Studying Nitric Oxide in the Developing Retina: Neuromodulatory Functions and Signaling Mechanisms

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Abstract: The retina is a highly organized structure responsible for transducing light stimulation into electrical responses. Retinal organization is conserved in all vertebrate species and the generation of retinal cell types is chronologically determined and fairly documented from amphibians to humans. Furthermore, the chick retina is a well-established experimental paradigm for neurochemical and developmental studies regarding the nervous system. Among many signaling molecules regulating retinal physiology, nitric oxide (NO) is likely to play a prominent role within the retina. NO is a gaseous signaling transmitter, which regulates a plethora of physiological functions within an organism, including high-order signaling events both in the developing and mature nervous system. In this review we focus on different aspects of NO signaling in regulating retinal cell neurochemistry, focusing mainly on developing chick retina as a prevalent experimental model. Based on literature and data gathered from our group we conclude that NO is a major atypical neurotransmitter in the retina, regulating signaling events associated with the development of embryonic retinal neurons and glial cells.

Keywords: AKT, AMPA, ascorbate, CREB, glutamate, L-arginine, PKG, SVCT2.

1. THE CHICK EMBRYO RETINA AS AN EXQUISITE SYSTEM FOR NEUROCHEMICAL STUDIES

The retina is a specialized tissue of the central nervous system (CNS), which is responsible for the reception and transduction of light stimuli derived from the outside environment. Within this tissue, the first processing of visual input takes place, which will be further processed in higher brain structures such as the optic tectum, thalamus and visual cortex. The cell types present in the retina are very well known and comprise the photoreceptors (rods and cones), horizontal, bipolar, amacrine and ganglion cells, as well as the Müller glial cells, and in some species interplexiform cells and the microglia. Most, if not all, neurotransmitter and neuromodulator molecules present in other areas of the CNS are also present in the retina, such as glutamate, dopamine, GABA, acetylcholine, adenosine, etc.

The avian retina, especially from *Gallus gallus*, is a very convenient model for neurochemical studies of the CNS because it is very easy to isolate during most of the embryonic period of development. Moreover, the neurogenesis in the chick retina is well known and the cells from the early developing tissue can be dissociated to prepare cultures where many of the neurochemical properties are maintained as in the intact tissue.

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1.1. Early Studies Using the Retina

Acetylcholine was the first neurotransmitter identified in the chick retina [1] but a detailed study of chick retinal development and neurogenesis occurred in the 50`s and 60`s [2-4]. From this period are the pioneer studies from Moscona and collaborators who used the chicken retina to study the regulation of glutamine syntethase activity induced by corticoids [5-8].

Chick retinal cultures were developed and used in a variety of studies. Cultures of retinal cell aggregates were first developed [9,10] and used to study glutamine synthethase induction and its dependence on cellular interactions [11,12]. Thereafter, different studies using distinct types of retinal cultures, including monolayer cultures showed the properties of GABA uptake and synthesis [13,14]. In the same period, other studies established the sequential appearance of neurons and the formation of synapses during chick retinal development [15,16]. The presence of receptors for neurotransmitters like acetylcholine, GABA and glutamate was detected in this tissue and studied during development [17-19]. Dopamine-coupled cAMP production was studied during chick development and also in monolayer cultures [20,21]. Adenosine and different types of adenosine receptors, modulating adenylyl cyclase activity, were also detected in the developing chick retina [22,23]. Adenosine-elicited cAMP accumulation was detected in the chick retina since embryonic day 14, attaining a maximum effect at day 17 and

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decreasing in the post-hatching period [22]. On the other hand, dopamine-dependent cAMP accumulation, modulated by A1 adenosine receptors, was detected at earlier stages (E10) [24] and completely disappeared in the post-hatching period, although these receptors were detected by binding and could modulate the effect of forskolin at this period [25].

1.2. Neurochemistry of the Chick Retina

As specified above, many neurotransmitters and neuromodulatory molecules are expressed in the chick retina. Several groups studied the major excitatory and inhibitory neurotransmitters, respectively glutamate and GABA, in the intact chick retina as well as in cultures of chick retinal cells. Studies of GABA release as well as other amino acids and acetylcholine, induced by glutamate, highlighted the importance of this amino acid in retinal physiology [26,27]. Studies using purified cultures of chick retinal neurons showed the uptake of adenosine by neurons and photoreceptors as well as its release induced by membrane depolarization in a Ca^{2+}-dependent manner [25]. Glutamate was also shown to induce the release of adenosine in chick retinal monolayer cultures [28]. Adenosine receptors were localized in the plexiform layers in the chick retina [29] and recent studies showed the regulation of A1 adenosine receptor expression by cell-cell contacts and activation of A2a adenosine receptors [30,31].

1.3. Why Study Nitric Oxide (NO) in the Retina?

As stated by Garthwaite and Boulton, in a seminal review published in 1995, "the brain contains by far the highest activity of nitric oxide synthase (NOS) of any tissue so far examined, and the widespread distribution of the enzyme therein indicates that NO could be involved in practically all aspects of CNS function" [32]. Pioneer works established a clear linkage between NMDA-type glutamate receptors, NO and cGMP production in the CNS [33,34]. These studies were followed by the demonstration of NO production from L-arginine (L-Arg), NO-mediated activation of soluble guanylyl cyclase (sGC) [35] and the isolation and cloning of NOS [36-38]. An "explosion" of interest followed these findings and several groups described the localization and function of the machinery for NO production in diverse CNS regions, including the retina [39,40]. One important finding was that NADPH diaphorase, a histochemical marker used to label specific set of neurons, corresponded to NOS [41]. This was followed by studies showing the localization of NOS and citrulline in the brain and their colocalization with NADPH diaphorase [42]. Moreover, different authors have also shown the presence of NADPH diaphorase and NOS in the retina of several vertebrate species (further discussed in section 2).

We became very interested in studying the development of the NO system in the chick retina for several reasons. First, Gally and colleagues in 1990 proposed a hypothesis that NO could play an important role during development as a small and diffusible signal [43]. Other studies have shown that NMDA receptors and NO have important functions during cerebellum and motor neuron development [44-46]. Additionally, important studies demonstrated the effects of NO on growth cones and retinotectal projections [47, 48]. Still, the pioneering studies of Peunova and Enikolopov on the effects of NO on gene expression and cell cycle arrest during neuronal differentiation had a tremendous impact and opened a new field on cell signaling pathways activated by NO during nerve cell development [49-51].

1.4. NO in the Developing Chick Retina: An Overview

An extensive amount of literature arose since NO was elected the molecule of the year in 1992 [52]. After an amazing burst of research focusing in NO biology and chemistry in the CNS, our group was also seduced by the signaling of NO in the developing avian retina. However, to study the signaling events regulated by NO throughout the developing and mature CNS would require a very extensive review of the literature. Hereafter we will discuss data regarding signaling mechanisms of NO mainly in the chick, using findings from our group as the building blocks for further signaling roles within the retina. Inside the specific sections of this review, we will cover methodological advances for detecting and studying NO in the retina in a chronological point of view. Furthermore, a special emphasis will be given to the roles played by NO in the regulation of developmental aspects of embryonic retinal neuronal cells, such as neurotransmitter release, proliferation and cell death.

2. DETECTING NO IN THE RETINA: THROUGHOUT THE METHODOLOGICAL EVOLUTION

2.1. The Pale-Blue NOS

The pioneer studies of Thomas and Pearce (1961) described a particular enzymatic activity known as NADPH-diaphorase in brain tissues. NADPH diaphorase can be detected by a histochemical technique based on the presence, in certain neurons, of an enzyme that catalyzed the NADPH-dependent conversion of a soluble tetrazolium salt into an insoluble blue formazan precipitate [53]. In theory, any NADPH-requiring enzyme could exhibit diaphorase activity. However, when NADPH diaphorase histochemistry was applied to aldehyde-fixed brain tissues, only a specific population of neurons was stained [54]. This method was a useful tool for the examination of selective neuronal populations and was referred to be a selective marker for forebrain neurons containing specialized neurotransmitter systems (somatostatin, neuropeptide Y and cholinergic neurons) forming the ascending reticular system. Interestingly, cerebral NADPH-diaphorase activity remained a complete mystery until the early 90's, when Hope and colleagues (1991) showed that neuronal NADPH-diaphorase activity corresponds to NOS activity; henceforth, NADPH diaphorase histochemistry was employed as a specific histochemical marker for neurons producing NO.

In the late 90`s, our lab became interested in relating the activity of NADPH-diaphorase with enzymatic NO production in the chicken embryo retina. Using the colorimetric property of formazan absorption, it was established that NADPH-diaphorase could be measured in retinal homogenates and its activity partially corresponded to NOS in the developing chick retina. The enzymatic activity could be measured within the early stages of retinal development (E8, E11 and E14), and was determined to be Ca^{2+}-dependent.

Indeed, the enzymatic activity increased 50% in the presence of Ca^{2+} and was completely abolished by EGTA or EDTA [55,56]. Remarkably, Ca^{2+}-dependent activation was reduced at E17 and became almost void in the post-hatching retina. When NADPH-diaphorase activity was measured in the presence of high concentrations of L-Arg analogs, around 35% of the activity remained, and this was related with the existence of other NADPH-dependent enzymes in normal retinal metabolism. Therefore, approximately 65% of NADPH-diaphorase activity was related to NOS and was predominant in early stages of retinal embryonic development [55]. Moreover, histochemical analysis revealed the presence of several stained cells over the whole extent of retinal tissue. At E12 and E14, stained cells were associated with photoreceptors, amacrine and ganglion cells. At E17, the staining was very similar to that found at post-hatching retinas, with a significant staining in the inner segments of photoreceptors and in few amacrine and ganglion cells. A similar staining pattern was previously observed for NADPH-diaphorase as well as NOS immunocytochemistry in retinas from other species, including rats [57-59], rabbits [60,61]and primates [62]. Although the staining appeared more intense in mature retinas, NADPH-diaphorase activity was approximately the same throughout development. However, the inhibition by L-Arg analogues was smaller in mature retinas, a fact that could indicate an increased presence of non-NOS NADPH-diaphorase in some cells at this stage.

2.2. L-Citrulline: A Stoichiometric Partner for NO Production

In 1982, Deguchi and Yoshiokag showed for the first time that L-Arg was an endogenous activator of soluble guanylyl cyclase (sGC) in neuroblastoma cells [63]. Indeed, in the late 70's, Murad and his associates demonstrated that several nitroso compounds could stimulate sGC activity, causing an increase in cGMP accumulation [64,65]. As a sequence of these fundamental studies, Moncada's group showed that sGC stimulation by L-Arg (as previously proposed by Deguchi and Yoshiokag, 1982) precisely matched L-Citrulline (L-Cit) formation in the CNS, with a concomitant NADPH and Ca^{2+}-dependence. Hence, L-Cit produced in the CNS was in fact a stoichiometric co-product of NO synthesis catalyzed by NOS [35]. Interestingly, nerve tissues exhibit L-Cit formation despite the absence of ornithine carbamoyltransferase (OCT) and carbamoyl phosphate syntethase I (CPS-I) expression, two important enzymes involved in the urea cycle [66]. Therefore, all L-Cit formed in the CNS was strongly associated with NOS activity and NO production [67].

Besides the NADPH-diaphorase histochemical localization and biochemical determination by the formazan absorption assay, we were motivated to find alternative methodologies which could detect NO formation. We then started to use a series of scintillation-based spectrophotometric methods to evaluate the conversion of L-[^{3}H]-Arg into L-[^{3}H]-Cit as an indicative of NOS activity. We initially used a Dowex AG50-WX8 resin packed into a chromatographic column to separate L-[^{3}H]-Cit from L-[^{3}H]-Arg in experiments employing homogenates from the retina. Our lab, and Macaione's group, concomitantly showed that NOS activity was high at earlier stages of retinal development (E8 and E9), decreasing until E13-14 and attaining minimal levels at E15 up to the post-hatching period [55,68]. At that time, experiments using retinal homogenates were instructive in providing details regarding NOS enzymatic activity during development, Ca^{2+}/CaM dependence and relative efficacy for different inhibitors [69]. To better evaluate the contribution of some signaling systems involved in NO generation, we developed a precise chemical analysis based on thin layer chromatography and scintillation [40]. Protein-free samples either of extracellular or intracellular medium was added to silica gel plates, together with a standard solution containing L-Arg, L-ornithine (L-Orn) and L-Cit [70]. A mixture of chloroform, methanol and ammonium hydroxide (2:3:2; v/v/v) was used as an eluent. For the determination of retention factors (Rfs), we sprinkled a solution containing ninhidrin (0.1% ethanol/acetic acid, 5:1; v/v). The Rfs appeared at different spots (L-Arg, 0.20; L-Orn, 0.40; L-Cit, 0.69) and silica areas containing the selected amino acids were scraped off from plates for radioactivity determination by liquid scintillation (hereafter we refer to this entire process as TLC analysis). With this procedure, we measured L-[^{3}H]-Cit formation and associated it with NOS activity [70]. Moreover, we verified the amounts of L-[^{3}H]-Arg and L-[^{3}H]-Cit present in retinal cells, as well as in the extracellular fraction. At the same period, we were also interested in evaluating L-[^{3}H]-Arg uptake in neuronal and glial cells and in determining the cellular target related with L-Cit/L-Arg mobilization. For such, we performed L-[^{3}H]-Arg autoradiography and visualized a high density of autoradiographic grains in cultured glial cells [70]. We noticed that the labeling was weak in neurons, especially in the ones close to or above glial cells. Interestingly, retinal neurons, including photoreceptors, exhibited an extensive labeling when grown in the absence of glial cells [70]. These observations suggested that glial cells could take up L-Arg with high affinity and promptly release it to neurons. To test this hypothesis, we used purified glial cell cultures pre-loaded with L-[^{3}H]-Arg. Then, coverslips containing purified neurons were transferred to dishes containing the radiolabeled glial cells and accommodated onto a plastic apparatus, which avoided the direct contact between the two cellular populations but allowed medium sharing. Using this procedure we demonstrated that glial cells released L-[^{3}H]-Arg in response to depolarization induced by high potassium. When released material was analyzed by TLC, most of the radioactivity was found as L-Arg, with smaller amounts of L-Orn and L-Cit, indicating that glial cells were capable of retaining and releasing L-Arg that could be taken up by neurons [70]. Using a similar methodological approach, Bolaños group showed that rat astrocytes could release L-[^{3}H]-Arg, which could be taken up by neurons upon peroxynitrite ($ONOO^-$) treatment [71]. These findings indicated an interesting mechanism for the regulation of neuronal NO production, since the enzymatic activity of NOS with low availability of L-Arg leads to O_2^- production. This reactive oxygen species, in the presence of residual NO, gives rise to $ONOO^-$, which in turn would be the trigger for glial L-Arg supply [71]. Prior to that, Grima and colleagues demonstrated that

the stimulation of non-NMDA receptors promoted L-Arg release from rat astroglial cultures [72,73].

At that time, it was clear that both neuronal and glial cells could take up L-[^{3}H]-Arg, but glial cells appeared to regulate the demand for neuronal L-Arg uptake. Since L-Arg is an important amino acid present in proteins, we decided to use the protein synthesis inhibitor cyclohexymide (CHX) during uptake assays to avoid the protein synthesis-dependent component in L-Arg uptake [23]. Interestingly, we demonstrated that protein synthesis represented an important driving force for L-Arg uptake [23]. More than 70% of intracellular taken up L-Arg was incorporated into proteins and when this incorporation was blocked by CHX, the free intracellular L-Arg pool increased significantly. Unexpectedly, when protein synthesis was inhibited, the intracellular L-Cit production increased and the extracellular content of L-Cit increased even more [23]. This particular phenomenon drew our attention to two facts: (1) High levels of L-Cit found in the extracellular environment could be related with some releasing system working in concert with intercellular recycling pathways. Indeed, glial cells express the enzymes involved in the *de novo* formation of L-Arg by transamination (ASS and ASL) [74-77]. (2) Protein synthesis could play an active role as an intermediate step between L-Arg influx and the control of its intracellular availability for NO synthesis. Actually, we found that activation of NMDA receptors increased L-[^{3}H]-Cit formation, but it also inhibited protein synthesis and increased intracellular free L-Arg. Moreover, these effects were mediated by Ca^{2+}/CaM-dependent activation of the enzyme eukaryotic elongation factor 2 kinase (eEF2K). In such way, stimulation of NMDA receptors would be the trigger not only for NOS enhanced activity, but also for increased L-Arg availability produced by inhibiting protein synthesis [23].

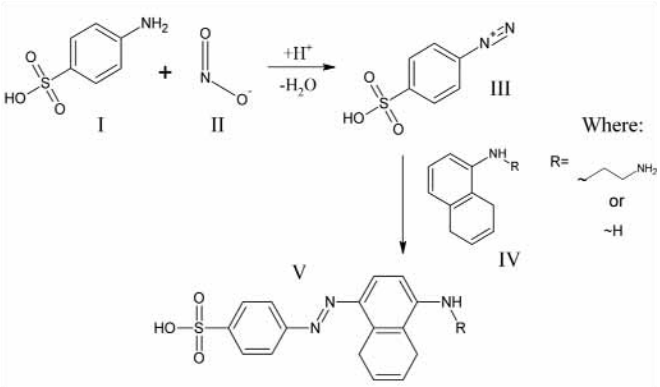
2.3. Painting NO with Antibodies

Later on, we decided to look for the cell type that could produce NO in retinal cultures. At the late 90's, Dr. S. Vincent from the University of British Columbia in Canada kindly supplied antibodies against L-Cit which were tested extensively, and its colocalization with NOS was determined in several structures throughout the CNS [78]. Using avidin-biotin-peroxidase complex with a diaminobenzidine-developed staining, we visualized a large proportion of neurons, including photoreceptors, which were immunolabeled, with no staining observed in glial cells. The immunoreactivity for L-Cit was greatly reduced when cultures were pre-incubated with the NOS inhibitor L-nitroarginine (L-NA). Moreover, using antibodies against neuronal NOS, we observed the same labeling pattern in neuronal cells, while glial cells were devoid of staining. The antibodies against neuronal NOS were kindly supplied by Dr. V. Riveros-Moreno (Wellcome Research Laboratories, Beckenham, Kent, U.K.). This antibody was raised against the sequence 519–540 (LPLLLQANGNDPELFQIPPELC) of the rat neuronal enzyme. Despite being produced for rat NOS, the antibody worked perfectly in our cellular model (the chick retina). Therefore, based on our experimental data we suggested that, in the retina, most of the NO synthesis constitutively occurs in neurons but not in glial cells. Nonetheless, under special circumstances, retinal glial cells also produce NO (further discussed in the next section).

2.4. NO More Mysteries with the Griess Method

When Johann Peter Griess in 1879 described an analytical detection method for nitrite (NO_2^-), he could never have guessed it would be one of the most propagated methods in experimental biology [79]. NO, as it is now known, oxidizes quickly in biological environments producing NO_2^- and NO_3, which in the past was "invisible" in several experimental protocols. For more than a century, the Griess reaction has been used analytically to identify NO_2^- produced by bacterial reduction of NO_3^-. This procedure was related with the diagnosis of bacterial infection in the urogenital tract [80].

The Griess reaction is inexpensive and marked by its experimental simplicity. Since this reaction is specific for NO_2^-, the analysis of the final oxidized product NO_3^- requires either chemical or enzymatic reduction for its determination. The procedure consists of a diazotization reaction using NO_2^- (II) and sulfanilic acid (I) to form a diazonium cation (III) under acidic conditions. After cation formation, an aromatic electrophilic addition reaction occurs with aromatic amine (IV) to produce water-soluble azo dye (V), according to the following scheme:



Nowadays, it is known several variations of this method and every laboratory in different areas use their own adapted Griess assay [80]. In that sense, we decided to use a copper-plated cadmium reduction, as described by Green and colleagues (1982) [81,52,48], with modifications [82,53,49]. Briefly, samples were deproteinized with zinc sulfate ($ZnSO_4$) and NaOH, reduced with activated cadmium by copper sulfate ($CuSO_4$) and the nitrite content measured with the Griess reagent. We used this technique in studies showing that NO displays a neuroprotective effect in the chick embryo retina. We demonstrated that re-feeding purified neuronal cultures with fresh medium caused intense cell death (further discussed in section 3), an effect blocked by pre-treatment of cultures with adenosine and activation of A2a receptors [83]. We explored the mechanism by which NO, produced from the NO donor S-nitroso-acetyl-D-L-penicillamine (SNAP), showed a similar neuroprotective effect in such experimental paradigm. Our measurements of NO_2^- production showed that NO release from SNAP is complete after 3 h either in the presence or absence of cells and nitrite production is linear as a function of SNAP concentration. Interestingly, SNAP not only reduced the neu-

ronal death promoted by re-feeding but also produced an increase in neurite outgrowth [84].

In a different work, Socodato and colleagues [114] used a selective NO sensor to detect the release of NO in cultured retinal cells stimulated with glutamate. Interestingly, it has been demonstrated that the recovery of NO from NO_2^- using iodide was possible. The authors took advantage of this chemical property and performed this transformation, according to the following scheme.

$$2NO_2^- + 2I^- + 4H^+ \longrightarrow 2NO + I_2 + 2H_2O$$

Moreover, this chemical reaction shows the mole conversion of $NO2^-$ to NO with a 1:1 ratio and thus a very reliable quantification of released NO content in our culture samples could be resolved either under non-stimulated or stimulated conditions. This work showed that NO was quickly generated in cultured retinal neurons when those cells were stimulated by glutamate [114]. Taking into account previous immunocytochemistry data from L-Cit labeling, where only neurons were stained [70], Socodato and cols [114] demonstrated that cultured Müller glial cells could not release NO, even when stimulated with glutamate. Therefore, we strongly suggested that in retinal tissues there is an important endogenous nitrergic activity and that neuronal-glial interplay directly modulates bioavailability of the NOS substrate (L-Arg) for NO production and release in early-developing retinal neurons (Fig. **3**).

2.5. The Shining DAF

Kojima and colleagues (1998) proposed a very innovative way for determining NO content within a cell. For the first time it was available a specific NO-trapping tool with the possibility of tracking and visualizing intracellular NO with temporal and spatial resolution. The authors synthesized a series of diaminofluoresceins (DAFs), which displayed high selectivity for NO, differently from previous 2,7-dichlrofluorescein that could react with different ROS, including NO. The main characteristic of DAF-based compounds is that they present aromatic vicinal diamines, which are capable of reacting with NO in the presence of oxygen, forming a triazolic ring that expands its conjugating bound-system, thus absorbing blue light at 495nm and emitting fluorescence at 515nm [85]. In such way, the substance that produced better results regarding fluorescence stability and pH range was diacetate DAF-2 (DAF-2 DA), which has two additional ester bounds in a two-phenolic ring. It is well known however that intracellular environments display high esterase activity and promotes the hydrolysis of ester bounds to create esterate anionic compounds. Those compounds therefore present a strong anionic characteristic and are likely to be trapped within a cell upon hydrolysis. Kojima and colleagues (1998) demonstrated that NO could be fairly quantified and visualized in biological samples using DAF-2 DA. Subsequently, the same group demonstrated that DAF-2 fluorescence stability could be improved towards a broader pH range by the addition of a methyl group in a triazole-forming amine and two fluor atoms in the vicinal acetate groups [86]. The synthesized substance was named diacetate difluormethyl DAF (DAF-FM-DA), and had a widespread use in different cellular systems, including in cultured neuronal cells (Fig. **1**).

Using the quantitative characteristics of DAF-FM-DA fluorescence in cultured retinal neurons, Socodato and colleagues demonstrated that activation of glutamate receptors could strongly increase DAF-FM-DA fluorescence intensity

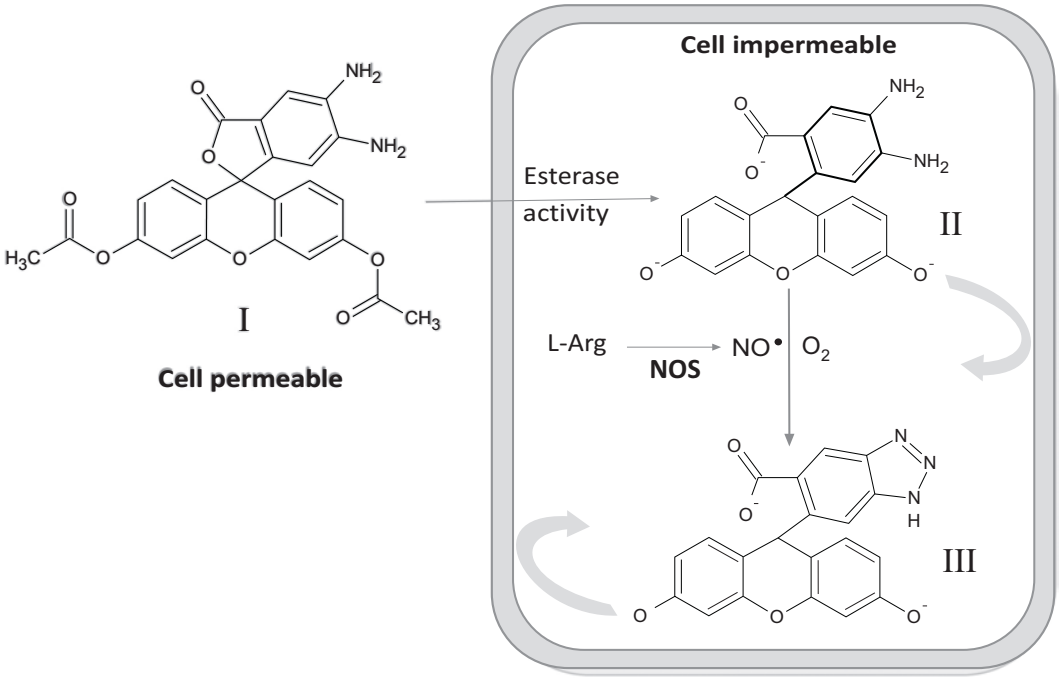


Fig. (1). Schematic representation of DAF-FM-DA action mechanism. DAF-FM-DA is a lipophilic permeable compound (I) that is hydrolyzed by intracellular esterases. Since the anionic form (II) is trapped within the cell, the newly NO produced could immediately react with the aromatic vicinal diamines forming a triazolic ring. This newly formed compound (III) could be detected by fluorescence methods.

[87]. Moreover, the authors specifically showed that neuronal stimulation of Ca^{2+}-permeable AMPA receptors was the initial trigger for the activation of nNOS and NO production [87]. Different from indirect NO measurements and L-Cit immunostaining, the remarkable feature of using DAF-FM-DA was that we could observe that although NO was exclusively produced in neurons, it could also be found in the Müller glia (Fig. **2**). This finding definitely demonstrated the existence of glial targets, modulated by neuronal-derived NO, as suggested by previous data from our group (Fig. **3**). So, we were ultimately convinced that NO neurochemistry depended on both cell types in the developing retina: neurons (producers) and Müller glial cells (substrate suppliers).

3. NO SIGNALING AND DEVELOPMENT: FOCUSING IN THE EMBRYONIC RETINA.

3.1. NO and the Release of Neuromodulators in the Retina

Nitric oxide is an important molecule, which regulates the release of several neuromodulators in the chick retina, such as GABA, glutamate, glutamine, ascorbate and others [88-90].

3.1.1. GABA and Glutamate

Ientile and colleagues showed that NMDA receptor stimulation was capable of stimulating glutamate, GABA and glutamine release in the retina [88, 91]. It has also been observed that the NMDA effect on the release of neuromodulators was NO-dependent since L-NA, a selective NOS inhibitor, totally blocked the NMDA effect in the E9 chick retina [88]. To corroborate this finding, two other NO donors were used, sodium nitroprusside (SNP) and SNAP, and both increased GABA, glutamate and glutamine release. Important data documented in the 1996 article by Ientile and colleagues was the demonstration that NMDA effect was not potentiated by zaprinast, a cGMP phosphodiesterase inhibitor, thus suggesting that the canonical pathway is not involved in the NMDA effect. Furthermore, 8-Br-cGMP, a membrane permeable and phosphodiesterase-resistant cGMP analogue, could not induce GABA, glutamate or glutamine release. The authors showed that this NO-induced GABA and glutamate release occurred before the period of synaptogenesis and they suggested that these phenomena might contribute to important functions during retinal development [88].

In a different experimental model, using the paradigm disseminated by the group of de Mello [92], Ientile and colleagues [91] in 1997 tested GABA release in cultured retinal cells. In this work, they showed that NMDA promoted an increase in [^{3}H]-GABA release and that this effect was mediated by NO, since it was blocked by L-NA and mimicked by the NO donor spermine/NO complex (SpNO). On the other hand, the authors documented that the NMDA effect depended on cGMP, since 8-Br-cGMP mimicked this NO-induced GABA release, and zaprinast amplified both NMDA and SpNO-mediated GABA release [91]. The discrepancy between these two studies (from 1996 and 1997) could be

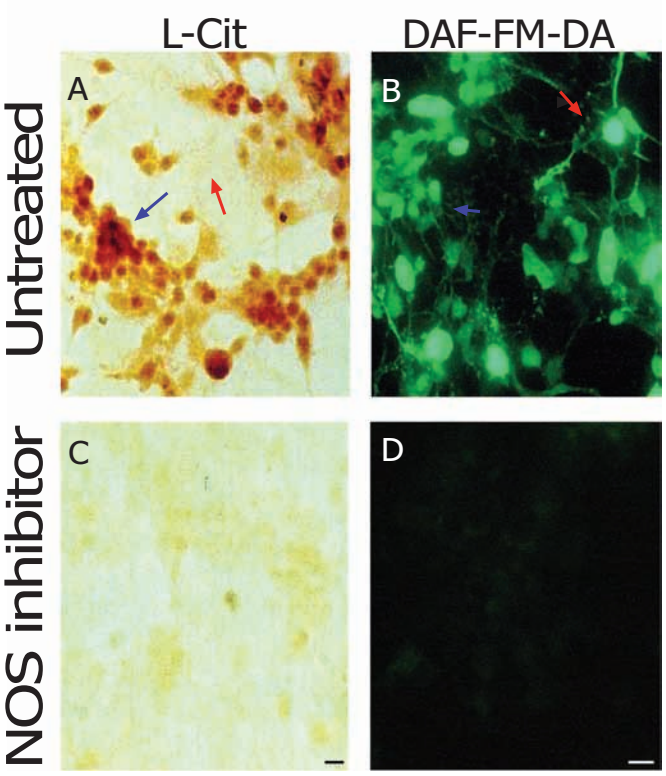


Fig. (2). Determination and cellular localization of NO in embryonic chick retinal cultures. The left photomicrographs (**A** and **C**) represent the immunocytochemistry for L-citrulline using diaminobenzidine. The right photomicrographs (**B** and **D**) represents the triazolic compound fluorescence formed by NO and DAF-FM-DA reaction. Note the L-citrulline immunostaining in neurons (blue arrows) and total absence of this staining in glial cells (red arrows). The DAF-fluorescence was also verified in neurons and unexpectedly in glial cells. The labeling was totally prevented by NOS inhibitors (L-NA 500μM in C; 7-NI 200μM in D). Scale bar 10 μm.

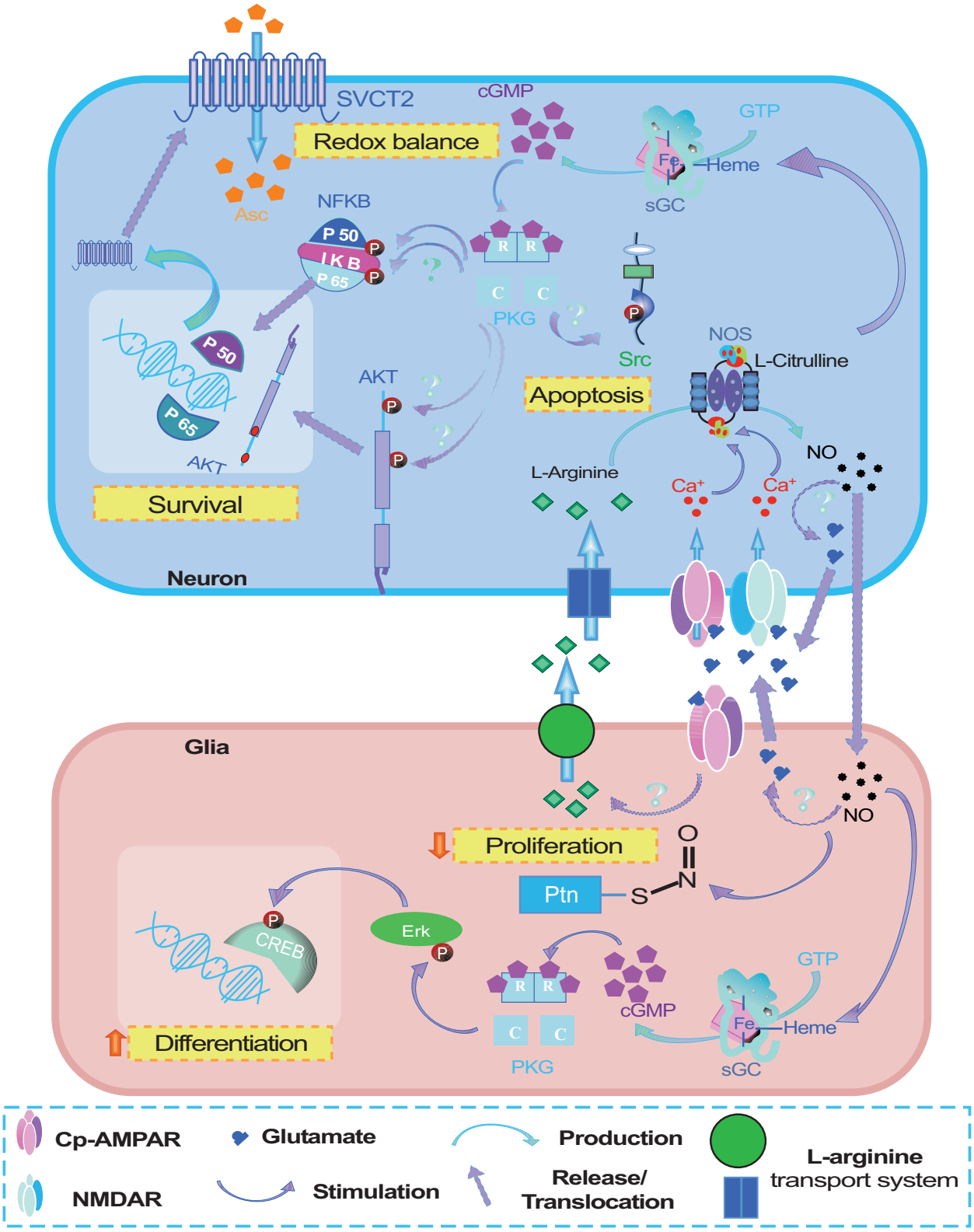


Fig. (3). Schematic representation of multiple NO pathways described throughout this review.

explained by the use of different experimental models, and the authors suggested that the chick retina cell culture was a more suitable model than acute retinal segments for the characterization of neurochemical processes related with retinal development and differentiation [91].

In line with this, it was later shown that NO could also stimulate GABA release in a different experimental model, the adult turtle retina [93]. The authors observed that the NO effect was cGMP-dependent. Another important finding described in that work was the mechanism by which the NO

donor DETA NONOate could stimulate GABA release. It was demonstrated that DETA NONOate-induced GABA release was mediated by the reversal of GABA transporters (GAT) in horizontal cells, which depended on Ca^{2+} ions in the inner plexiform layer [93].

Regarding GABA release, an opposite finding was described [89]. The authors demonstrated that NO inhibited GABA release in the post-hatched chick retina in a Ca^{2+}-independent and Na^{+}-dependent fashion by the reversal of GAT. This finding was corroborated by experiments utilizing NO-711, a GAT inhibitor [89]. Besides the animal model, a significant difference between these works was that Maggesissi *et al*. (2009) treated chicken retinas with L-Arg while Yu and Eldred (2005) used DETA NONOate, a NO donor, in the turtle retina. Accordingly, the differences between these studies could be explained by the differences between NO bioavailability generated by L-Arg and DETA NONOate within the retinas. It was observed that L-Arg exerted a biphasic modulation in the ganglion cell layer, as low concentrations of NO led to inhibition of GABA release whilst high NO concentrations increased GABA release. On the contrary, in amacrine cells, NO could only inhibit GABA release regardless the concentration used [89].

3.1.2. Glycine

NO is also capable of regulating the transport of glycine, another inhibitory retinal neuromodulator [93,94]. It was first observed, in rabbit retinas, that NO donors (SNP and SNAP) inhibited high potassium-mediated glycine release [94]. Additionally, it was observed in the adult turtle retina that DETA NONOate could enhance glycine uptake and inhibit its release. To better evaluate this NO effect, Yu and Eldred used 8-Br-cGMP and SIN-1, a $ONOO^{-}$ donor, and observed that SIN-1, but not 8-Br-cGMP, mimicked DETA NONOate effect, suggesting therefore that NO may work through $ONOO^{-}$ to stimulate glycine uptake and inhibit its release [93]. In summary, different groups showed that NO is capable of modulating the release of inhibitory neurotransmitters in many retinal cell types and at different developmental stages of the vertebrate retina.

3.1.3. Acetylcholine

NO is also capable of regulating the release of other retinal modulators such as the stimulatory neurotransmitters acetylcholine [94,95] and dopamine [96-98].

Regarding acetylcholine release, it was described in the rabbit retina that NO donors (SNP and SNAP) enhanced its release induced by high potassium and flickering light. Likewise, the NOS inhibitors, L-nitromonomethylarginine (L-NMMA) and L-NA, reduced light-evoked acetylcholine release. Moreover, as the NO effect was inhibited by strychnine, it was postulated that the increase of acetylcholine release was indirectly mediated by the inhibition in glycine release [94].

In line with this, Okada and colleagues (2001) demonstrated that NO regulates acetylcholine release in the isolated rat retina. Nonetheless, they observed that NO donors (SNAP and NOR3) inhibited high potassium evoked-acetylcholine release [95]. This inhibition seemed to be mediated specifically by NO since carboxy-PTIO (a NO scavenger) abolished SNAP-induced inhibition of KCl-evoked acetylcholine release. Additionally, bicuculline, a $GABA_A$ receptor antagonist, abolished the effect of NO on KCl-mediated release, suggesting that NO potentiates GABA efflux leading to the concomitant inhibition of acetylcholine release [95]. Taken together, these findings strongly suggest that acetylcholine release is related with regulatory effects mediated by NO upon the bioavailability of inhibitory neurotransmitters, as accessed either by their uptake or release from neuronal cells.

3.1.4. Dopamine

Regarding dopamine release, it was observed in the intact bovine retina that a NO donor, hydroxylamine, decreased both basal and potassium-induced dopamine release [96]. In the same report, Bugnon and colleagues also tested whether the NO effect was mediated by cGMP. In order to answer this question, dibutyryl cGMP was used and it was ineffective, suggesting therefore that endogenous NO regulates dopamine release by a cGMP-independent mechanism.

In agreement with Bugnon and colleagues, it was demonstrated that NO leads to a striking decrease in dopamine release stimulated by high potassium in the rabbit retina [97]. In this work, three NO donors were tested (hydroxylamine, SNP and SNAP), and all of them displayed the same effect, i.e. the inhibition of dopamine release. Djamgoz and colleagues in 1995 [99] also evaluated the possible participation of GABA in this NO effect. Likewise, they observed that bicuculline had no significant effect on dopamine release, suggesting that NO had a direct signaling role upon dopaminergic cells. The same group also reported that the decrease in dopamine release observed was not related to SNP-mediated dopamine oxidation at the extracellular environment [99].

Likewise, Pottek and colleagues in 1997 observed in intact carp retinas that SNP did not interfere with basal dopamine levels but could prevent high potassium-evoked dopamine release [98]. In summary, it was demonstrated, in different animal models (bovine, rabbit and carp), that NO inhibits dopamine release mediated by depolarizing stimulus in retinal cells. Additionally, this NO modulation of dopamine release could represent a high-order function in the process of light transduction within the retina since dopamine is a putative transmitter associated with light adaptation.

3.1.5. Ascorbate

Our group described for the first time that NO was capable of stimulating ascorbate uptake in cultured chick retinal cells [90]. Since the early 90's, ascorbate was claimed as an important molecule in the CNS acting as a neuromodulator [100]. Additionally, ascorbate has been described as an important factor for glutamatergic neuronal maturation in the brain [101]. Ascorbate has been shown to be present in a variety of vertebrate retinas [102, 103], including the chick retina [103]. In this tissue, ascorbate displays many functional roles such as: (1) prevention of dopamine oxidation; (2) prolongation of dopamine actions in the extracellular environment [99]; (3) regulation of voltage-dependent potassium currents in ON-centered mixed bipolar cells [104]; and (4) inhibition of dark-induced GABA efflux [105]. In line with this, high-affinity proteins called sodium vitamin C co-

transporters (SVCTs) stereospecifically transport ascorbate in a Na^+-dependent manner using two Na^+ ions for each transported ascorbate molecule [106, 107]. Previous studies showed the presence of SVCT-2 transcripts in the inner nuclear layer of the rat retina [107] and our group demonstrated the presence of SVCT-2 in both cultured chick retinal cells and post-hatched chick retina [90, 108].

Regarding the importance of ascorbate and NO to retinal physiology, our group tested the effect of NO donors (SNAP and Noc-5) and L-Arg on ascorbate uptake and observed that these compounds could stimulate the uptake of ascorbate in cultured retinal cells [90]. In order to confirm the participation of NO, the scavenger carboxy-PTIO was tested and it completely blocked the effect of SNAP. Moreover, we evaluated whether the classical NO pathway could stimulate ascorbate uptake. In such way, we treated cultured retinal cells with sGC and PKG inhibitors and observed that these compounds completely blocked NO-stimulated ascorbate uptake [90]. Additionally, we performed a kinetic characterization of this effect and observed that NO increased the V_{max} for ascorbate uptake, suggesting that it modulates the SVCT-2 transport capacity. This increase in transport capacity induced by NO could reflect an increase in SVCT-2 expression. To test this hypothesis we performed qRT-PCR, western blotting and immunocytochemistry and observed that NO augmented SVCT-2 transcription and expression *via* its classical sGC/cGMP/PKG pathway [90].

As we observed an increase in SVCT-2 mRNA, we decided to evaluate SVCT-2 synthesis. It has already been described that the transcription factor NF-κB was capable of modulating SVCT-2 expression [109]. We then tested whether this transcription factor was involved in NO-induced SVCT-2 expression and observed that NF-κB inhibitors (PDTC and sulfasalazine) completely blocked NO or L-Arg-induced SVCT-2 expression and ascorbate uptake. These data suggested that NO positively regulates SVCT-2 expression and this enhanced expression modulates ascorbate uptake in cultured retinal cells [90] (Fig. **3**). In order to verify whether this effect only occurred in cultured cells, we used the intact chick retina and observed that SNAP or L-Arg robustly increased SVCT-2 expression and ascorbate uptake, indicating that NO-induced stimulation of SVCT-2 expression and ascorbate uptake also occurs *in vivo*.

Overall, within this whole section, it was reported that NO regulates the increase or decrease in evoked and endogenous release of neurotransmitters, probably profoundly impacting on retinal physiology and promoting drastic effects on visual responses. Moreover, considering the entire panel of NO actions within the developing retina, one can conclude that NO-regulated transmitter release directly impacts on the development of retinal cells.

3.2. NO and Proliferation of Developing Retinal Cells

Our group demonstrated in 2006 that NO donors such as the S-nitrosothiol SNAP and SpNO were capable of inhibiting [^{3}H] thymidine incorporation in retinal cells [110]. In those experiments, we took advantage of the high proliferation rate of embryonic retinal cells in culture to evaluate radiolabeled thymidine incorporation into the DNA as a direct index of mitotic progression. The data clearly showed that SNAP and SpNO decreased thymidine incorporation in cultured retinal cells and in the *in vivo* embryonic retina. Those drugs, on the other hand, were not effective in increasing retinal cell death. Differently from data where NO was released from NO donors, blockade of basal NOS activity by a broad spectrum NOS inhibitor (L-NA in that case) had no significant effect in diminishing thymidine incorporation in retinal cultures [110], which was suggestive that endogenous NO production played no significant role in regulating the proliferation of cultured retinal cells.

Seminal works relating NO with nerve cell proliferation [51] and fly development [49] have clearly established that in developing neurons, throughout the embryonic CNS, NO signals to induce cell cycle arrest. Data gathered up to 2006, however, did not convey the embryonic retina as a working model for the anti-proliferative effects of NO. Furthermore, although NO usually stimulates sGC to produce cGMP, this NO-induced proliferation arrest in retinal cells was independent of this canonical pathway [110]. In the same study, Magalhães and colleagues demonstrated that this decrease of NO-induced thymidine incorporation was neither inhibited by ODQ (a blocker of sGC) nor mimicked by cGMP analogs, but was completely blocked by the anti-oxidant DTT, suggesting that S-nitrosylation was operative in regulating the decrease of NO-mediated proliferation in developing retinal cells. Ientile and colleagues demonstrated that NO donors such as SNAP and SNP promoted glutamate, GABA and glutamine release from cultured embryonic retinal neurons in a way independent of cGMP accumulation. Although data from Ientile *et al*. was not directly related with retinal proliferation, they demonstrated a NO-dependent and cGMP-independent release of important neurotransmitter molecules for retinal physiology. On the other hand, it was recently shown that NO was capable of enhancing the proliferation rate of endothelial cells through an S-nitrosylation-dependent mechanism [111].

An attractive hypothesis presented in the paper of Magalhães *et al*. [110] was that NO-mediated inhibition of retinal cell proliferation was associated with the capacity of NO to S-nitrosylate ornithine decarboxylase (ODC) to consequently decrease polyamine synthesis. This premise took into account an earlier finding by the group of Ignarro [112] showing that NO donors, including S-nitrosoglutathione (GSNO), inhibited the activity of purified ODC and this effect was prevented by DTT (as in the paper of Magalhães *et al*. [110]) or glutathione. However, Magalhães and colleagues found no evidence of ODC inhibition, since an ODC blocker could not reproduce the SNAP effect in decreasing retinal cell proliferation.

Besides inhibiting retinal cell proliferation, it was also observed that SNAP could block the increase of ATP-induced proliferation in the retina [110], which in turn was MAP kinase-dependent [113]. However, inhibition of cell proliferation induced by NO did not involve an upstream inhibition of the MAPK pathway, since SNAP strongly increased ERK phosphorylation [110]. In later studies, Socodato and colleagues (2009 and 2012) explored these phenomena and demonstrated that NO regulates ERK pathway through the cGMP/PKG/Src signaling network (discussed in the next sub-session).

The main contribution in the work carried out by Magalhães and colleagues in 2006 was the elegant demonstration that glial cells, but not neurons, were the main *in vitro* targets of NO (Fig. **3**). Using thymidine incorporation coupled to image-based autoradiography, the authors showed that cultured Müller glial cells were densely labeled within the nuclei with autoradiographic grains in control groups. Importantly, SNAP-treated cells were completely devoid of this labeling pattern, indicating that NO inhibited Müller glial cell proliferation.

3.3. NO and Transcription Factor Activation: CREB as a Model in the Retina

In a different work, our group showed that glutamate could induce a transitory ERK2 phosphorylation through the activation of AMPA/Kainate ionotropic glutamate receptors [114]. These data were supported by findings showing that DNQX, an AMPA/Kainate receptor antagonist, but not MK-801, an NMDA receptor blocker, suppressed this glutamate-induced ERK2 phosphorylation [114]. Interestingly, the glutamate effect was completely abrogated by nNOS inhibition. Therefore, this evidence suggested that ERK2 phosphorylation induced by AMPA/Kainate receptors was indeed coupled to NO production, pointing toward a direct interaction between these receptors and nNOS [114]. Later on, we managed to demonstrate that this nNOS-mediated NO increase involved the activation of Ca2+-permeable AMPA receptors in retinal neurons [87].

Differently from Magalhães *et al*. (2006) data, NO-induced ERK2 phosphorylation relied exclusively on the canonical NO pathway [114]. Again, pharmacologically mapping the classical NO pathway showed that sGC activity, cGMP accumulation, PKG modulation and PKG-induced Src activation were the main signaling events involved in glutamate and NO-induced ERK2 activation [87]. Socodato and colleagues validated this Src-mediated effect, downstream of the canonical NO pathway, in regulating ERK2 phosphorylation using shRNA-mediated Src loss-of-function and retroviral-dependent Src gain-of-function in primary retinal neuronal cultures [87]. As a direct evidence for the importance of this concatenated signaling, it has been reported that cGMP/PKG/ERK pathway is a key signaling step in the expression of proteins involved in nNOS-dependent neuronal plasticity [115].

Furthermore, it has been shown that glutamate/AMPA/-NOS/PKG/ERK was an upstream regulatory pathway for the transcription factor cAMP-responsive element binding protein (CREB) in cultured retinal cells [114]. Intriguingly, immunocytochemistry and fluorescence microscopy experiments demonstrated that glutamate and NO could stimulate CREB phosphorylation exclusively in Müller glia with no effect in retinal neurons [114]. These data were unexpected since neurons were the classical and well-established targets in coupling glutamatergic neurotransmission to CREB activation in the CNS. In subsequent experiments, the authors demonstrated that neuronal-derived NO could diffuse from neurons to reach the Müller glial cells and regulate the phosphorylation of CREB [114], indicating a direct interplay between neurons and Müller glial cells in coupling glutamate-induced NO release to CREB-mediated transcriptional activation.

In these experiments, Socodato and colleagues (2009) used both mixed neuron-glia cultures and purified glial cultures to measure glutamate-evoked NO release using a selective NO electrode. The authors documented that glutamate could only evoke NO release and CREB phosphorylation in neuron-containing cultures, while in purified glial cultures NO release induced by glutamate was not observed [114]. However, once reached the Müller glia, NO transduction relay promptly induced CREB phosphorylation since sGC and ERK2 were expressed in cultured Müller cells (Fig. **3**). However, contrasting results from Riccio and colleagues indicated that NO could also trigger a cGMP-independent and S-nitrosylation-dependent CREB-DNA binding activity and CREB-regulated transcription in cortical neurons in the absence of CREB phosphorylation [116]. Later on, Riccio and associates characterized that BDNF-mediated NO production in cortical neurons could regulate HDAC2-DNA binding activity and consequent CREB-dependent promoter activation by an S-nitrosylation mechanism [117]. In that sense, it was shown that S-nitrosylation of HDAC2 at Cys 262 and 274 uncoupled this enzyme from the DNA, maintaining histone acetylation and promoter activity in BDNF-induced genes [117].

Although neuronal CREB activation is classically known to play important physiological roles within the brain, conclusive reports also suggested key signaling roles of CREB in glial cells. For instance, ATP and noradrenaline may cooperate to regulate the transcription of *bdnf* gene in cortical astrocytes, which could directly influence activity-dependent synaptic plasticity in cortical cells [118]. Besides, it has also been shown that CREB could partially mediate a cGMP/PKG-dependent anti-apoptotic signal in R28 neuroglial progenitor cells [119], and that NO/Ca^{2+}-responsive CREB signaling pathway played an important role in regulating endoplasmic reticulum-related cell death in human glioma cells [120].

Therefore, it would be interesting to speculate that a cooperative signaling between neuronal-produced NO and glial CREB activation may orchestrate a development-based differentiation of Müller cell progenitors, since NO itself could directly inhibit glial proliferation through S-nitrosylation [110] while, in a cGMP/PKG/ERK2-dependent fashion, NO could trigger CREB-dependent transcription of genes related with cell differentiation, such as *c-fos* [114], and cell survival (Fig. **3**).

3.4. NO and Retinal Cell Death or Survival

Within neuronal cells, NO may be generated postsynaptically through the activity of NMDA receptors, which couple activity-dependent excitatory neurotransmission to nNOS activation by the Ca^{2+}/CaM complex. NO may also diffuse back into the presynaptic terminal and act as a retrograde messenger associated with synaptic strengthening and plasticity, a mechanism that plays an import role in long-term sensitization. Due to its chemical nature in biological systems, NO quickly diffuses through the lipid membrane, activating signaling cascades at different cellular compartments. NO is widely studied in biological systems including the

CNS. However, its actions and signaling mechanisms with respect to the modulation of neuronal survival and death are still controversial. Literature data demonstrate that in several cases NO displays cytotoxic effects but in lesser cases it has been described as a neuroprotective agent.

Specifically in the retina, our group has long been interested in the effects of NO regarding the mechanistic regulation of neuronal cell survival and death. It has been shown that NO was a neurotoxic agent for retinal neurons when released from LPS-primed Müller glial cells [121]. In this scenario, neurons co-cultured with Müller cells derived from iNOS null animals were preserved even when glial cells were stimulated with LPS. The authors readily concluded that iNOS-produced NO mediated retinal neuronal loss [121]. Further on, the use either of a sGC inhibitor or a caspase inhibitor rescued this NO-induced cell death, while a peroxynitrite scavenger prevented this effect [121]. Additional evidence from iNOS knockout mice clearly suggested that in an *in vivo* model of retinopathy, NO generated from this pathway critically contributed for neuronal degeneration during the course of disease [122]. In a different report, it was shown that iNOS-mediated NO production, in an *in vivo* model of experimental autoimmune uveoretinitis, dramatically contributed to photoreceptor cell death [123]. An interesting finding however suggested that the cellular NO source within the retina in this *in vivo* paradigm were infiltrating monocytic cells and not resident retinal immune cells [123]. In line with this, anoxia-induced cultured retinal ganglion cell (RGC) loss was prevented by pharmacological blockade of NOS [124]. In another set of experiments, it was demonstrated that broad-spectrum NOS inhibitors could significantly delay optic nerve axotomy-induced RGC loss [125].

Furthermore, RGC are highly sensitive to NO in the absence of glial cells. In purified rat RGC cultures, NO promoted a dose-dependent increase in cell death, while in RGC co-cultured with Müller glial cells this effect was prevented [126]. NOS activity has also been claimed to play a prominent role in cell death during transient retinal ischemia in rats [127]. Injection of a NOS inhibitor alleviated the deleterious effects of ischemic insult in the photoreceptor layer [127]. In the mouse retina, nNOS-induced NO production and sGC activity has been demonstrated to participate in light-induced photoreceptor degeneration [128]. In that work, the authors documented that intense light stimulation increased Ca^{2+} dynamics in retinal cells, which could lead to nNOS activation and concomitant NO-induced photoreceptor apoptosis [128]. However, Donovan and colleagues (2001) found no direct evidence of a caspase-3-mediated apoptosis in this NO-induced neuronal loss in their *in vivo* paradigm of light-mediated retinal damage. In another report, it was demonstrated that albino rats also displayed a severe NOS-dependent component in light-induced photoreceptor damage and retinal impairment [129]. In that report, the authors showed that inhibiting NOS with L-NAME rescued light-induced photoreceptor loss, accompanied by a robust retinal gain of function [129].

In vivo injections of a NO donor (NOC-12 in this case) in rat eyes produced a robust cell death, mainly in the ganglion cell layer, which was also accompanied by a significant decrease of inner plexiform layer thickness [130]. Interestingly, injection of trophic factors such as CNTF, BDNF or erythropoietin completely prevented this NO-induced retinal damage [130,131]. In another set of experiments, it was demonstrated that NOS inhibition played a significant role both in high glucose and advanced glycation end products (AGEs)-induced loss of cultured retinal cells [132, 133]. Furthermore, endothelin-1 has been shown to increase NO production in cultured retinal cells and this effect was directly associated with endothelin-1-mediated retinal neuronal death [134]. Besides, NOS blockade prevented cone photoreceptor degeneration in the rd-1 mouse model of retinitis pigmentosa [135]. Moreover, NO has been recently associated with TRPV-1-mediated retinal cell death in the mature rodent retina [136]. On the other hand, it was demonstrated that NO displayed an anti-apoptotic action upon neuronal precursor cells in the rat retina [137]. The authors used an *in vitro* system to demonstrate that endogenous NO release could protect cells in the retinal neuroblastic layer from anisomycin-induced apoptosis [137]. Besides, using a diaphorase-based histochemical labeling, the authors concluded that NO exerted its neuroprotective role in developing retinal cells in a paracrine fashion (from the internal retina towards the neuroblastic layer) [137].

Our group characterized a paradigm of neuronal cell death induced by fresh medium re-feeding [83]. In 2007, using this same model of retinal neuronal death, we showed that the NO donor SNAP was capable of preventing this re-feeding-induced cell loss [84]. Besides, release of NO from other donors like GSNO, or the increase of endogenous NO production in cultured neurons using L-Arg also abrogated neuronal death [84].

Many of the neurotoxic effects observed in retinal cells were associated with the capacity of NO in contributing to ROS generation. However, Mejía-García and Paes-de-Carvalho in 2007 showed that the neuroprotection induced by NO in retinal neurons was completely dependent on sGC and cGMP accumulation [84]. Furthermore, the protective effect of NO on retinal neurons involved different protein kinases, as for example the PI3K/AKT and MEK/ERK pathways, since inhibiting both pathways NO-induced neuronal survival was prevented. Nonetheless, it is not possible to exclude the involvement of CREB in the survival mechanism mediated by NO since we also have demonstrated the regulation of CREB phosphorylation by NO as a consequence of excitatory signaling in retinal neurons [114], and CREB activity is likely to be involved in the developmental regulation of retinal cell survival [138]. However, in 2012, Socodato and colleagues published a work showing the involvement of Ca^{2+}-permeable AMPA receptor activation in increasing apoptotic cell death in the retina by the downstream production of NO. It has been shown that activation of Ca^{2+}-permeable AMPA receptors could activate nNOS, which in turn, *via* the sGC/PKG, increased the (activation of Src) tyrosine kinase, leading to retinal neuronal apoptosis (Fig. **3**).

Interestingly, the regulation of Src activation by AMPA/-NO/PKG occurs in neurons [87], corroborating previous findings which indicate that neurons, but not Müller glial cells, in retinal cultures were capable of producing NO in response to glutamate stimulation. In that sense, it is sugges-

tive that cultured neurons treated with glutamate could not activate a CREB-dependent neuroprotective pathway [114]. Therefore, due to excitotoxic stimulation of AMPA receptors, retinal neurons undergo nNOS-dependent apoptotic cell death, while Müller cells may upregulate CREB activation and were preserved from this NO-mediated cell damage (Fig. **3**).

More recently, our group showed that glutamate, NMDA and the NO classical pathway is also involved in AKT phosphorylation at both Ser 473 and Thr 308 residues. This AKT phosphorylation was required to its translocation to neuronal cell nucleus in cultured retinal cells through a phosphatidylinositol 3' kinase (PI3K) pathway [143]. Recent findings indicate that this nuclear localization of AKT is related to important cellular functions such as proliferation [139] and neuronal differentiation [140]. In addition, increase in NO levels was linked with increased AKT phosphorylation and neuronal cell survival [141]. Along with AKT, it was also observed that inhibition of NOS decreases the phosphorylation of CREB, which may lead to neuronal damage [142]. Still, our previous findings showing that the survival of retinal neurons mediated by NO in retinal cultures is dependent on PI3K activity [84] prompted us to presume that AKT activation, and its consequent nuclear translocation, were responsible for the activation of transcription factors to consequently increase retinal neuronal survival (Fig. **3**).

4. CONCLUDING REMARKS

In this review, we navigated through the deep sea represented by the signaling of NO in the developing retina. We hope we have convinced the reader that NO controls intricate neurochemical networks within retinal neuronal and glial cells and should be regarded as an essential atypical retinal messenger. The release and uptake of transmitter molecules, cellular proliferation and neuronal survival are fundamental aspects for proper development of nerve tissues and, based in solid literature background, we argue that NO is tightly correlated with such regulatory aspects in the developing retinal tissue. Overall, the understanding of NO actions, and its related downstream pathways, may shed light into the network of events modulating the development of the vertebrate retina either under normal or pathophysiological conditions that may culminate in the onset of retinal degenerative diseases.

5. EPILOGUE

Current effort is now being carried out to comprehend the concrete NO participation in the early retinal development. Preliminary data suggest that endogenous NO either can turn on or switch-off the pro-apoptotic program of developing retinal precursor cells in the early retina. Surprisingly, these effects rely exclusively on the activity of PKG-II during retinal development.

CONFLICT OF INTEREST

*The authors declare no conflict of interest.

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