. The disturbance of Ca^{2+} homeostasis is a significant cause of RGC degeneration, as it leads to changes in synaptic plasticity, the dysfunction of neurons, and, eventually, apoptosis [54]. Oxidative stress also plays a key role in the development and progression of diabetes and its complications, by increasing the production of free radicals and impairing antioxidant defenses [55]. Hyperglycemia-induced overproduction of superoxide in the mitochondrial electron-transport chain seems to be the key event in the pathogenesis of all diabetic complications [56]. Diabetes mellitus can thus be considered to be among major metabolic disorders causing neuropathy via various mechanisms, including Ca^{2+} dysregulation.

Hyperglycemia combined with a high-fat diet as a model of metabolic stress has been observed to induce RGC axonal degeneration and apoptosis within 4 weeks [11]. In this model, Ca^{2+} dysregulation induced by hyperglycemia causes calpain activation and oxidative stress, leading to RGC apoptosis. Calpastatin-knockout mice, animals in which endogenous calpain inhibitors are defective, undergo enhanced RGC death in hyperglycemic conditions. Furthermore, RGC apoptosis and axonal degeneration are significantly prevented by SNJ-1945, a potent exogenous calpain inhibitor that can cross the blood–retinal barrier [57]. Together, these results indicate that calpain activation is a critical part of RGC degeneration in animal models.

2.3.4 Axonal Flow Damage and Optic Nerve Crush-Induced RGC Degeneration

Recent evidence indicates that the causes of RGC degeneration are compartmentalized into processes such as deficits in axonal transport, remodeling of dendrites and synapses, and physiological dysfunction. These new findings are changing the way we think about glaucoma [6].

It has been known for some time that glaucoma involves deficits in axonal transport. Since the 1970s [58, 59], research on this subject had focused on events in the optic nerve head (ONH) because that is the site of the mechanical blockade that causes axonal impairment [60, 61]. Blockade of the ONH causes retrograde

transport impairment, and the resulting deficit of neuronal survival factors (e.g., BDNF), which normally come from the brain, results in apoptosis [8].

Axonal transport impairment precedes and eventually causes axonal and somatic degeneration in glaucoma [62]. The disorders involved in this axonal transport deficit include changes in molecular motors [63] and cytoskeletal structures [64]. ONC is a common animal model of axonal impairment and is often used to research the pathologies involved in glaucoma and axonal degeneration [65–67]. In addition to ONC, the administration of vinblastine to the optic nerve also prevents axoplasmic flow [10]. The authors successfully established a new glaucoma model not involving mechanical damage to the axons. Vinblastine (VB) is a microtubule disassembly chemical that inhibits mitosis and stops axonal transport in RGCs by binding microtubular protein [68, 69]. Results obtained with this model indicated that RGC apoptosis involves BDNF deficits, impairment of the mitochondria, and calpain activation, a finding that agrees with a number of other reports [8, 70, 71].

2.4 Neuroprotective Therapy

All clinical treatments for glaucoma, including eyedrops and surgery, aim at lowering IOP. However, IOP-lowering drugs do not prevent the progress of visual field defects in all glaucoma patients, especially patients with normal IOP. It is therefore imperative to establish new neuroprotective treatments for these patients.

Neuroprotective therapy is directed specifically at the neurons. In glaucoma, neuroprotection is directed at the neurons in the central visual pathway, particularly at the RGCs, and does not target IOP or any other risk factors.

2.4.1 Calpain Inhibitors (Fig. 2.2)

Calpain activation is implicated in numerous mechanisms underlying glaucomatous RGC degeneration, suggesting that suppression of overactivated calpain pathways may be a good candidate for neuroprotection in glaucoma. This alternative treatment would be especially useful for patients with normal IOP.

The calpain inhibitor ((1S)-1-((((1S)-1-benzyl-3-cyclopropylamino-2,3-di-oxopropyl)amino)carbonyl)-3-methylbutyl)carbamic acid 5-methoxy-3-oxapentyl ester (SNJ-1945) is an agent with high cellular permeability and high metabolic stability [72], which has the potential to reduce retinal cell death in vivo and in vitro [10, 11, 73, 74] (Fig. 2.2). Compared to other calpain inhibitors, such as 4-fluorophenylsulfonyl-Val-Leu-CHO (SJA6017) and *N*-acetyl-Leu-Leu-Nle-CHO (ALLN), SNJ-1945 is readily administered orally [72]. In one report, SNJ-1945 reached a maximum concentration in the retina 15 min after oral administration of 10 mg/kg

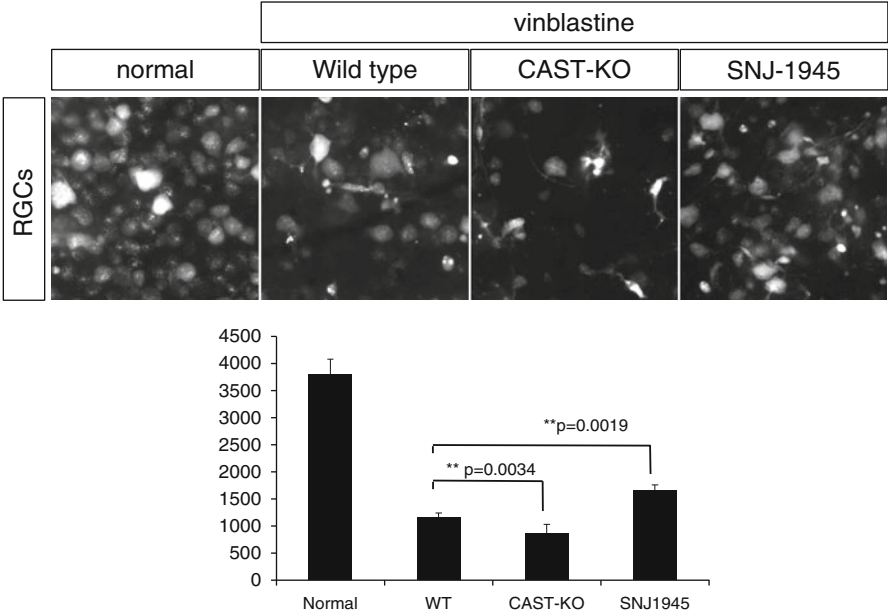


Fig. 2.2 Calpain inhibitor suppressed RGC death from axonal damage induced by vinblastine (CAST-KO, calpastatin-knockout mice; SNJ-1945, a novel calpain inhibitor)

and then gradually decreased over 8 h. The concentration of SNJ-1945 in the retina still maintained IC₅₀ against calpains 1 and 2 even 4 h after oral administration (Y. Shirasaki, Senju Pharmaceuticals, unpublished data).

2.4.2 Calcium Channel Antagonist

Adrenoceptor antagonists for lowering IOP also suppress the influx of Na⁺ and Ca²⁺ into the RGCs [75, 76]. This effect may explain the ability of beta-blockers to protect RGCs in DBA/2J mice, which are animals with inherited glaucoma [77]. The selective beta-blockers betaxolol and levobetaxolol have a greater suppressive effect on Ca²⁺ influx [78] than timolol, a nonselective beta-blocker. Furthermore, research in animals on the topical application of betaxolol and levobetaxolol has shown the necessary quantity at the back of the eye to effectively protect RGCs from various insults [79].

A number of independent clinical trials have compared the effect of betaxolol with timolol on both IOP and the progression of visual field (VF) loss in glaucoma patients [80, 81]. Some have reported that betaxolol provides greater suppression of the progression of VF loss than timolol, despite the greater reduction of IOP by timolol. The association between IOP reduction and VF preservation remains

unclear, but may involve neuroprotective mechanisms. In addition to reducing ischemic injury by acting as a vasodilator or Ca^{2+} influx suppressor, betaxolol can also increase the expression of BDNF mRNA in the retina [82].

The primary effect of calcium channel blockers (CCBs) is the inhibition of Ca^{2+} influx, resulting in the relaxation of vascular smooth muscle and consequent increased blood flow [83]. In an ischemic model, CCBs can reduce vascular constriction in the retina and optic nerve head [84].

Although CCBs primarily protect ischemic RGCs by restoring impaired blood flow through vasodilation, they also directly suppress Ca^{2+} influx-related cell death pathways, including ischemia- and excitotoxicity-induced apoptosis and necrosis [85].

An investigation exploring the effect of nimodipine (a Ca^{2+} channel antagonist) on ocular blood flow in NTG [86] found that while the drug did not affect blood flow or IOP, contrast sensitivity was dramatically improved in the treatment group. The researchers concluded that nimodipine's efficacy was a direct result of decreasing Ca^{2+} influx to the RGCs and their axons, a finding that agreed with other studies of Ca^{2+} channel antagonists.

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