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# A biochemical basis for induction of retina regeneration by antioxidants

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## Abstract

The use of antioxidants in tissue regeneration has been studied, but their mechanism of action is not well understood. Here, we analyze the role of the antioxidant N-acetylcysteine (NAC) in retina regeneration. Embryonic chicks are able to regenerate their retina after its complete removal from retinal stem/progenitor cells present in the ciliary margin (CM) of the eye only if a source of exogenous factors, such as FGF2, is present. This study shows that NAC modifies the redox status of the CM, initiates self-renewal of the stem/progenitor cells, and induces regeneration in the absence of FGF2. NAC works as an antioxidant by scavenging free radicals either independently or through the synthesis of glutathione (GSH), and/or by reducing oxidized proteins through a thiol disulfide exchange activity. We dissected the mechanism used by NAC to induce regeneration through the use of inhibitors of GSH synthesis and the use of other antioxidants with different biochemical structures and modes of action, and found that NAC induces regeneration through its thiol disulfide exchange activity. Thus, our results provide, for the first time, a biochemical basis for induction of retina regeneration. Furthermore, NAC induction was independent of FGF receptor signaling, but dependent on the MAPK (pErk1/2) pathway.

### Keywords

Regeneration; NAC; Retina



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**Competing interests**

No competing interests declared.

**Author contributions**

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## 1. Introduction

Regeneration of damaged tissue would be the ultimate cure for many degenerative diseases. While some simple organisms and lower vertebrates are able to regenerate lost structures, or even an entirely new organism in the case of hydra and planarians, higher vertebrates do not have the innate ability to regenerate most tissues. Unfortunately, with increased organismal complexity, regenerative potential has been lost (Bely and Nyberg, 2010).

Stem cells are a common source for replenishing cells after injury or cell death in regenerating organisms. Specifically in the eye, stem cells present in the ciliary margin (CM) of several organisms are induced to proliferate and differentiate to replace damaged tissue (Fischer et al., 2013). In addition, stem cells must perform self-renewal to promote the maintenance of the stem cell niche, otherwise, exhaustion of these cells would hinder healing processes (Jopling et al., 2011). Understanding the mechanism of stem cell activation and maintenance will help contribute to the induction of the lost regenerative potential in higher vertebrates including humans.

Redox status, the balance of reactive oxygen species (ROS) and cellular antioxidants, is one of the main regulators of stem cell self-renewal (Sart et al., 2015). A hypoxic niche and a low level of intracellular ROS is important for the maintenance of stem cells (Lonergan et al., 2007) because, as the levels of ROS increase, mainly due to shifts in metabolism, ROS can act as important second messengers enhancing cell differentiation (Sart et al., 2015). This differentiation is accompanied with changes in expression of redox sensitive stemness factors such as, Sox2, Oct4, Nanog, Klf4, Tra-160, and an increased number of mature mitochondria (Ji et al., 2010; Lonergan et al., 2007).

NAC is a well-documented antioxidant that controls the redox status of cells through scavenging free radicals and/or reducing oxidized proteins and lipids (Zafarullah et al., 2003). NAC is able to reduce free radicals directly (scavenging activity) or by serving as a precursor for cysteine, which is necessary for the synthesis of glutathione (GSH) (Cotgreave, 1997; Laragione et al., 2003). Additionally, NAC has the ability to reduce cellular proteins through its thiol-disulfide exchange activity (Laragione et al., 2003). Specifically, NAC has been reported to directly interact with target proteins that contain cysteine residues or thiol groups such as Raf-1, MEK, and ERK (Kim et al., 2001).

NAC has been shown to play a role in regeneration in several model systems (Drowley et al., 2010; Uzun et al., 2009; Welin et al., 2009; Xiong et al., 2012; Yamada et al., 2013), however, the specific inductive mechanism is not clear. Here, we analyze the properties of NAC to determine how it induces retina regeneration in the embryonic chick. Retina regeneration is normally induced in the embryonic chick following complete retina removal at embryonic day (E) 4–4.5 using ectopic factors such as fibroblast growth factor 2 (FGF2) which activates retinal stem/progenitor cells present in the CM of the eye or induces transdifferentiation of the retinal pigmented epithelium (RPE) (Spence et al., 2004). Here, we report that NAC is able to induce retina regeneration in the absence of any exogenous factor. Even though NAC decreases the level of ROS induced by injury, its regenerative potential is not dependent on its free radical scavenging ability. Our results support a model

in which NAC activates the MAPK pathway independently of FGF receptor signaling, through its thiol-disulfide exchange activity.

## 2. Results

### 2.1. Redox status changes in the CM in response to injury

Since low levels of ROS have been shown to create/maintain an optimal redox status conducive for stem cell self-renewal (Urao and Ushio-Fukai, 2013), we first investigated the redox status in the CM (the retinal stem/progenitor cell niche of the embryonic chick) following retinectomy. Changes in redox status were documented by measuring levels of immunofluorescence when using an antibody against 5,5-dimethyl-1-pyrroline-N-oxide (DMPO) which covalently binds to oxidized adducts in proteins. Previous work in our laboratory determined that activation of transcription factors necessary for induction of regeneration occurs in response to injury by 6 hours (h) post-retinectomy (PR) (Luz-Madrigal et al., 2014). Therefore, we investigated changes in the redox status within the CM at both 6h PR and 24h PR to be certain measurements were within the window of induction. DMPO immunofluorescence shows the level of oxidized proteins is increased significantly in the CM of retinectomized eyes at both 6h and 24h PR compared to uninjured developing eyes at E4 and E5 respectively (Fig. 1 B–E, N). We, then, added various antioxidants to determine their effect on the increased ROS that occurs in response to injury. The addition of either NAC, XJB 5–131, or Vitamin C at the time of retinectomy leads to a reduction in the level of ROS compared to retinectomy only, with the reduction by NAC and XJB 5–131 being significant after quantification of the DMPO immunofluorescence (Fig. 1 F–K, O). Interestingly, FGF2 also decreased (marginally significant) the level of oxidized proteins at 6 and 24h PR compared to eyes receiving retinectomy only (Fig. 1L, M, O). This suggests that changes in the redox status are necessary for induction of regeneration since regeneration will not occur in retinectomized eyes without the addition of exogenous factors such as FGF2. The redox status results for NAC were corroborated with the fluorescent probe, (6)-carboxy-2′,7′dichlorodihydrofluorescein diacetate, acetyl ester (CM-H2DCFDA), which oxidizes in the presence of ROS (Fig. S1).

### 2.2. NAC is able to induce retina regeneration

To examine if the change in redox status is indeed critical for induction of regeneration, NAC, XJB 5–131, or Vitamin C was added into the eye cups following retinectomy at E4 and the eyes were collected 7d PR (the time when differentiation of regenerated retina is apparent in the presence of FGF2). We found that only NAC was able to induce regeneration from the CM (Fig. 2A–C, E, F) and this regeneration occurred in the absence of exogenous FGF2. As a matter of fact, NAC was able to induce regeneration from the CM (referred to as CR) at a comparable level to that of FGF2, as no significant difference was observed in the level of regeneration induced by both treatments (Fig. 2D). Coincidently, transdifferentiation (TD) of the RPE was also induced, however, it was significantly less in the NAC treated eyes when compared to FGF2 treated eyes (Fig. 2D). Because of these observations, we focused on investigating the retina regeneration induced from the CM by NAC.

### 2.3. NAC induces proliferation

To test if NAC does indeed induce stem cell proliferation and maintenance, we compared the number of proliferating cells present in the CM after retinectomy in response to treatments with NAC or FGF2. EdU was added to eyes one hour before collection to detect cells in S phase. Then, double immunohistochemistry was performed using antibodies against EdU as well as phospho-histone 3 (PH3), which detects cells in G2/M phase (Figs. 3, S2). We found a significantly higher level of EdU+ cells at 6h, 24h and 3d PR and PH3+ cells at 3d PR in the CM exposed to NAC compared to eyes receiving no treatment (retinectomy only). The number of proliferating cells in the NAC treated eyes was comparable to the number of proliferating cells in the FGF2 treated eyes, showing that in the presence of NAC, the retinal stem/progenitor cells proliferate at a similar level as eyes exposed to FGF2 (Fig. 3). This is in agreement with the results above that show NAC and FGF2 induce a comparable level of regeneration from the CM (Fig. 2D). Additionally, we noted there was no increase in apoptosis during NAC-induced regeneration as no observable TUNEL positive cells were seen in eyes treated with either NAC or FGF2 (Fig. S3).

### 2.4. Cell differentiation is delayed in NAC-induced regeneration

Next, we examined if NAC induced the neuroepithelium to differentiate analogous to FGF2. During retina development, the retinal neurons are formed in a specific order. Ganglion cells, are formed first followed by horizontal cells, cone photoreceptors and amacrine cells, and lastly by rod photoreceptors, bipolar and Müller glia (Cepko, 2014). We evaluated the differentiation of each retinal cell type by immunohistochemistry using antibodies to detect Brn3a (ganglion cells), Napa (ganglion cell axons), Visinin (photoreceptors), Ap2 and Pax6 (amacrine cells), Vimentin (Müller glia), Vsx2 (bipolar cells) and Lim 1/2 (horizontal cells). In response to FGF2 induction, all major retinal cell types were detected by 7d PR (Fig. 4A, E, I, M, Q, U, Y) while NAC treated eyes at 7d PR had significantly less number of each of the cell types (Fig. 4B, F, J, N, R, V, Y). However, by 11d PR, NAC did begin to promote differentiation of most of the cell types (Fig. 4D, H, L, P, T, X, Y). It is important to note that Lim1/2 is expressed in differentiating horizontal cells before they reach the horizontal cell layer of the laminated retina which is most likely why Lim1/2 positive cells are present near the RPE at 7d PR (Edqvist et al., 2006).

The decrease in differentiated cells at 7d PR in NAC treated eyes led us to hypothesize that treatment with NAC results in a delay in differentiation because NAC prolongs stem cell self-renewal and maintenance compared to FGF2. To test this, we performed immunohistochemistry against the stage-specific embryonic antigen-1 (SSEA-1), a marker for immature retinal progenitor cells in mice (Koso et al., 2006). In NAC treated eyes, SSEA-1 was present in the anterior retina at higher levels than in FGF2- treated eyes 7d PR (Fig. 5A, B, I). Co-expression of Vsx2/Pax6 is also indicative of neural progenitor cells and these markers were also significantly increased in the NAC treated eyes at 7d PR in the anterior and posterior regions of the regenerated retina when compared to FGF2 treated eyes (Fig. 5E–I). Taken together these results show that while NAC and FGF2 induce comparable proliferation of retinal stem/progenitor cells during the early stages of regeneration (Fig. 3), the proliferating cells differentiate at different rates. NAC induces extended maintenance

of progenitor cells during chick retina regeneration resulting in a delay in differentiation compared to treatment with FGF2.

### 2.5. NAC-induced regeneration is not dependent on GSH activity

We next sought to identify the mechanism of NAC induction of retina regeneration. NAC works as a scavenger of free radicals both independently and through GSH synthesis, and as a reducer of proteins through its thiol disulfide exchange activity. The inability of Vitamin C or XJB 5–131 to induce regeneration (Fig. 2E and F) suggests that the direct scavenger activity of NAC is not sufficient to induce regeneration since Vitamin C and XJB 5–131 decrease the levels of ROS by scavenging but do not serve as precursors of the GSH synthesis or contain a thiol group (Niki, 1991; Wipf et al., 2005).

Therefore, we investigated if GSH synthesis occurs in response to NAC treatment. One of the mechanisms cells use to lower the levels of ROS is the synthesis of the small molecule, glutathione (GSH). GSH works as an antioxidant because the enzyme glutathione peroxidase can oxidize GSH to GSSH while reducing $H_2O_2$ to $H_2O$. Therefore, the ratio of GSH/GSSH is the main indicator of redox status in the cell. Increased GSH will lead to a reduced state, while high levels of GSSH are indicative of oxidative state (Forman et al., 2004). NAC is a precursor of GSH, so it is possible that NAC enhances a reduced state by increasing the level of GSH (Fig. 6A). We measured the levels of GSH in the CM in the presence or absence of NAC after retinectomy and found that NAC indeed increased the levels of GSH (after 6h PR: GSH/GSSH = 29:1; after 24h PR: GSH/GSSH = 9:1) compared to untreated controls (after 6h PR GSH/GSSH = 1:4; after 24h PR GSH/GSSH = 0.2:8) (Fig. 6B). In order to evaluate the role of GSH during NAC-induced regeneration, two inhibitors of the GSH synthesis pathway were used: L-Buthionine sulfoximine (BSO) which reversibly inhibits γ-glutamyl-cysteine synthase, and diethyl maleate (DEM) which conjugates with GSH inhibiting its binding with antioxidant enzymes (Fig. 6A). While these inhibitors significantly diminished the ratio of GHS/GSSH in the presence of NAC (at 6h PR BSO+NAC: GSH/GSSH = 1:2.33; DEM+NAC: GSH/GSSH = 1:8; at 24h PR BSO+NAC: GSH/GSSH = 2.8:2; DEM+NAC: GSH/GSSH = 1:4) (Fig. 6B), they did not affect the level of regeneration induced by NAC. Histological analysis performed with eyes collected 3d PR showed that similar levels of regeneration were induced with NAC as with NAC + BSO or NAC + DEM (Fig. 6C–F). These results indicate that NAC's regenerative ability is not dependent on GSH synthesis.

### 2.6. The MAPK pathway is necessary for NAC induced regeneration

In addition to GSH synthesis, NAC is also able to reduce proteins at cysteine residues through its thiol sulfate group. Interestingly, MAPK is one of the target proteins documented to be reduced by NAC (Kim et al., 2001; Sun, 2010) and MAPK activation is also necessary for chick retina regeneration induced by FGF2, BMPs, and C3a (Haynes et al., 2007, 2013; Spence et al., 2007a). Immunohistochemistry results showed that pErk, a MAPK, is transiently activated 6h PR in response to injury, however the activation of pERK is sustained only in the presence of NAC or FGF2. NAC is able to activate Erk to a comparable level as that of FGF2 (Fig. 7A and Fig. S4). These results suggest NAC could be inducing regeneration through activation of the MAPK pathway. To test this, we added

the MAPK inhibitor PD98059 along with NAC in retinectomized eyes and collected 3d PR. Histological analysis of these eyes showed there was a significant decrease in regeneration in the embryos treated with the PD98059 and NAC when compared to NAC in presence of DMSO (Fig. 7B, D, H). However, the addition of the FGFR inhibitor, PD173074, along with NAC, did not show a significant decrease in regeneration suggesting that NAC-induced regeneration depends on the activation of the MAPK pathway in an FGF-independent manner (Fig. 7C, D, H). Since NAC has been shown to reduce the protein MAPK through its thiol disulfide exchange activity (Kim et al., 2001; Sun, 2010), we tested the importance of this activity next. We used N-acetylglycine (NAG), which is able to induce GSH synthesis but lacks a thiol group (Fig. 8A), and N-acetylserine (NAS), which is structurally similar to NAC except it lacks the thiol group (Fig. 8C), and found that neither NAG nor NAS are able to induce regeneration (Fig. 8A and C) supporting the importance of the thiol disulfide exchange activity of NAC (Fig. 8B) in the induction of retina regeneration. Thus, our results support a model in which the thiol disulfide exchange activity of NAC, and not its GSH synthesis or ROS scavenging activity, is critical for the induction of retina regeneration (Fig. 9)

## 3. Discussion

Our results indicate that the antioxidant NAC is able to induce retina regeneration in the embryonic chick primarily from retinal stem/progenitor cells in the CM. NAC has been shown to promote or enhance tissue repair or regeneration in other systems. For example, in rats, NAC enhanced liver regeneration after partial hepatectomy (Uzun et al., 2009), it enhances peripheral sensory neuron growth after injury (Welin et al., 2009), and it induces bone regeneration and osteogenesis (Yamada et al., 2013). In these reports, the antioxidant property of NAC is thought to be critical for its role in regeneration, but specific mechanisms were not defined. Here, we separately examined the GSH synthesis, the free radical scavenger activity and the thiol disulfide exchange activities of NAC in the induction of chick retina regeneration, and concluded that the thiol disulfide exchange activity is critical to induce retina regeneration. We suggest that this activity is responsible for activating the MAPK pathway.

We show that NAC does increase the level of GSH and modifies the redox status by decreasing the level of ROS after retinectomy. However, inhibition of GSH synthesis does not affect the induction of regeneration by NAC. Also other antioxidants such as Vitamin C and XJB 5–131, which are scavengers, are unable to induce regeneration supporting that low levels of ROS are not sufficient for induction of regeneration. Instead, we show that induction of regeneration requires the thiol-disulfide exchange activity of NAC since NAS and NAG, two structurally similar molecules to NAC which lack the thiol-sulfate group, are unable to induce regeneration. Although NAG does not have the thiol-sulfate group, it has been reported to have the ability to increase GSH levels (Bloch, 1949), further highlighting the importance of the thiol-disulfide group in induction of chick retina regeneration.

Oxidation of the thiol group in cysteine residues of proteins occurs when levels of ROS increase and consequently change the redox status thereby affecting different signaling pathways and changing gene expression. A large variety of proteins including transcription

factors, chaperones, protein tyrosine phosphatases and protein kinases have been shown to be tightly regulated via redox processes (Barford, 2004; Gupta and Carroll, 2014; Leonard and Carroll, 2011). MAPK family, most notably Erk1/2, is one protein that has been shown to be sensitive to the redox status (Murray et al., 2015). The MAPK pathway has been identified as being critical for retina regeneration induced by several signaling molecules including FGF2, BMPs, and C3a (Haynes et al., 2007, 2013; Spence et al., 2007a), and now we show that NAC-induced regeneration is also dependent on the MAPK pathway but independent of FGFR activation. Therefore, activation of MAPK by the thiol disulfide exchange activity is a likely possibility although we cannot rule out that NAC is not affecting other signaling molecules (Fig. 9). A cysteine residue at position 166 of Erk1/2 lies within an important domain for ATP binding, and modifications of this domain with an irreversible inhibitor at this site decreases its interactions with other kinases (i.e. MEK1/2) as well as its translocation to the nucleus (Corcoran and Cotter, 2013; Galli et al., 2008; Ohori et al., 2007), so it is possible that the reduction of this particular residue by NAC can enhance Erk1/2 activation. NAC has been reported to enhance activation of Erk1/2 in cultured bovine and human chondrocytes (Zafarullah et al., 2003) as well as in sympathetic neurons and PC-12 cells further supporting our results (Yan and Greene, 1998). In fact, Yan and Greene (1998) suggested that the thiol-disulfide exchange activity of NAC was necessary for the in vitro activation of Erk1/2. Activation of Erk1/2 mediates basal and stimulus-activated gene expression and controls different cellular processes including proliferation, differentiation, and cell death (Ohori et al., 2007; Young et al., 2003) which are all critical cellular events for regeneration.

Tissue regeneration in vertebrates relies mainly on the activation and self-renewal of stem/progenitor cells (Lane et al., 2014) as we see in the embryonic chick. However, regeneration is limited in higher vertebrates. It is possible that stem cells in higher vertebrates are particularly sensitive to changes in redox status. After injury, the production of ROS increases as a consequence of the inflammatory response, and this increase in ROS production modifies the redox status, affecting the process of regeneration by diminishing stem/progenitor cell self-renewal as well as the presence of active stemness factors dictating cell differentiation (Kobayashi and Suda, 2012). While our system supports that a decrease in ROS is necessary to induce proliferation of retinal stem/progenitors and subsequent regeneration, it is worth noting that it has also been reported that an initial increase in ROS is necessary for regeneration in some species (Sehring et al., 2016; Serras, 2016). This apparent contradiction will have to be resolved through future experiments but it is possible that an initial increase in ROS is necessary but without the activation of signaling pathways, the redox status is not changed, and regeneration will not ensue. Therefore, the initial increase in ROS observed at 6h PR may be the first step in the regeneration process (Fig. 1 N).

Our work shows NAC modifies the redox status as well as induces proliferation and subsequently the number of retinal progenitor cells in the CM as shown by the prolonged expression of Vsx2/Pax6 and SSEA. NAC has been shown to increase proliferation and reduce apoptosis and necrosis in adipose-derived stem cells from human subcutaneous adipose tissue (Xiong et al., 2012) as well as to enhance the ability of muscle-derived stem cells used in implantation to improve cardiac function in a myocardial infarction murine

model (Drowley et al., 2010) supporting our results. Previous studies have also shown that NAC delays multipotent adult progenitor cell differentiation in rat bone marrow (Xiao et al., 2014). These results agree with our observations since the NAC-induced retina has delayed cell differentiation compared with FGF2 treated eyes. However, by 11d PR, the retina induced by NAC had increased differentiated photoreceptors, amacrine and bipolar cells suggesting that differentiation was not inhibited but delayed by the presence of NAC, as expansion of retinal stem/progenitor cells continued beyond that observed for FGF2. However, differentiation of ganglion cells as well as Müller Glia was significantly low even at later times. While it is possible that the differentiation of these cell types is not supported by NAC, we hypothesize that degeneration is beginning to occur in these retinas because at 11d PR fewer ganglion and ganglionic axons are present in the regenerated retina induced by FGF2 as well. The delivery method of NAC and FGF2 could account for the degeneration at 11d PR. For this study, NAC and FGF2 were both injected directly into the eye cup as opposed to delivery via heparin beads, which has been used in the past to deliver FGF2 (Spence et al., 2004). The heparin beads allow a slow release of FGF2 which sustains the treatment longer resulting in the maintenance of the differentiated cells at 11d PR (Spence et al., 2004). Since NAC is not able to be absorbed by the heparin beads, this delivery system was not applicable to this study.

The results presented here not only show that NAC has the ability to induce retina regeneration and can therefore be a potential therapeutic treatment for retinal degenerative diseases, but also increases our overall understanding about the role antioxidants play in the regulation of stem cells. Antioxidants have diverse mechanisms, and this study shows that antioxidants can have different effects in different contexts as other antioxidants were not able to induce retina regeneration. The fact that antioxidants can modify signaling pathways is of great importance, not only because manipulation of signaling pathways can lead to induction of stem cells and a potential repair mechanism, but also to avoid off target effects from signaling molecules during future treatments involving antioxidants, including cancer treatments.

## 4. Material and methods

### 4.1. Chick embryos

Fertilized chicken eggs were purchased from Michigan State University, poultry farm. The eggs were incubated at 38 °C in approximately 40–70% humidity until they reached the developmental stage HH 22–24 (Hamburger and Hamilton, 1951) (E4–E4.5).

### 4.2. Surgical Procedure

Microsurgical removal of the retina was carried out at E4 as previously described (Coulombre and Coulombre, 1965; Park and Hollenberg, 1989; Spence et al., 2004). After retina removal, 4 μl of the different treatments were added in the eye cup; 100 mM NAC (Sigma A9165), 100 mM NAG (Sigma-Aldrich A16300), 100 mM NAS (Sigma-Aldrich A2638), 10 mg/ml vitamin C (Sigma A4544), 5 mM XJB 5–131 (obtained from Dr. Wipf from Pittsburg University), or 1 μg/ul of FGF2 (R & D Systems) was added inside the eyecup. Controls for NAC and FGF2 included retinectomy plus vehicle for NAC and

FGF2 (1% glycerol in 1X PBS sterile). To determine the optimal concentration of NAC to use in this study, varying concentrations ranging from 1 mM to 100 mM were tested. Concentrations below 100 mM did not consistently induce regeneration.

### 4.3. Tissue fixation and sectioning

Embryos were collected at specified times after retinectomy and fixed according to the procedure. For histology, the tissue was fixed in 10% buffered formalin (Richard-Allan Scientific. Ref: 5701) at 4 °C for at least 24h, and then transferred to 70% ethanol for dehydration. Finally, the tissues were placed in a tissue processor (Leica TP 1020) and embedded (Thermo electron corporation, Shandon histocentre 3) in paraffin wax. The tissues were sectioned at 10 μm thickness using a Microm HM 355 s microtome, and subsequently stained using hematoxylin and eosin (H & E).

For immunohistochemistry and Click iT EdU analysis, the tissue was fixed in 4% paraformaldehyde overnight (O/N) at 4 °C, rinsed in 1X PBS three times for 5 min, and cryopreserved in 30% sucrose for 2 days at 4 °C. The tissues were embedded in optimal cutting temperature media (O.C.T, from Tissue tek. Ref: 4583) and sectioned at 10 μm thickness using a Microm HM 505 N cryotome. The slides are kept at −20 °C until use.

### 4.4. Immunohistochemistry

Immunohistochemistry was performed on frozen sections. Three different biological samples were used in triplicate for each experiment. The tissues were permeabilized with 1% saponin for 5 min, blocked for 30 min in 10% goat or donkey serum followed by an incubation with the primary antibody O/N at 4 °C. The secondary antibody was incubated for 2h in the dark. The primary antibodies used for immunohistochemistry include: Vsx2 (1:50; Exalpha biological, ×1180P), DMPO (1:1500; Cayman chemical #10006170), phospho histone 3 (Ser 10) (1:500. Cell signaling # 06-570), phospho-P44/42 (1:500; Erk1/2) (Cell signaling # 4370), Brn3a (1:50; Chemicon International) and Sox2 (1:1000; Santa Cruz, sc # 17319). SSEA1, Vimentin, Visinin, Ap2, Pax6, and Napa (1:100) were obtained from the Developmental Studies Hybridoma Bank, created by the NICHD of the NIH and maintained at The University of Iowa, Department of Biology, Iowa City, IA 52242. Secondary antibodies include: donkey anti sheep IgG 488 (Invitrogen A11015), Goat anti mouse IgG 546 (Invitrogen A11003), Goat anti mouse IgM 488 (Invitrogen A21042), Goat anti rabbit IgG 488 (Invitrogen A11008), Goat anti rabbit IgG 546 (Invitrogen A11010), and Goat anti mouse IgG (Invitrogen A11001). Fluorescence was evaluated using a laser scanning confocal microscope (Olympus FV500).

### 4.5. Cell proliferation

The Click iT EdU Alexa fluor 488 image kit (Invitrogen # C10337) was used. One hour before collecting the embryo, 30 μl of 5 mM EdU (Component A) was added on the top of the eye (making sure there were no membranes on top). We performed the click reaction as per manufacturer instructions.

### 4.6. Quantification of Immunopositive cells

Quantification of the posterior differentiated retina was done by counting the number of immunopositive cells within a defined square drawn with Image Pro 7.4. The square was moved to 3 different posterior regions of each eye to determine the average number of immunopositive cells. For quantification of the CM, a square was drawn with Image Pro 7.4 and copied to the CM region of each eye and the number of immunopositive cells counted.

### 4.7. ROS quantification

Two different methods were used to measure ROS: the chemiluminescent probe (6)-carboxy-2′, 7′dichlorodihydrofluorescein diacetate, acetyl ester (CM-$H_2$DCFDA) and the immuno-spin trapping 5,5-dimethyl-1-pyrroline-N-oxide (DMPO). The CM-$H_2$DCFDA (Invitrogen Molecular Probes # C6827) was used as described by Owusu-Ansah et al. (2008). The CM was collected after 6h, washed in 1X PBS at room temperature (RT)/5 min. In a glass bottom microwell dish (MatTek Part No-P35G-1.5–14-C) the tissue was incubated for 15 min with a final concentration of 1 μM CM-$H_2$DCFDA followed by 3 washes in 1X PBS, and immediately photographed using confocal microscopy. The immuno-spin trapping DMPO (Cayman chemical Item No: 10006170) was performed using immunohistochemistry as per manufacturer instructions. The intensity of the signal was quantified using Image Pro 7.4 and reported as a ratio between the intensity/area selected.

### 4.8. Inhibitors

For inhibition studies, inhibitors were added after retinectomy 30 min before the treatments with either FGF2 or NAC. The following inhibitors were added in a volume of 4 μl: 1 μM FGFR inhibitor PD173074 (Selleckchem Cat No S1264); 1 μM MAPK inhibitor PD98059 (Cell Signaling Cat No 9900); and the GHS inhibitors: 0.9 mM conjugating agent Diethyl Maleate (DEM) (Sigma-Aldrich Cat No D97703) and 10 mM L-Buthionine sulfoximine (Cayman chemical Item No: 14484). According to manufacturer's specifications, PD98059 specifically inhibits MEK1 and MEK2 while PD173074 is a potent inhibitor for FGFR1, however it can also inhibit VEGFR2, PDGFR and c-Src but at a much higher IC value. We have used this inhibitor to specifically inhibit FGF2 treated eyes in the past (Spence et al., 2007b, 2004). Likewise, DEM and BSO have previously been shown to inhibit GHS (Kang et al., 1999). All inhibitors were prepared in 1X PBS, 20% glycerol and 5% DMSO, except for BSO which was prepared in 1X PBS and 20% glycerol.

### 4.9. Glutathione quantification (GSH)

The embryos were collected after 6h and 24h PR in 1X PBS at RT. The CM was collected and processed immediately for GSH quantification. GSH quantification was determined following the instructions from Rahman et al. (Rahman et al., 2006). The samples were read in a spectrophotometer at 412 nm at 0, 1 and 2 min. Data was analyzed using the following formulas: $y = AX+B$. $[GSH]_{TOTAL} = 2*((\Delta OD_{412}/\text{min-B})/A)*\text{Sample dilution}$, $[GSSH] = ((\Delta OD_{412}/\text{min-B})/A)*\text{Sample dilution}$. $[GSH/GSSH] = ([GSH]_{TOTAL} - 2[GSSH])/[GSSH]$.

### 4.10. Statistical analysis

The statistical analysis was performed using SAS/STAT software, Version 9.4 of the SAS System for Windows. Copyright © 2016 SAS Institute Inc. SAS and all other SAS Institute Inc. product or service names are registered trademarks or trademarks of SAS Institute Inc., Cary, NC, USA. Mixed model analyses of variance (ANOVA) were performed for examining treatment comparisons (Figs. 4, 5), with Dunnett-adjusted multiple comparisons to control Type I error in treatment comparisons against a control performed of analyses in Figs. 1, 3, 6, and 7. All model assumptions of normality and homoscedasticity were checked and verified, with occasional implementation of log transformations to stabilize error variance. Mann-Whitney-Wilcoxon rank-based tests were used for analyses of skewed response data in Fig. 2. Means and standard deviations for measured responses for all experiments are given in Tables S1–9.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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## Appendix A. Supplementary material

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.ydbio.2017.08.013.

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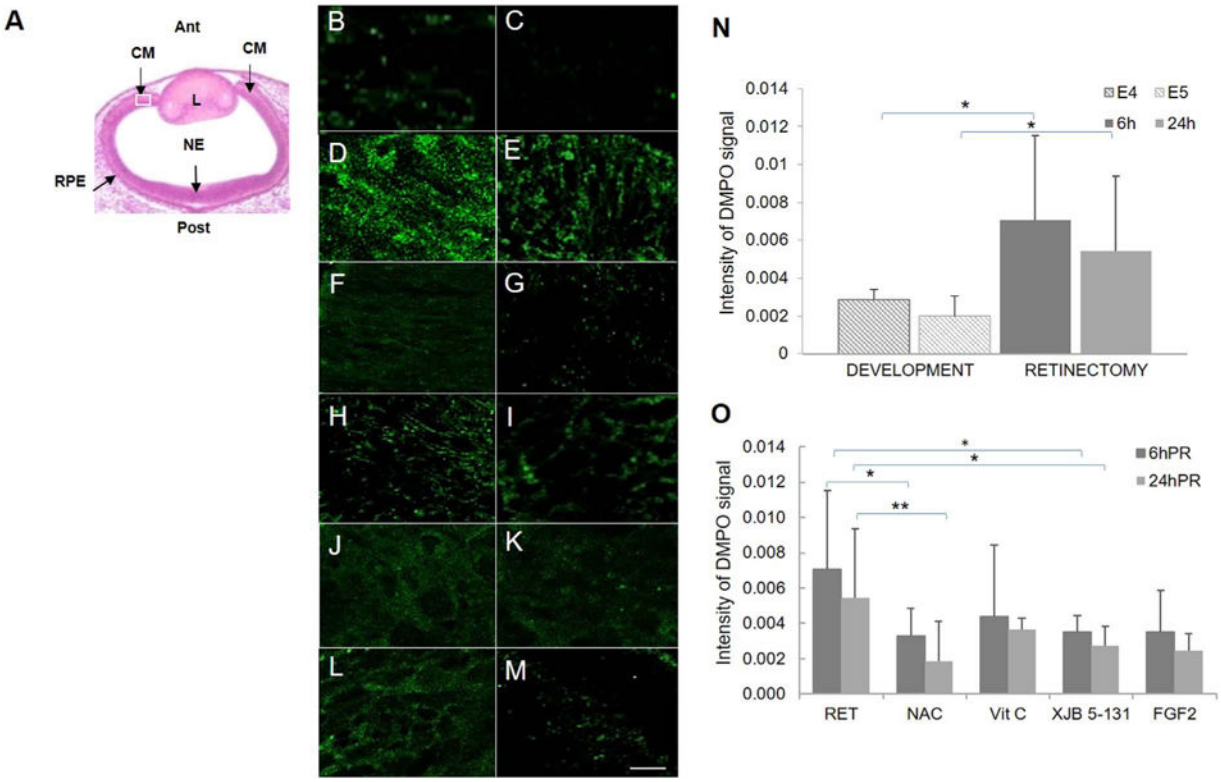


**Fig. 1.**

Redox status in the chick ciliary margin post-retinectomy (PR). (A) Histological section of an embryonic day 4 (E4) chick eye showing the structures of the eye. Anterior (Ant) and posterior (Post) regions. L: Lens; CM: Ciliary margin; NE: neuro epithelium; RPE: Retinal pigmented epithelium. (B–M) Immunohistochemistry using the immunospin trap 5,5-dimethyl-1-pyrroline-N-oxide (DMPO) antibody on sections of the CM of chick eyes at (B) E4, (C) E5, (D) 6h and (E) 24h post-retinectomy (PR) only, as well as with the following treatments: (F, G) NAC at 6h and 24h PR respectively, (H, I) Vitamin C at 6h and 24h PR respectively (J, K) XJB 5–131 at 6h and 24h PR respectively, and (L, M) FGF2 at 6h and 24h PR respectively. Scale bar in M is 125 μm and applies to all. (N, O) Graphical representations of the ratio (intensity/area) of the signals detected in B–E and F–M respectively. Statistical analysis was performed using Dunnett multiple comparisons. The first analysis compared developmental samples and retinectomy samples and are shown in (N). Retinectomy significantly increased the level of oxidized proteins at both 6h and 24h PR (Dunnett 6h * = 0.05 and 24h * = 0.04). The second analysis compared NAC, Vitamin C, XJB 5–131, and FGF2 to Ret and results are shown in (O). NAC significantly reduced the level of oxidized proteins at both 6h and 24h PR (For NAC compared to Ret, Dunnett 6h * = 0.04 and 24h ** = 0.005; for XJB 5–131 compared to Ret, Dunnet 6h *= 0.05 and 24h *= 0.05). As an aside test, FGF2 was compared to Ret and showed marginal significant differences (Dunnett 6h = 0.07 and 24h = 0.06).

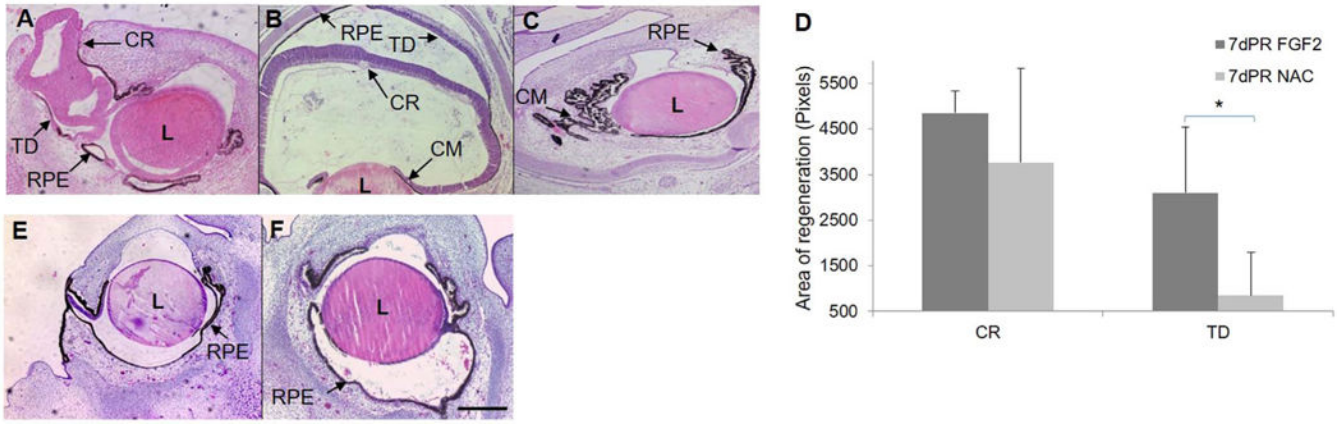


**Fig. 2.**

The antioxidant NAC induces chick retina regeneration. (A–C, E, F) Histological analysis of chick eyes collected 7d PR treated with (A) NAC, (B) FGF2, (C) retinectomy + vector (PBS), (E) Vitamin C, or (F) XJB 5–131. Scale bar in F is 125 μm and applies to all. L: Lens; RPE: Retinal pigmented epithelium; ciliary regeneration (CR) and RPE transdifferentiation (TD). (D) Quantitative analysis of the mean level of regeneration for each treatment: NAC (n = 10), FGF2 (n = 10). Statistical analysis was performed using nonparametric Mann-Whitney-Wilcoxon tests for both CR and TD. There was no significant difference in the regeneration induced from the CM by NAC and FGF2 treatments (Wilcoxon S = 115.0, P = 0.4813), however, FGF2 treated eyes showed a significant increase in RPE TD when compared to NAC (Wilcoxon S = 138.0, *P = 0.0107).

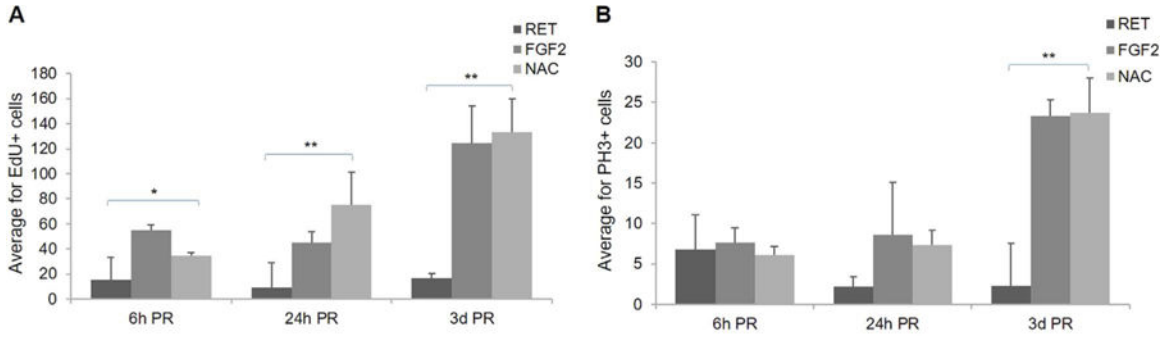


**Fig. 3.**

NAC-treatment enhances cell proliferation in the CM after retina removal. (A) Graphical representation of the mean level of $EdU^+$ or (B) $PH3^+$ cells after treatment with NAC for indicated times. Statistical analysis was performed using Dunnett multiple comparisons. Treatment with NAC resulted in a significant number of $EdU^+$ cells compared to eyes receiving retinectomy only at 6h, 24h, and 3d PR (Dunnett for $EdU^+$ cells: $*P_{6hPR} = 0.0170$, $**P_{24hPR} = 0.0061$ and $**P_{3dPR} = 0.0065$; and for PH3 it was only significant at 3d PR. Dunnett for $PH3^+$ cells: $P_{6hPR} = 0.3694$, $^{M}P_{24hPR} = 0.0587$ and $**P_{3dPR} = 0.0024$). M = marginal significance.

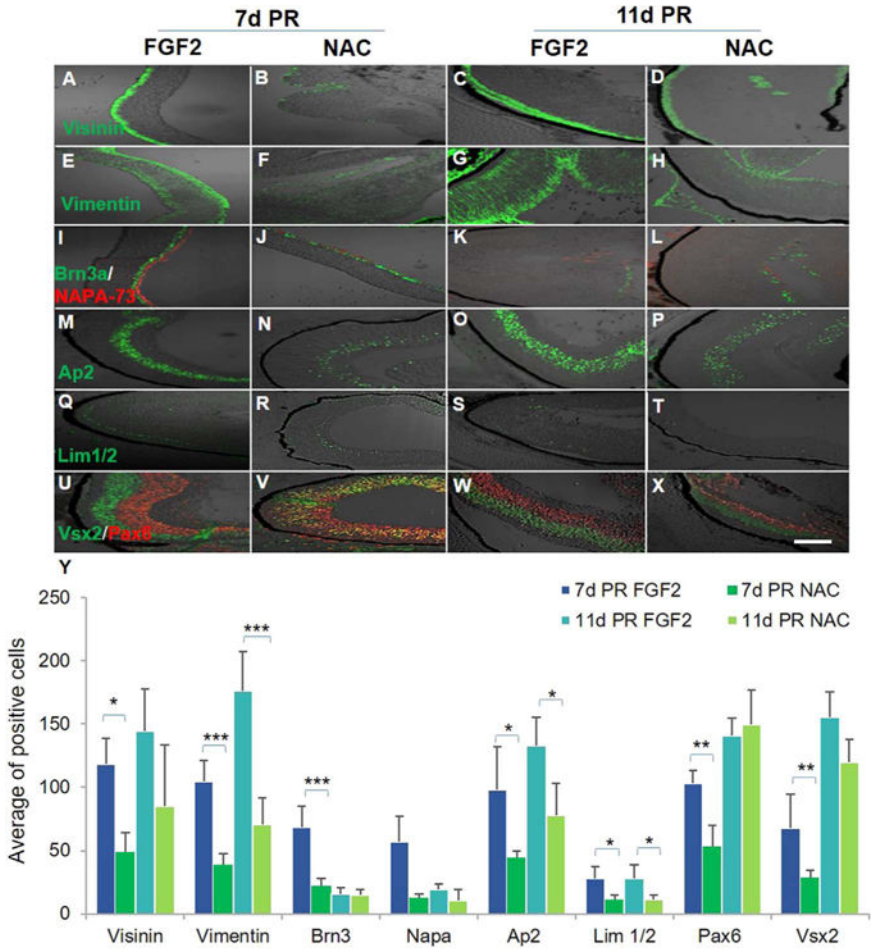


**Fig. 4.**

Cell differentiation during retinal regeneration. (A–X) Immunofluorescence for cell makers for the different cell types of the retina at 7d PR and 11d PR: (A–D) Visinin labeled photoreceptors, (E–H) Vimentin labeled Müller glia, (I–L) Brn3a labeled ganglion cells and NAPA 73 labeled ganglion axons, (M–P) Ap2 labeled amacrine cells, (Q–T) Lim 1/2 labeled horizontal cells, (U–X) Pax6 labeled amacrine, horizontal and ganglion cells and Vsx2 labeled bipolar cells. Scale bar in X is 125 μm and it applies to all panels. (Y) Graphical representation of the number of positive cells for each marker. After 7d PR the number of immunofluorescent$^{+}$ cells present in NAC treated eyes was significantly lower compared to eyes treated with FGF2 for Visinin (*P = 0.0118), Vimentin (***P = 0.0006), Brn3a (***P = 0.0008), Ap2 (*P = 0.0123), Lim1/2 (*P = 0.0119), Pax6 (**P = 0.0026) and Vsx2 (**P = 0.0051) and marginal for Napa 73 ($^{M}$P = 0.0672). After 11d PR the number of immunofluorescent$^{+}$ cells present in NAC treated eyes was significantly lower compared to eyes treated with FGF2 for Vimentin (***P = 0.0009), Ap2 (*P=0.0415) and Lim1/2 (*P = 0.0115), marginal for Visinin ($^{M}$P = 0.0641), but was not significant for Brn3a (P = 0.9721), Napa 73 (P = 0.1300), Pax6 (P = 0.7596) and Vsx2 (P = 0.2537). The number of immunofluorescent$^{+}$ cells was also compared between 7d PR with 11d PR in the NAC treated eyes, and there was significance difference for Vimentin (*P = 0.0120), Pax6 (***P = 0.0002) and Vsx2 (***P = 0.0001), marginal for Ap2 ($^{M}$P = 0.0529), but not for Visinin (P =

0.1141), Brn3a (P = 0.1360), Napa 73 (P = 0.3078), and Lim1/2 (P = 1.00). The significance is not shown in the graph for the comparison of 7d PR and 11d PR NAC treated eyes. Note that the $Pax6^{+}$ cells used for the comparisons were only the ones present in the INL excluding horizontal cells, so it is representative of amacrine cells. Mixed model ANOVA was used for the statistical analysis.

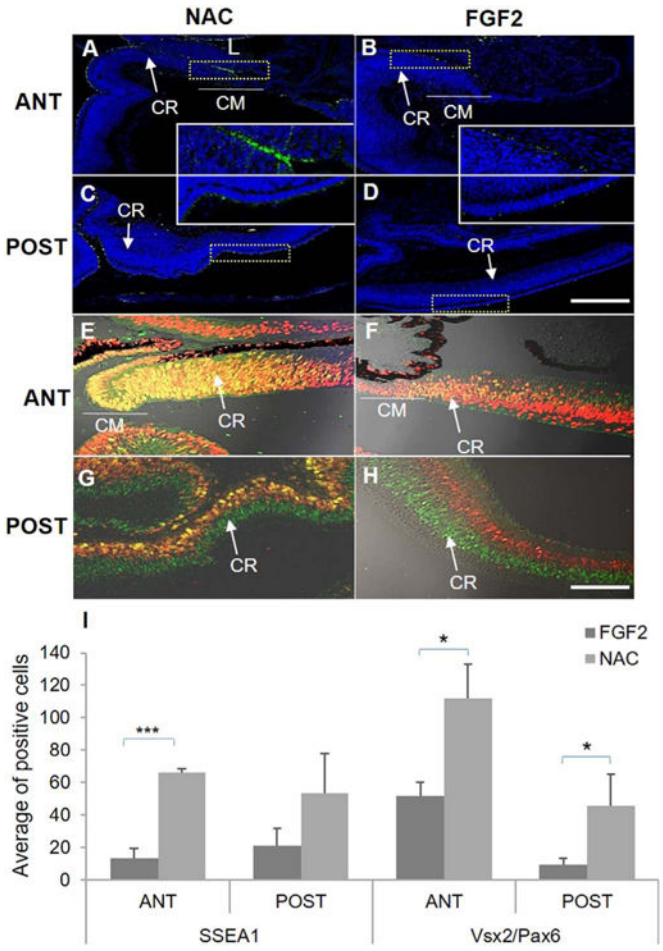


**Fig. 5.**

The presence of stem/progenitor markers are enhanced during NAC-induced retina regeneration. (A–D) Immunofluorescence for the cell surface antigen SSEA1 indicates the presence of progenitor cells in the (A, B) anterior (ANT) and (C, D) posterior retina (POST) at 7d PR. Eyes were treated with (A, C) NAC or (B, D) FGF2. (E–H) Double immunofluorescence for the transcription factors, Vsx2 (green) and Pax6 (red) indicates the presence of progenitor cells in the (E, F) anterior and (G, H) posterior retina at 7d PR in eyes treated with (E, G) NAC or (F, H) FGF2. Scale bar in D is 125 μm and it applies to A–D, and in H, it is 60 μm and it applies to E–H. CM: ciliary margin; CR: ciliary regeneration; L: lens. (I) Quantitative analysis of the mean presence of SSEA-1 and Vsx2/Pax6$^{+}$ cells in NAC and FGF2 treated eyes at 7d PR. There is a significant difference in the ANT, between eyes treated with NAC and FGF2 for both markers SSEA1: ($F(1,4) = 212.64$, ***$P = 0.0001$); Vsx2/Pax6: ($F(1,4) = 14.77$, *$P = 0.0184$). In the POST, there was a significant difference in eyes treated with NAC for Vsx2/Pax6 ($F(1,4) = 8.82$, *$P = 0.0412$) but not for SSEA1 ($F(1,4) = 4.62$, $P = 0.0981$). Mixed model ANOVA was used for the statistical analysis.

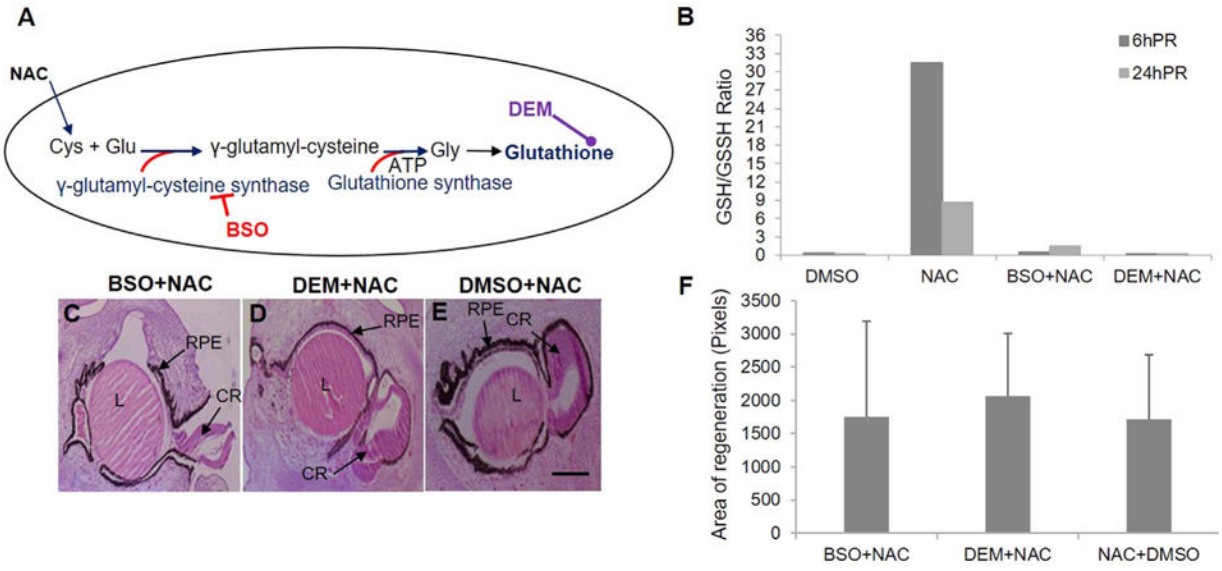


**Fig. 6.**

Increased levels of glutathione (GSH) are not required during NAC-induced retina regeneration. (A) Schematic representation of the GHS pathway indicating the inhibitory mechanism of L-Buthionine sulfoximine (BSO) on γ-glutamyl-cysteine synthase, and diethyl maleate (DEM) on GSH. (B) Graphical representation of the GSH/GSSH ratio in the CM after 6h and 24h PR in eyes treated with DMSO (n = 10), NAC (n = 10), NAC + BSO (n = 10), and NAC + DEM (n = 10). Values were: at 6h PR with NAC: GSH/GSSH = 29:1, after 24h PR: NAC: GSH/GSSH = 9:1, compared to untreated controls after 6h PR: GSH/GSSH = 1:4, after 24h PR: GSH/GSSH = 0.2:8. The use of the inhibitors decreased the levels of GHS in presence of NAC. At 6h PR BSO+NAC: GSH/GSSH = 1:2.33, DEM+NAC: GSH/GSSH= 1:8, at 24h PR BSO+NAC: GSH/GSSH = 2.8:2, DEM+NAC: GSH/GSSH = 1:4. (C–E) Histological analysis of eyes collected 3d PR and treated with NAC and (C) BSO, (D) DEM, or (E) DMSO. Scale bar in E is 250 μm and it applies to all panels. L: lens; RPE: retinal pigmented epithelium; CR: ciliary regeneration. (F) Dunnett multiple comparisons was used as statistical analysis. Quantitative analysis of the regenerated area showing no statistical significance in regeneration between DMSO+NAC and BSO+NAC (P = 0.949) nor with DMSO+NAC and DEM+NAC (P = 0.4639).

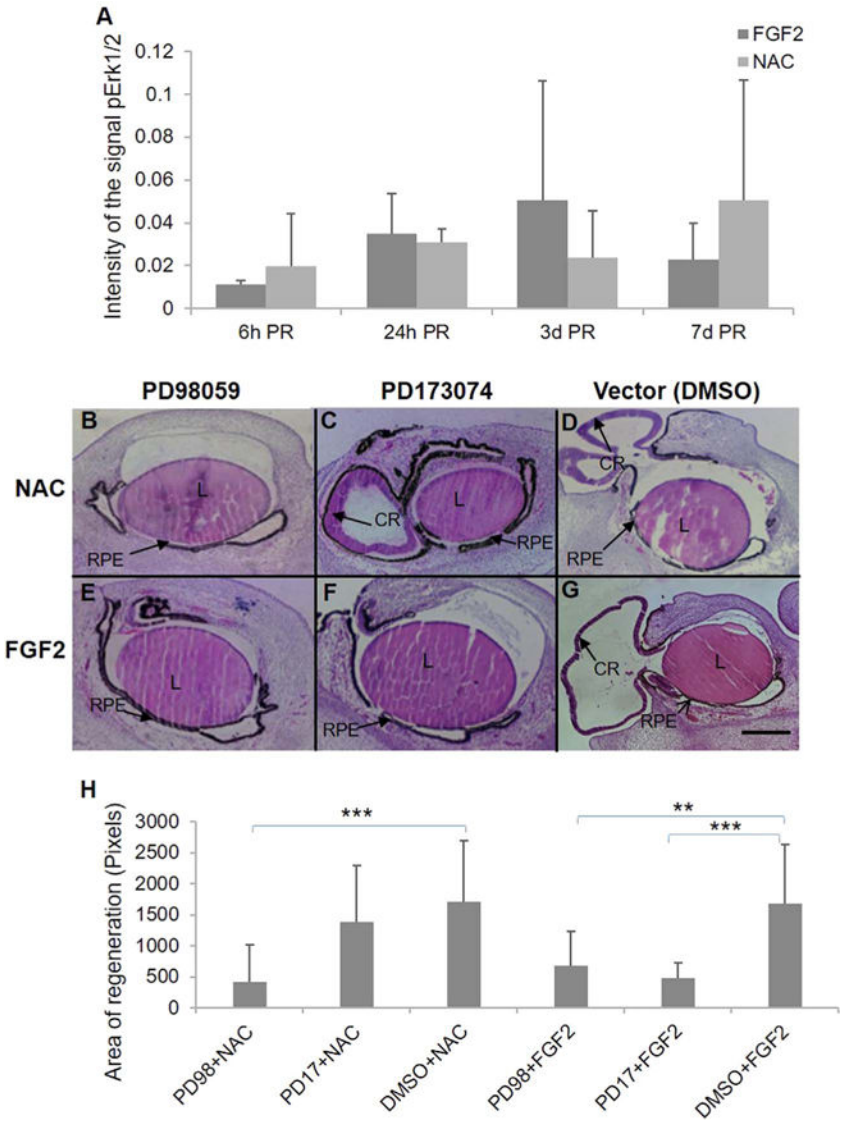


**Fig. 7.**

pErk activation is necessary for NAC-induced regeneration. (A) Quantitative analysis of pErk$^{+}$ cells at 6h, 24h, 3d and 7d PR (intensity of the signal/area). Statistical analysis was done using Dunnett multiple comparisons. After 6h PR, 24h PR, 3d PR and 7d PR no significance in the intensity of pErk signal was observed when compared with FGF2 (Dunnett $P_{6h} = 0.40$, $P_{24h} = 0.81$, $P_{3d} = 0.38$, $P_{7d} = 0.25$). (B–G) Histological analysis 3d PR in eyes treated with (B) NAC + PD98059, (C) NAC + PD 173074, (D) NAC + DMSO, (E) FGF2 + PD98059, (F) FGF2 + PD173074, (G) FGF2 + DMSO. Scale bar in G is 250 μm and applies to all panels. L: lens; RPE: retinal pigmented epithelium; CR: ciliary regeneration. (H) Quantitative analysis of eyes that regenerated in presence of the different inhibitors at 3d PR. The area of the CM was included in the measurement of the amount of regeneration. The statistical analysis for the inhibitors was performed using Dunnett multiple comparisons. The amount of regeneration is significantly higher in the DMSO+NAC treated eyes compared to the eyes treated with PD98059 (MEK inhibitor)

+ NAC (***P = 0.0008). There is no significant difference between the DMSO+NAC and PD173074 (FGFR inhibitor) + NAC (P = 0.4973) treated eyes. However, there is a significant difference in the DMSO+FGF2 treated eyes compared to eyes treated with PD173074+FGF2 (***P = 0.0001) and PD98059+FGF (**P = 0.0013) that were used as controls for the treatments.

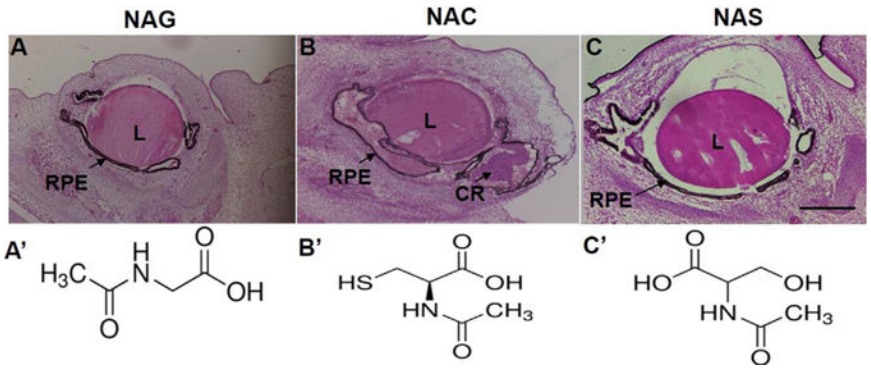


**Fig. 8.**

The thiol group is critical for induction of retina regeneration. (A–C) Histological analysis of eyes collected 3d PR and treated with (A) N-acetylglycine (NAG), n = 12, (A′) NAG structure; (B) NAC, n = 10, (B′) NAC structure; or (C) N-acetylserine (NAS), n = 10, (C′) NAS structure. Scale bar in C is 180 μm and it applies to A and B. Histology after 3d PR of embryos treated with different antioxidants. L: lens; RPE: retinal pigmented epithelium; CR: ciliary regeneration.

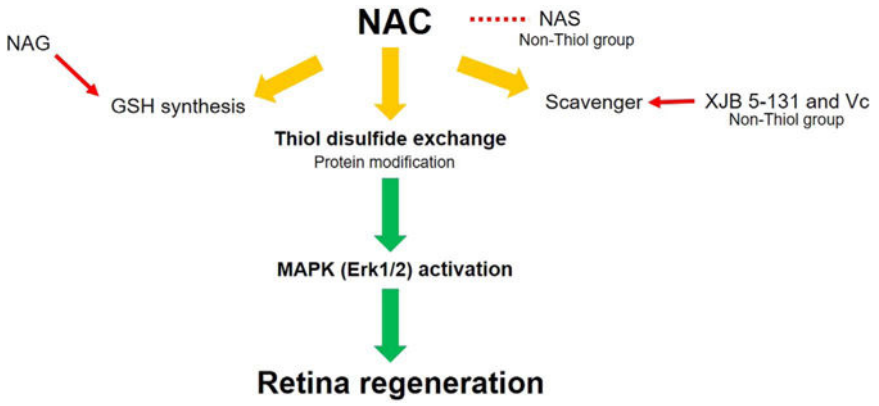


**Fig. 9.**

A model for the induction of chick retina regeneration by NAC. Arrows in yellow represent the different roles of the antioxidant NAC. Arrows in red represent the different molecules that feed into the specific roles of NAC. When the GSH synthesis role of NAC was tested through the use of specific inhibitors, regeneration still occurred. The use of another N-acetyl amino acid, N-acetylglycine (NAG), which can feed into the GSH synthesis pathway, did not result in regeneration. When different molecules that can scavenge ROS were used, no regeneration occurred. In addition, N-acetylserine (NAS) which is similar in structure to NAC but lacks the thiol group in carbon 3, and instead has a hydroxyl group, was unable to induce regeneration. Our data suggests that NAC's thiol group is essential for the activation of Erk and subsequent induction of retina regeneration.