**Chapter XX**

**Retinal Caveolin-1 Modulates Neuroprotective Signaling**

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**Abstract**: Caveolin-1 (Cav-1), the scaffolding protein of caveolae, is expressed in several retinal cell types and is associated with ocular pathologies. Cav-1 modulates neuroinflammatory/neuroprotective responses to central nervous system injury. We have shown that loss of Cav-1 results in a blunted cytokine response in retinas challenged with inflammatory stimuli. As neuroinflammatory and neuroprotective signaling overlap in their cytokine production and downstream signaling pathways, we hypothesized that loss of Cav-1 may also suppress neuroprotective signaling in the retina. To test this, we subjected mice in which Cav-1 was deleted specifically in the retina to a neurodegenerative insult induced by sodium iodate (NaIO3) and measured STAT3 activation, a measure of neuroprotective signaling. Our results show that Cav-1 ablation blunts STAT3 activation induced by NaIO3. STAT3 activation in response to intravitreal administration of the IL-6 family cytokine, leukemia inhibitory factor (LIF), was not affected by Cav-1 deletion indicating a competent gp130 receptor response. Thus, Cav-1 modulates neuroprotective signaling by regulating the endogenous production of neuroprotective factors.

**Keywords:** Caveolin-1, Cre/lox, Conditional knockout, Neuroprotection, Cytokines, Sodium Iodate, STAT3

**XX.1 Introduction**

Cav-1 is the principal protein of caveolae and is involved in cellular functions including endocytosis, mechanotransduction, and cell signaling (Parton and Simons, 2007). Cav-1 is expressed in several retinal cell types including retinal vasculature, retinal pigment epithelium (RPE) and Müller glia (Gu et al., 2014b; Gu et al., 2014a; Li et al., 2014). Cav-1 is linked to diseases with significant retinal pathologies including diabetic retinopathy and glaucoma (Klaassen et al., 2013; Thorleifsson et al., 2010) but its role in retinal neuroprotection is unknown. Retinal cells express several toll-like receptors (TLRs) that recognize and respond to pathogenic stimuli and initiate pro-inflammatory cytokine responses. Cav-1 associates with TLRs and regulates TLR signaling (Jiao et al., 2013). In addition to recruiting circulating leukocytes during inflammation, cytokines also act as ligands for neuroprotective signaling. In particular, IL-6 family cytokines including ciliary neurotrophic factor (CNTF) and LIF activate the JAK/STAT pathway, which upregulates anti-apoptotic factors to prevent retinal neuronal death (Chucair-Elliott et al., 2012; Lavail et al., 1992).

The purpose of this study was to determine if retina-specific ablation of Cav-1 alters expression of downstream neuroprotective signaling after insult. We subjected retina-specific Cav-1 knockout and littermate control mice to NaIO3 treatment which induces RPE damage and secondary retinal degeneration (Carido et al., 2014). We show that loss of Cav-1 dampens injury-induced STAT3 activation in the retina.

**XX.2 Materials and Methods**

***XX.2.1 Mice***

All procedures were carried out according to the Association for Research in Vision and Ophthalmology Statement for the Use of Animals in Ophthalmic and Vision Research and were approved by Institutional Animal Care and Use Committee of the University of Oklahoma Health Sciences Center. Retina specific Cav-1 KO mice (Retina-Cav-1-KO) were generated by crossing floxed Cav-1 mice carrying loxP sites inserted in intronic regions flanking exon 2 of the *Cav1* gene (Cao et al., 2003) with mice carrying Cre recombinase driven by the Chx10 promoter (Rowan and Cepko, 2004). Mice were backcrossed to generate littermate mice homozygous for the *Cav1* floxed allele that carried either Chx10-Cre (Retina-Cav-1-KO) or did not (littermate controls wild type for Cav-1 expression). Recombined retinal cells in Retina-Cav-1-KO mice were null for Cav-1 protein (Fig. XX.1b) as previously described for global Cav-1 KO mice generated using the same floxed construct (Cao et al., 2003).

***XX.2.2 Sodium Iodate and LIF injection***

Adult male and female retina-Cav-1-KO mice and littermate controls were systemically injected with 25mg/kg NaIO3 (Sigma-Aldrich, St. Louis, MO). In other experiments, 0.5 µg LIF in 1 µL PBS (Millipore, Billerica, MA) was injected intravitreally. Seven days after NaIO3 treatment or 24 h after LIF injection mice were euthanized by CO2 inhalation, eyes were prepared for eyecup flatmounts, histology, immunohistochemistry, and Western blot analysis.

***XX.2.3 Retinal Flatmount Preparation, Immunohistochemistry***

For eyecup flatmounts, enucleated eyes were fixed in 4% paraformaldahyde (PFA; Electron Microscopy Sciences, Hattfield, PA) in PBS for 10 min after a small incision was made at the limbus. Anterior segments, lens and vitreous were removed and eyecups were fixed for an additional 40 min. Retinas were then removed and resulting eyecups with RPE intact were permeabilized in PBS containing 1% Triton X-100. Immunohistochemistry was performed as previously described for retinas (Gu et al., 2014a). Eyecups were stained with FITC-Phalloidin (Life Technologies, Grand Island, NY) to label the actin cytoskeleton at RPE cell borders. Immunohistochemistry of retinal paraffin sections fixed with Prefer fixative (Anatech, Ltd., Battlefield, MI) was performed as described (Gu et al., 2014b) using rabbit polyclonal rabbit anti-Cav-1 (1:400, BD Biosciences, San Jose, CA) and monoclonal anti-α1-Na/K-ATPase (clone a6f; 1:100, DSHB, University of Iowa, Iowa City, IA). Imaging was performed on an FV1200 (Olympus, Tokyo, Japan) confocal microscope.

***XX.2.4 Western blotting***

Retinas were lysed in buffer containing 60 mM octylglucoside, 10 mM Tris-HCl, pH 7.4, 100 mM NaCl, 0.5 mM EDTA and 1 mM orthovanadate and Western blots were probed with: mouse monoclonal antibodies against β-actin (Sigma, 1:7500) and α-tubulin (Sigma, 1:500) and rabbit polyclonal antibodies against Cav-1 (BD Biosciences, 1:3000) and pSTAT3 (Cell Signaling, 1:1000). Imaging and densitometry were performed on an In Vivo F-Pro Image System (Carestream Health, Inc., Rochester, NY).

**XX.3 Results**:

***XX.3.1 Efficient Cav-1 deletion in Retina-Cav-1-KO mice***

The Chx10 promoter is expressed in neuroretinal progenitor cells during development (Rowan and Cepko, 2004) and Chx10-driven Cre expression promotes efficient recombination in retinal neurons and Müller glia (Chucair-Elliott et al., 2012). Recombination of floxed Cav-1 (Fig. XX.1a) resulted in loss of Cav-1 protein in the neural retina except for a small number of cells with Müller glial morphology (Fig. XX.1b). Cav-1 expression is retained in retinal vasculature and RPE as these cells are not targeted by Chx10-driven Cre. By quantitative mass spectrometry and Western blot densitometric analysis (not shown), Cav-1 protein was reduced by 70%. As the non-targeted retinal vasculature contributes to the remaining 30% of Cav-1 protein in whole retinal lysates, we estimate the deletion in targeted cells to be even more efficient.

***XX.3.2 Sodium iodate induces similar damage to the RPE of both genotypes.***

Intraperitoneal injection of NaIO3 specifically destroys the RPE resulting in secondary retinal injury resembling that observed in macular degenerations. As the RPE is not targeted by Chx10-Cre, Cav-1 expression in the RPE is retained similarly in both genotypes. Thus, RPE damage from NaIO3 should not differ between genotypes and any effects on the retina should be derived only from deletion of Cav-1 in the neural retina. In undamaged eyes, the hexagonal RPE cells are consistent in size and shape and are, in many cases, binucleated. Fig. XX.2a shows a typical WT RPE monolayer from an untreated Retina-Cav-1-KO mouse stained with Phalloidin and DAPI. As expected, 7 days after NaIO3 treatment, RPE damage was not different between genotypes (Fig. XX.2b,c). Fig. XX.2d,e show representative retinal sections from n = 4 eyes per genotype that also display similar damage. Quantitative assessment of retinal neuronal loss is difficult at this relatively early post-NaIO3 time, so we are not yet certain if loss of Cav-1 specifically in the neural retina/Müller glia results in enhanced neurodegeneration. Of note, retinal function as assessed by electroretinography was also virtually lost in both genotypes treated with NaIO3 (data not shown). As NaIO3 induced similar insults to the RPE in both genotypes, we next assessed whether Cav-1 deletion specifically in the retina resulted in altered endogenous neuroprotective signaling.

***XX.3.3 STAT3 activation is suppressed in NaIO3-treated Retina-Cav-1-KO retinas***

The typical retinal damage response results in local production of endogenous neuroprotective molecules including IL-6 family cytokines (Lavail et al., 1992; Chucair-Elliott et al., 2012). This results in activation of the IL-6 family signaling receptor, gp130, and a downstream STAT3 response. Thus, we assessed STAT3 activation in NaIO3-treated Retina-Cav-1-KO and control retinas by Western blot analysis for phosphorylated STAT3 as previously described (Chucair-Elliott et al., 2012). Sodium iodate treatment resulted in characteristic STAT3 activation in littermate controls which was dramatically suppressed in Retina-Cav-1-KO retinas (Fig. XX.3a). These results suggest that retinal Cav-1 modulates either the production of neuroprotective cytokines and/or the downstream activation of the gp130 receptor pathway.

To directly determine whether the gp130/STAT3 pathway is competent in Retina-Cav-1-KO retinas, we intravitreally injected LIF and assessed STAT3 activation. As shown in Fig. XX.3b, LIF induced equivalent STAT3 activation in both genotypes suggesting that the blunted neuroprotective response to NaIO3 is upstream of gp130.

**XX.4 Discussion**

Here we demonstrate the first successful generation of a conditional knockout mouse with efficient retina-specific Cav-1 deletion. Because Cav-1 has previously been linked to ocular pathologies (Klaassen et al., 2013; Thorleifsson et al., 2010), this mouse model allows us to test the retina-intrinsic roles of Cav-1 in retinal neuroprotection in a variety of disease-relevant insults. Using this unique mouse model we show here that retinal Cav-1 plays a critical role in modulating stress-induced neuroprotective signaling. As inducers of retinal STAT3 activation (e.g., CNTF) are currently in clinical trials for retinal degenerative diseases, understanding the endogenous signaling cascades that mediate retinal neuroprotection is essential. Our results provide evidence that retina-intrinsic Cav-1 promotes neuroprotective signaling upstream of the gp130 receptor. We have recently shown that Cav-1 supports the production of inflammatory cytokines such as IL-6 in response to inflammatory challenge (Li et al., 2014). In the context of these published results, the findings presented herein suggest that Cav-1 may also promote the damage-associated induction of neuroprotective cytokines but this remains to be determined directly. Intriguingly, Cav-1 regulates TLR4 activity (Jiao et al., 2013) outside of the eye and our results suggest that similar Cav-1-modulated innate immune receptors may also initiate damage responses in the retina. Because retinal pathology so often results in functional and/or morphological neuronal loss, the identification of Cav-1 as a potential neuroprotective modulator may be significant.

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# Figure Captions

**Fig. XX.1 a:** Cartoon illustrating deletion of exon 2 of Cav-1 by tissue-specific Cre recombinase expression. **b:** Cav-1 localization in retinal sections of conditional KO and the control retinas. Cav-1 (green), Na-K ATPase (red) and DAPI (blue).

**Figure XX.2** **a-c:** RPE damage is similar between genotypes at 7 day post-NaIO3 treatment. Eyecups were stained with Phalloidin (green) and DAPI (blue). **d, e:** Hematoxylin/eosin stained sections from Retina-Cav-1-KO and littermate controls after NaIO3 treatment.

**Figure XX.3** **a:** Blunted STAT3 activation in Retina-Cav-1 KO mice after NaIO3 treatment. **b:** Activation of STAT3 pathway by exogenous administration of LIF is not affected by retina-specific Cav-1 ablation.

Figure 1



Figure 2



Figure 3

