Calcium-permeable α-Amino-3-hydroxy-5-methyl-4-isoxazolepropionic Acid Receptors Trigger Neuronal Nitric-oxide Synthase Activation to Promote Nerve Cell Death in an Src Kinase-dependent Fashion*

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Background: Src is a non-receptor tyrosine kinase that tightly modulates neuronal function in the CNS. AMPAR-mediated signaling regulates Src activation and retinal cell death through the canonical nitric oxide signaling pathway.

Results: AMPAR-mediated signaling regulates Src activation and retinal cell death through the canonical nitric oxide signaling pathway.

Conclusion: Nitric oxide coupling to Src transduces AMPARs signaling in retinal neurons.

Significance: Src activity may integrate death-associated signaling in retinal neurons.

In the retina information decoding is dependent on excitatory neurotransmission and is critically modulated by AMPA glutamate receptors. The Src-tyrosine kinase has been implicated in modulating neurotransmission in CNS. Thus, our main goal was to correlate AMPA-mediated excitatory neurotransmission with the modulation of Src activity in retinal neurons. Cultured retinal cells were used to access the effects of AMPA stimulation on nitric oxide (NO) production and Src phosphorylation. 4-Amino-5-methylamino-2′,7′-difluorofluoresceindiacetatefluorescence mainly determined NO production, and immunocytochemistry and Western blotting evaluated Src activation. AMPA receptors activation rapidly up-regulated Src phosphorylation at tyrosine 416 (stimulatory site) and down-regulated phosphotyrosine 527 (inhibitory site) in retinal cells, an effect mainly mediated by calcium-permeable AMPA receptors. Interestingly, experiments confirmed that neuronal NOS was activated in response to calcium-permeable AMPA receptor stimulation. Moreover, data suggest NO pathway as a key regulatory signaling in AMPA-induced Src activation in neurons but not in glial cells. The NO donor SNAP (S-nitroso-N-acetyl-dl-penicillamine) and a soluble guanylyl cyclase agonist (YC-1) mimicked AMPA effect in Src Tyr-416 phosphorylation, reinforcing that Src activation is indeed modulated by the NO pathway. Gain and loss-of-function data demonstrated that ERK is a downstream target of AMPA-induced Src activation and NO signaling. Furthermore, AMPA stimulated NO production in organotypic retinal cultures and increased Src activity in the in vivo retina. Additionally, AMPA-induced apoptotic retinal cell death was regulated by both NOS and Src activity. Because Src activity is pivotal in several CNS regions, the data presented herein highlight that Src modulation is a critical step in excitatory retinal cell death.

The retina is a structure highly specialized in processing environmental signals. It is derived from the anterior neural tube during embryogenesis, and all major classes of neurotransmitter systems are as expressed in the mature tissue. Within mature retinal tissue there are different populations of neuronal and glial cells, including the microglia.

In the CNS, fast glutamatergic neurotransmission is mediated by ionotropic receptors, mainly NMDA- and AMPA-type glutamate receptors. NMDA receptors are calcium channels that function in an activity-dependent manner, whereas the permeability of AMPA receptors to calcium ions is mainly dictated by the presence or not of GluR2 subunit in receptors composition (1). Moreover, nerve cell loss induced by the activity of calcium-permeable AMPA receptors (CP-AMPARs) has been described in diverse CNS neurodegenerative processes such as Alzheimer’s disease, Huntington’s disease, and multiple sclerosis.

The abbreviations used are: CP-AMPAR, calcium-permeable AMPA receptor; AKT, v-akt murine thymoma viral oncogene homolog 1; CREB, cAMP response element-binding protein; Src, v-src sarcoma (Schmidt-Ruppin A-2) viral oncogene homolog (avian); CSK, C-terminal Src kinase; EB 8, day-old chick embryo retina; FAK, focal adhesion kinase; HBSS, Hanks’ balanced salt solution; NASPM, 1-naphthylacetyl spermine trihydrochloride; NOS, nitric-oxide synthase; nNOS, neuronal NOS; PKG, cGMP-dependent protein kinase; SFK, Src family kinase; sGC, soluble guanylyl cyclase; SH, Src homology; SNAP, S-nitroso-N-acetyl-dl-penicillamine; APV, 2-amino-5-phosphono pentanoic acid; L-NAME, Nω-nitro-L-arginine methyl ester hydrochloride; DAF-FM, 4-amino-5-methylamino-2′,7′-difluorofluorescein diacetate; cPTIO, 2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide, potassium salt; PP1, 4-amino-5-(4-methylphenyl)-7-[(t-butyl)pyrazolo-d-3,4-pyrimidine; CT, control.

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amyotrophic lateral sclerosis, stroke, and ischemia (1). On the other hand, neurotransmission mediated by CP-AMPARs in the retina occurs under physiological conditions in a large scale and controls diverse signaling events (2).

Nitric oxide (NO) is a gaseous signaling molecule that displays ubiquitous roles within neuronal cells, modulating several aspects of their physiology (3, 4). NO is produced by nitric-oxide synthase enzymes (NOS) using l-arginine as substrate (5). Besides, several effects of NO are mediated by activating its intracellular receptor, soluble guanylyl cyclase (sGC), which couples to the production of the second messenger cGMP (5). In the retina, a high affinity transport system for l-arginine has already been described (6), and neuronal NOS (nNOS) is abundantly expressed as well (7). In line with this, endogenous NO levels in cultured retinal cells have been demonstrated to regulate the activity of NFkB and the dynamics of ascorbate transport (8). Furthermore, NO has been shown to have both an antiproliferative role (9) and to modulate the phosphorylation of ERK MAP kinases and CREB transcription factor in the retina (10). Moreover, correlations between glutamatergic neurotransmission and NO signaling have been described for several neuronal cells paradigms (3).

The Src family kinases (SFKs) are a class of cytoplasmic protein-tyrosine kinases that are highly conserved among metazoan. Classically, nine members represent this family: Src itself, Yes, Fyn, Lyn, Lck, Hck, Blk, York, and Egr (11). All members display a highly conserved structure with four basic domains: Src homology (SH) 4; SH3; SH2, and SH1 domains (11). SH3 domain interacts with proline-rich motifs (PPXX) in related proteins (11). SH2 domain interacts with phosphotyrosine residues in specific set of proteins creating modular scaffold conformations (12). SH1 is the domain that lies within the activation loop of SFKs; therefore, it directly participates in SFKs activation/inactivation cycle. Activation of SFKs is dictated by the phosphorylation status of both Tyr-416 (in the activation loop at SH1 domain) and Tyr-527 (at the C terminus) (13). When Tyr-527 is phosphorylated, SFKs are in their inactive state. However, when Tyr-527 dephosphorylation is induced, the enzyme autophosphorylates Tyr-416, which promotes its full catalytic activity (13).

SFKs exhibit several important functions in the nervous system. For instance, they modulate long term potentiation (LTP) induction in the hippocampus (14), directly control NMDA-induced LTP in the olfactory bulb (15), regulate the process of myelin formation (16), and control BDNF-induced AMPA receptors expression in neocortical neurons (17).

Although many efforts have already been made elucidating the mechanisms of both NO signaling pathway and SFKs in controlling neuronal functioning in the CNS, an accurate interplay between these systems in neuronal cells remains elusive. To the best of our knowledge, only one report has correlated NO-SFKs in neuronal cells, but this correlation took into account cortical neurons in a hypoxic microenvironment (18). Therefore, given that NO and SFKs systems are extremely important for the normal functioning of CNS cells and that Src is highly expressed in developing retinal neurons (19), we investigated the correlation between AMPAR activation, NO, and Src in neuronal cells. We demonstrate that CP-AMPARs and NO strictly control Src-tyrosine kinase activation in cultured retinal neurons through a cGMP- and PKG-dependent manner. Still, NO production is tightly regulated by AMPA-type glutamate receptors through a calcium-dependent manner, thus consecutively regulating Src activation. In addition, concatenated AMPA-NO-sGC-PKG-Src signaling directly control ERK MAP kinase functioning in this paradigm. Furthermore, AMPA-regulated Src phosphorylation is related to excitatory retinal cell death and can also be reproducible at the mature retina in vivo by intravitreal injections of AMPA.

**EXPERIMENTAL PROCEDURES**

**Animals**

Fertilized White Leghorn chicken eggs were obtained from a local hatchery and incubated at 38 °C in a humidified atmosphere. Wistar and Long Evans rats were purchased from Charles River (Barcelona, Spain) and housed under a 12-h light/12-h dark cycle with standard chow and water ad libitum. All procedures involving animals were conducted in accordance with the Association for Research in Vision and Ophthalmology (ARVO) statement for the use of animals in ophthalmic and vision research.

**Reagents and Drugs**

2-Amino-5-phosphono pentanoic acid (APV) was from Biomol (Plymouth Meeting, PA). 6-Anilinoquinoline-5,8-quinone (LY83583), 4-amino-5-(4-methylphenyl)-7-(t-butyl)pyrazolo-d-3,4-pyrimidine (PP1), 7-nitroindazole, and KT5823 were from Calbiochem. S-Nitroso-N-acetyl-dl- penicillamine (SNAP), 3-(5-hydroxymethyl-2′-furyl)-1-benzyl indazole (YC1), ionomycin calcium salt, S-AMPA, 4′,6-diamidino-2-phenylindole (DAPI), penicillin, streptomycin, EGTA, Heps, 1,2-bis(dimethylamino)ethane (TEMED), β-mercaptoethanol, poly-l-ornithine, l-glutamic acid, 6,7-in tro o xoquinolazine-2,3-dione, (SR235)-1,5-methyl-10,11-dihydro-5H-dibenzo[a,d]cyclohepten-5,10-imine hydron maleate (MK-801), DTT, 1,2-bis(2-aminophenoxy)-ethane-N,N′,N″,N‴-tetraacetic acid tetrakis(acetoxy-methyl ester) (BAPTA-AM), Nω-nitro-l-arginine methyl ester hydrochloride (L-NAME), SKI-1 (Src inhibitor 1), 1-naphthylacetyl spermine trihydrochloride, and BSA were from Sigma. 2-(4Carboxyphenyl)-4,4,5,5-tetramethylimidazole-1-oxyl-3-oxide, potassium salt (cPTIO), anti-rabbit Alexa488, anti-rabbit and anti-mouse Alexa568, anti-rabbit peroxidase-conjugated secondary antibody, and ProLong Gold reagent were from Molecular Probes (Eugene, OR). DMEM/F-12 GlutaMAX™ I medium, Invitrogen B-27 supplement, Neurobasal-A medium, gentamicin (50 mg/ml), 4-amino-5-methylamino-2′,7′-difuoro-rofluorescin diacetate (DAF-FM diacetate), trypsin, minimum essential medium, 199 medium, glutamine, and FBS were from Invitrogen. An ECL kit, PVDF membranes, alkaline phosphatase anti-rabbit secondary antibody, the enhanced chemiluminescence reagent, and anti-mouse/anti-rabbit peroxidase-conjugated secondary antibodies were from GE Healthcare. AKT, Src monolosomal, phospho-Src family (Tyr-416), phospho-Src family (Tyr-527), phospho FAK (Tyr-925), CSK, ERK, and phospho-ERK antibodies were from Cell Signaling (Beverly, MA). Phospho-nNOS Ser-1417 antibody was from Millipore. Phospho-CSK Ser(P)-364 antibody was from Abcam. Fyn...
**AMPAs Controls Src Activation**

and phospho-Fyn Thr-12 antibodies were from Santa Cruz. Mouse monoclonal 2M6 antibody was kindly supplied by B. Schloshauer (NMI Naturwissenschaftliches und Medizinisches Institut an der Universität Tübingen). Complete Mini protease inhibitor mixture tablets were from Roche Diagnostics. The bicinechonic acid (BCA) protein assay kit was from Pierce. Ketamine was from Merial Portuguesa (Rio de Mouro, Portugal), Xylazine was from Bayer Health Care (Carnaxide, Portugal). All other reagents were of analytical grade.

**Plasmids**

pLNCX chick Src Y527F (plasmid 13660), pLNCX chick Src K295R (plasmid 13659), pUMVC (Plasmid 8449), psPAX2 (plasmid 12260), and pMD2.G (plasmid 12259) were from Addgene; Src Mission® shRNA clones TRCN000023597 and TRCN000023598, CSK Mission® shRNA clones TRCN000023735 and TRCN000023736 were from Sigma.

**Cell Culture**

*Chicken Retina Mixed Cultures*—E8 chick embryo retinas were dissected from other ocular tissues (including from the retinal pigmented epithelium) and digested with 0.1% trypsin (w/v) in Ca²⁺⁻ and Mg²⁺⁻ free calcium and magnesium-free HBSS for 17 min at 37 °C. Then cells were suspended in minimum essential medium (supplemented with 3% FBS (v/v), 2 mM l-glutamine, 100 units/ml penicillin, and 100 µg/ml streptomycin), dissociated using a Pasteur pipette, and seeded in 24- or 12-well culture plastic dishes in a density of 2 × 10⁵ cells/mm². Cells were maintained at 37 °C in a humidified incubator with 5% CO₂, 95% air. At C1 (1 day in culture) the medium was replaced with fresh medium, and at C2 or C3, cultures were serum-starved, and experiments were performed at C3 or C4, respectively.

*Chicken Retina Purified Neuronal Cultures*—E8 purified cultures were carried out precisely as previously described (20).

*Rat Retina Mixed Cultures*—Rat pups (3–4 days old) were sacrificed, their eyes were enucleated, and the retina was dissected in HBSS. Then the retinas were digested with 0.07% trypsin (w/v) for 12 min, centrifuged at 1280 × g at room temperature for 1 min, and resuspended and mechanically dissociated using a glass pipette. Cells were counted and seeded with DMEM/F-12 + GlutaMAX™-1 (with 10% FBS (v/v), 50 µg/ml gentamicin) at 6-well culture plates at a density of 2 × 10⁶ cells/cm². The cells were kept in a humidified incubator with 5% CO₂, 95% air for 7 days, but at C1 the medium was changed for fresh medium.

*Culture of Retinal Explants*—Male Wistar rats (9 weeks old) were sacrificed, and their eyes were enucleated. Retinas were carefully dissected completely free from other ocular tissues. Next, 4 radial cuts were performed in each retina using WPI scissors (World Precision Instruments, Inc.). Retinas were carefully transferred to 6-well culture plates with 0.4 µm polycarbonate filter inserts and cultured with Neurobasal A medium (with 50 µg/ml gentamicin, 1X B27 and 10 mM l-glutamine) for 4 days at 37 °C in a humidified incubator with 5% CO₂, 95% air ratio. The explants were checked every day, so they did neither dry nor did the retinas float.

**Lentiviruses/Retroviruses Production and Culture Transduction**

Low passage HEK293T cells were seeded in 90-mm culture dishes. When cultures reached ~80% confluence cells were co-transfected overnight with virus-producing plasmids with Lipofectamine 2000 (Invitrogen) according to the manufacturer’s instructions. Transfection ratios were as follows: 6 µg of shRNA plasmids to 3 µg of psPAX2 to 3 µg of VSVG (2:1:1) or 8 µg of Src constructs to 4 µg of pUMVC to 2 µg of VSVG (4:2:1). Normal growth media replaced the next day transfection media, and cells were cultivated for additional 48 h. Next, media with viral particles were collected and centrifuged at 1500 rpm for 5 min, and the supernatant was collected to new tubes. Cultured retinal cells were infected with viral supernatants at C0 (2 h after plating) overnight. At C1, infection media was replaced for normal growth media, and cells were cultivated up to C4. At C3, cultures were serum-starved for 24 h, and at C4 experiments were carried out.

*Sample Preparation*—Chicken retina mixed cultures extracts were prepared as previously described (10). For rat retina mixed cultures extracts, cells were washed twice with PBS and lysed with RIPA-DTT buffer (150 mM NaCl, 50 mM Tris base, 5 mM EGTA, 1% Triton X-100 (v/v), 0.5% DOC (w/v), 0.1% SDS (w/v), 1 mM DTT, and a Complete Mini protease inhibitor mixture, pH 7.5). Next, cells were scrapped off from the plates, transferred to tubes, sonicated, and centrifuged at 800 × g for 5 min at 4 °C. The supernatant was collected to new tubes, and the pellet was discarded. The amount of protein in each sample was determined using a BCA reagent kit according to the manufacturer’s instructions.

*Western Blot*—SDS-PAGE and Western blotting in cultures obtained from chicken retina were carried out exactly as described elsewhere (10). For cultures obtained from rat retina, proteins were separated by 8% SDS-PAGE and transferred to PVDF membranes. Membranes were blocked with 5% nonfat milk in Tris buffer saline (TBS)-Tween at room temperature, and primary antibodies were incubated overnight at 4 °C. Next, membranes were washed 3 × 10 min with TBS-Tween, and alkaline phosphatase-conjugated secondary antibodies were incubated for 1 h at room temperature. Then membranes were washed 6 × 5 min with TBS-Tween, and developed using an enhanced chemifluorescence detection kit following the manufacturer’s instructions. Fluorescence labeling was captured using Typhoon FLA 9000 system.

Western blots were carried out primarily using AKT as a loading control. Previous works from our group were conducted also using AKT as Western blots loading with reproducible results (10, 20). Furthermore, to rule out any discrepancies in the results generated for pSrc, we compared the variations induced by AMPA and SNAP treatment in total Src levels in relation to AKT and actin. We concluded that neither Src nor AKT protein varied in cultured retinal cells stimulated with AMPA and SNAP (supplemental Fig. 1).

**Immunocytochemistry**—Coverslips with 4% (w/v) paraformaldehyde-fixed E8C3 chicken retina cultures were washed 2 × 5 min in PBS and incubated for 1 h with blocking solution (5% BSA (w/v), 5% FBS (v/v), and 0.1% Triton X-100 (v/v) in PBS.
Next, primary antibodies were added, and the coverslips were maintained in a humidified chamber for 12 h. On the following day, coverslips were washed 3 x 10 min with PBS and incubated with secondary antibodies (anti-rabbit Alexa 568 or anti-mouse Alexa 488) for 2 h. After that, coverslips were washed 2 x 10 min with PBS, 1 x 10 min with TBS, incubated for 1 min with a solution of 1 μg/ml DAPI, and rinsed twice in TBS buffer. Then coverslips were mounted with ProLong Gold anti-fade reagent and visualized under a Nikon 80i fluorescence microscope. Quantification of the fluorescence intensity in experimental groups was determined using the ImageJ (Wayne Rasband, NIH) program. Briefly, 8-bit images were acquired at a resolution of 1024 x 1024 pixels using identical gain and offset parameters for all experimental groups; gamma values were never modified in any case for any group. Next, photomicrographs were converted to gray scale and thresholded in ImageJ software. Lower threshold measurements were modified accordingly within all experimental groups, and the upper threshold values were maintained at 255. Values corresponding accordingly within all experimental groups, and the upper threshold values were maintained at 255. Values corresponding to pixels intensity (mean pixel intensity and integrated intensity) in each group were developed in the ImageJ software and statistically analyzed using the GraphPad Prism Version 5.00 for Mac (GraphPad Software, CA).

DAF-FM Diacetate Labeling—Cultured E8C3 chicken retinal cells were incubated with DAF-FM-DA (5 μM) in the presence of NMDA receptor antagonist APV (100 μM) for 45 min. Incubation with specified drugs was performed after this preincubation period. Next, cultures were fixed with 1% paraformaldehyde (w/v) for 10 min, and coverslips were mounted in confocal mode slides using ProLong Gold and observed under a fluorescence microscope. Acquired images were processed and quantified using the ImageJ software as described above.

Quantification of pSrc in Neuronal Processes—Immunocytochemistry for pSrc Tyr-416 was performed as mentioned above in purified retinal neuronal cultures. Images were acquired using a Zeiss LSM 710 confocal microscope. Afterward, images were developed in xyz 8-bit mode at a resolution of 1024 x 1024 pixels in confocal mode. Next, images were thresholded and exported as raw tiff photomicrographs using the ZEN 2009 software (Carl Zeiss, Germany). Neurites and secondary branches in each neuron were converted to gray scale and individually studied using the ROI manager in the ImageJ software. Pixel intensities were quantified as described above.

Determination of Nitrites by Griess—Culture medium was collected to tubes and centrifuged at 16,000 x g at 4 °C for 5 min. The supernatant was transferred to Eppendorf tubes and kept on ice. Samples were added to an ELISA plate and mixed with an equal volume of Griess reagent (0.5% sulfanilamide (w/v), 2.5% H₃PO₄ (w/v), 0.05% N-1-naphthyl ethylenediamine dihydrochloride (w/v)). Plates were maintained on dark for 30 min at room temperature, and then the absorbance was measured at 550 nm.

Intravitreous Injections—Male Long Evans rats (8 weeks old) were anesthetized using a mix of ketamine (75 μg/kg) and xylazine (10 mg/kg) intraperitoneally administrated. AMPA (3 μl of a 2 mM stock solution; estimated final concentration to be 100 μM in the vitreous) was gently injected into the rat left eyes vitreous body. The control (right eye) was injected with PBS (3 μl). After 1 h of AMPA administration, the animals were sacrificed, and their eyes were enucleated. The retinas were carefully dissected in ice-cold HBSS and transferred to tubes containing 150 μl of RIPA-DTT buffer. Afterward, the samples were sonicated (6 pulses of 1 s) and centrifuged at 16,000 x g for 10 min at 4 °C. The pellet was discarded, and the supernatant was collected to new tubes and frozen at −80 °C. Total amount of protein in each sample was determined by the BCA method. The samples were subjected to 8% SDS-PAGE, and Western blot was carried out as described above.

Statistical Analysis—The data were evaluated by one-way analysis of variance followed by the Bonferroni post-test or unpaired two-tailed Student t test using the GraphPad Prism Version 5.00 for Mac (GraphPad).

RESULTS

We began trying to correlate excitatory activation mediated by glutamate with Src function. For this purpose we applied glutamate (250 μM) to cultured retinal cells and assessed the phosphorylation of Src at Tyr-416, which indicates that the enzyme is activated. The concentration of glutamate used was based on previous studies demonstrating that it is non-toxic to cultured retinal cells (10). Furthermore, cellular composition and proportions in our mixed culture paradigm has already been fully characterized elsewhere (8).

Glutamate induced an increase in Src Tyr-416 phosphorylation quickly and transiently (Fig. 1A) with an effect beginning at 5 min stimulation (285.3 ± 57.0%; n = 4) which was maintained up to 60 min (276.2 ± 27.1%; n = 5) and then decreased below basal levels at 90 min (66.3 ± 7.2%; n = 4). In line with this, we investigated whether glutamate effect on Src phosphorylation was triggered by activation of AMPA-type ionotropic glutamate receptors. Fig. 1B shows that when AMPA receptors were inhibited with 6,7-nitroquinoxaline-2,3-dione (100 μM), the increase in Src phosphorylation triggered by exposure to glutamate was prevented (from 254.7 ± 12.1%; n = 4 to 113.0 ± 13.2%; n = 4), indicating that the effect of glutamate in this process is primarily mediated by AMPA receptors. To confirm this observation, AMPA receptors were activated with AMPA instead of glutamate. As can be seen in Fig. 1C, AMPA (30 min) induced an increase in Src Tyr-416 phosphorylation in a concentration-dependent manner. The increase in Src Tyr-416 phosphorylation triggered by AMPA (50 μM) occurred as early as 5 min (Fig. 1D; 269.6 ± 44.3%; n = 6), an effect that persisted up to 30 min (227.1 ± 41.3%; n = 9), with phosphorylation restored to basal levels at 60 min (94.2 ± 8.8%; n = 9). Moreover, the effect triggered by AMPA was mediated exclusively by AMPA receptors and not by secondary activation of synaptic NMDA receptors, as neither MK-801 (NMDA blocker; Fig. 1E) nor APV (competitive NMDA antagonist; supplemental Fig. 2A) interfered with AMPA-induced increase in Src phosphorylation. Furthermore, several effects of AMPA-mediated neurotransmission in the retina, such as regulation of neurite outgrowth and neuronal cell death, are correlated with a high permeability of AMPA receptors to calcium ions (2, 21, 22). Therefore, we verified the relationship between CP-AMPARs and Src Tyr-416 phosphorylation. For this purpose we inhibited CP-AMPARs with NASPM, which is a synthetic analog of AMPA Controls Src Activation
the Joro spider toxin and selectively antagonizes CP-AMPARs (23). As can be observed in Fig. 1F, NASPM (100 μM) completely blocked AMPA-induced Src phosphorylation. On the other hand, nifedipine (an inhibitor of voltage-gated Ca2+ channels; supplemental Fig. 2B) had no effect in Src phosphorylation induced by AMPA receptors activation. These data suggest that CP-AMPARs directly modulate Src activation due to balancing Tyr-416/Tyr-527 phosphorylation ratio within Src (supplemental Fig. 2C). Moreover, to establish a functional significance between Src phosphorylation/dephosphorylation rate and its activity, we evaluated the phosphorylation of FAK at Tyr-925, which has already been described to be a specific and putative intracellular target of Src (24). As can be seen in Fig. 2B, cultured rat retinal cells treated with AMPA display increased levels of FAK phosphorylated at Tyr-925 both at 15 and 45 min of AMPA stimulation. To test for the relevance of AMPA stimulation in regulating Src activation in an in vivo system, we injected AMPA intravitreally in adult rats and checked for the phosphorylation status of Src at Tyr-416 and FAK at Tyr-925. As can be observed in Fig. 2C, intravitreous AMPA administration was sufficient to promote an increase in Src phosphorylation. Moreover, this increase was correlated...
with enhanced Src activity as an increase in FAK phosphorylation at Tyr-925 could be observed as well (Fig. 2D). Furthermore, CP-AMPARs stimulation was not coupled to Fyn kinase phosphorylation in cultured retinal cells (supplemental Fig. 2D). Importantly, AMPA-induced Src phosphorylation is independent of an AMPA-induced CSK down-regulation as CSK phosphorylation was not altered by AMPA stimulation (supplemental Fig. 2E). Collectively, these data suggest that CP-AMPARs specifically couple to Src activation (in a CSK-independent manner) and not to a global SFKs activation in cultured retinal cells.

There are many reports in the literature correlating ionotropic glutamate receptors with NO production. Therefore, we evaluated whether AMPA receptors directly modulated NO production in neuronal cells. To avoid any interference of NMDA-induced NO production in our primary effect, we abolished basal activity of NMDA receptors using their antagonist (APV) in the incubation saline during the course of AMPA stimulation. As can be observed in Fig. 3, A and C, AMPA strongly increased NO production in neuronal cells, as measured by DAF-FM-DA fluorescence. Furthermore, to correlate NO production with AMPA receptors activation in the intact retinal tissue, we measured the accumulation of nitrites by the Griess method, which is an indirect measure of NO production, in organotypic retinal cultures exposed to AMPA. As can be seen, AMPA also increased the accumulation of nitrites in this paradigm (Fig. 3B). In addition, AMPA-induced increase in neuronal NO production was totally dependent of calcium fluctuations (Fig. 3C), as it was blocked by EGTA, which chelates extracellular calcium, thus preventing its influx as well as by BAPTA-AM, which chelates intracellular calcium. Furthermore, to corroborate these findings, we directly augmented intracellular calcium using a calcium ionophore (ionomycin) and observed a rapid increase in DAF-FM-DA fluorescence in cultured retinal neurons (Fig. 3D). This effect was specifically coupled to a de novo synthesis of neuronal NO, as it was blocked by cPTIO, a NO scavenger, and L-NAME, a general NOS blocker (Fig. 3E). These data collectively suggest that AMPA receptors stimulate the production of NO in retinal neurons in a calcium-depen-
Moreover, to broaden our calcium data with AMPA-induced NO production, we blocked CP-AMPARs with NASPM, and AMPA-regulated NO production was prevented (Fig. 3F). Additionally, CP-AMPARs also increased nNOS phosphorylation in cultured retinal cells (Fig. 3G), suggesting that CP-AMPARs are tightly coupled to nNOS activation in cultured retinal neurons.

Afterward, we wanted to correlate both AMPA stimulation and calcium-induced NO production with Src activation. In this regard, ionomycin promoted a rapid increase in Src phos-
phorylation (Fig. 4A), and this effect was clearly mediated by NO production as both cPTIO and L-NAME blocked ionomycin-induced Src phosphorylation (Fig. 4B). Furthermore, AMPA-induced Src phosphorylation was totally dependent on intracellular calcium rise (Fig. 4C). In line with this, AMPA modulation of Src activation was directly controlled by NO (Fig. 4D) and by its canonical signaling pathway, namely sGC, cGMP, and PKG (Fig. 4E). Using selective inhibitors of NOS such as L-NAME and 7-nitroindazole (7-Ni), 5 μM LY83583 or 1 μM KT5823 and then stimulated with AMPA (50 μM) for 30 min. Data in A–E represent the mean ± S.E. of at least three independent experiments. **p < 0.01 in relation to both ionomycin (B) and AMPA (D); ###, p < 0.001 in relation to AMPA.

FIGURE 4. AMPA-triggered Src phosphorylation is mediated by the canonical NO pathway. A, ionomycin triggers the activation of Src, as assessed by the phosphorylation of Tyr-416 residue. Cultured E8C3 mixed retinal cells were treated with ionomycin (10 μM) for different time points. B, ionomycin (I) regulates Src phosphorylation in a NO-dependent manner. Cultured retinal cells were pretreated for 10 min with cPTIO (50 μM) or L-NAME (100 μM) and then exposed to ionomycin for 15 min. C–E, AMPA/calcium signaling triggers Src phosphorylation through the classical NO pathway. Cells were pretreated with 10 μM BAPTA-AM or 2 mM EGTA (C), 50 μM cPTIO (D), 100 μM L-NAME (E), 50 μM 7-nitroindazole (7-Ni), 5 μM LY83583 or 1 μM KT5823 and then stimulated with AMPA (50 μM) for 30 min. Data in A–E represent the mean ± S.E. of at least three independent experiments. *, p < 0.05; **, p < 0.01; ***, p < 0.001 in relation to control; ##, p < 0.01 in relation to both ionomycin (B) and AMPA (D); ###, p < 0.001 in relation to AMPA.

We have also investigated whether AMPA-triggered Src phosphorylation was restricted to either neurons or glial cells in culture. As can be observed in Fig. 6, AMPA receptors increased pSrc in both Muller glial cells and neurons. AMPA increased pSrc labeling in cultured Muller cells, which were labeled with 2M6 (Fig. 6, A and A.1). Furthermore, AMPA treatment also increased pSrc labeling throughout neuronal cells in culture (Fig. 6, C and C.1). Interestingly, AMPA modulates pSrc by the canonical NO pathway only in cultured retinal neurons, as both LY83583 and KT5823 completely abolished

To further extend these findings, we directly increased either NO or intracellular cGMP production in cultured retinal cells using the NO donor SNAP or the sGC activator YC-1. As shown in Fig. 5, both SNAP (Fig. 5, A and B) and YC-1 (Fig. 5, C and D) increased Src Tyr-416 phosphorylation in a time- and concentration-dependent manner. The specificity of both compounds to activate the canonical NO pathway and to modulate Src phosphorylation was also tested (Fig. 5E). As expected, the effects of SNAP and YC-1 on Src phosphorylation were mediated by activation of the classical NO pathway as both effects were completely blocked by LY83583 and KT5823.
AMPA-triggered Src phosphorylation in purified neuronal cultures (Fig. 6D), but they failed to inhibit AMPA-induced Src phosphorylation in 2M6^ Muller glial cells (Fig. 6B). To further explore this AMPA-induced pSrc increase in neurons, we used a quantitative confocal approach to verify whether Src phosphorylation was achieved in neuronal processes. As can be observed (Fig. 6, E–G), AMPA stimulated pSrc throughout the processes of cultured retinal neurons. Plot distribution and quantification of pSrc labeling confirmed that the intensity of Src phosphorylation was increased in the neurites when cultured neuronal cells were stimulated with AMPA (Fig. 6, F and G).

The ERK pathway is an intracellular target of Src kinase in several paradigms. Hence, we investigated whether NO-Src...
concatenation played a role in ERK MAPKs activation. Both AMPA and SNAP stimulated ERK phosphorylation in purified neuronal cultures (Fig. 7A). Besides, PP1, a general SFKs blocker, completely abolished both AMPA and SNAP-induced ERK phosphorylation (Fig. 7B), indicating that AMPA-NO-PKG signaling utilizes Src family kinases as an upstream mediator of ERK activation in cultured retinal neurons. In this regard AMPA-induced ERK phosphorylation was also blocked by BAPTA-AM (Fig. 7C) and mimicked by the calcium ionophore ionomycin (Fig. 7D), confirming its regulation by intracellular calcium rise. Moreover, the increase in ERK phosphorylation triggered by ionomycin was abolished when sGC or PKG were inhibited.
AMPAs inhibit neurotransmitter signaling pathways in retinal cells. In line with this, CP-AMPARs seem to be the primary source regulating the downstream signaling from NO/Src to ERK activation as NASPM completely prevented the phosphorylation of ERK induced by AMPA (Fig. 7F). To make our pharmacological data more robust, we decided to use genetic tools to regulate Src expression/activity and determine its specific participation in ERK activation. In the first experimental approach, we expressed in cultured retinal cells a constitutively active Src construct (Src Y527F) and observed that expression of active Src is sufficient to enhance the phosphorylation of ERK (Fig. 7G). Additionally, we knocked down CSK (supplemental Fig.
AMPARs have already been correlated with neuronal cell loss in different experimental paradigms (1). Hence, we wanted to correlate NO production and Src activation in CP-AMPARs-induced retinal cell loss. For this purpose we used a protocol to induce excitatory cell death by co-incubating cultured retinal cells with kainate and cyclothiazide. This procedure has been demonstrated to allow the potentiation of AMPA-mediated currents (27) and promoted a strong increase in the number of apoptotic cells in our cultured retinal cells paradigm (Fig. 8A). Importantly, preincubation of cultured cells with the NOS inhibitor L-NNAME or with the highly selective Src inhibitor SKI-1 prevented this kainate/cyclothiazide-induced retinal cell apoptosis. In line with this, incubation of cultures with the NO donor SNAP also enhanced the number of TUNEL-positive cells, an effect that was totally blocked by the selective Src inhibitor (Fig. 8B). Therefore, these data suggest that Src down-stream of NOS activity regulates AMPA-induced excitatory retinal cell death.

**DISCUSSION**

Here, we presented evidence that CP-AMPARs-mediated signaling regulates Src-tyrosine kinase activity and apoptotic cell death in cultured retinal neuronal cells. Moreover, AMPA-triggered Src phosphorylation is mediated by NO and its

**FIGURE 7. AMPA-calcium-NO-Src signaling step regulates ERK activation.** A, shown is AMPA and NO increase pERK levels in cultured retinal neurons. E8C3 purified neuronal cultures were treated with AMPA (50 μM) or SNAP (100 μM) for 30 min, and then immunocytochemistry for pERK (green staining) was carried out. Representative photomicrographs are depicted in the left; a quantification histogram is displayed on the right. B, AMPA and NO regulate pERK levels through a SFK-dependent mechanism. E8C3 mixed retinal cultures were pretreated with PP1 (5 μM) for 5 min and then stimulated with either AMPA (50 μM) or SNAP (100 μM) for 5 min. Note that both AMPA- and SNAP-induced pERK increase was completely abolished by inhibiting Src with PP1. C–E, calcium-triggered NO production increases pERK in retinal cells is shown. C, an intracellular calcium rise is linked with AMPA-regulated ERK phosphorylation. E8C3 retinal cultures were pretreated with BAPTA-AM (10 μM) for 5 min and then stimulated with AMPA (50 μM) for 5 min. D, rising calcium concentration directly triggers ERK phosphorylation. E8C3 cultures were treated with ionomycin (10 μM) for the indicated times. Observe that ionomycin regulates ERK phosphorylation in a time-dependent fashion. E, calcium-regulated ERK phosphorylation is mediated by the canonical NO pathway. Retinal cultures were pretreated with LY83558 (5 μM) or KT5823 (5 μM) for 5 min and then treated with ionomycin (10 μM) for 5 min. Note that both compounds totally prevented ERK phosphorylation induced by ionomycin. F, CP-AMPARs are directly involved in ERK phosphorylation. Cultured retinal cells were pretreated with NOSPM (100 μM) for 10 min and then stimulated with SNAP for 30 min. G, AMPA regulates ERK phosphorylation in a Src-dependent manner. G, cultured retinal cells were infected with viruses carrying and empty vector (pLKO), Src shRNA, or an Src kinase dead construct (Src K295R). At C4, cultures were transfected with pLKO or Src K295F and then stimulated with AMPA or NOSPM + AMPA as described above. Data in the graphs represent the mean ± S.E. of at least three independent experiments. *, p < 0.05; **, p < 0.01; ***, p < 0.001 in relation to control. #, p < 0.05 in relation to ionomycin or AMPA; ##, p < 0.01 in relation to AMPA; ###, p < 0.001 in relation to shCSK. &p < 0.05 in relation to SNAP. Calibration bar = 10 μm.
canonical signaling pathway, namely sGC/cGMP/PKG, in retinal neurons. Furthermore, intracellular calcium seems to be a key regulator of AMPA-induced NO production and consequently Src activation (Fig. 8C). Interestingly, AMPA also couples to NO production in retinal explants, suggesting that AMPA receptors activity exerts a tight control of NO availability to retinal cells population. Additionally, in vivo experiments suggest that Src activity is modulated by AMPA receptors in the fully organized retinal tissue.

**AMPA Receptors Couple to NO Production**—It is well established that glutamatergic neurotransmission mediated by NMDA receptors modulates nNOS activation and NO production in several CNS regions (3). On the other hand, much less evidence correlates AMPA receptors to enhanced NOS activity, even though recent findings point toward an AMPA-induced NO production in specialized CNS regions, such as the hippocampus (28). The data presented here consistently demonstrate that CP-AMPARs activation increases nNOS activity and modulates NO production in retinal neuronal cells. In this regard we have previously demonstrated that glutamate rapidly stimulates NO generation from neurons in retinal mixed cultures (10) and glutamate-induced nNOS phosphorylation has been demonstrated to couple nNOS activity with excitotoxic neuronal death (29). Herein, when either scavenging NO or blocking NOS activity, AMPA-induced DAF-FM-DA fluorescence increase was completely abolished in cultured retinal neurons. Furthermore, we also demonstrated that AMPA enhances NO production in the intact retinal tissue using cultured retinal explants. Collectively, these findings suggest that activation of AMPA receptors couples to NO production in retinal neurons, and they further suggest that this coupling also operates in the integral retinal tissue.

The permeability of AMPA receptors to calcium is dictated by either the presence or absence of GluR2 subunit in the receptors composition (30). In the chicken retina (our main experimental model), fluctuations in intracellular calcium concentrations during the early stages of development are primarily due to AMPA receptors activity (31) and CP-AMPARs have been correlated with retinal system functioning in several aspects (2). Additionally, it is known that nNOS is a calcium/calmodulin-dependent enzyme (32). Based on these evidences, we hypothesized that AMPA-coupled NO production in our paradigm was a calcium-dependent event. Interestingly, CP-AMPARs regulated the phosphorylation of nNOS (a hallmark of its enzymatic activation) in cultured retinal cells. Moreover, AMPA-induced NO production in cultured retinal neurons was totally dependent on intracellular calcium rise, as it was blocked when intracellular calcium was chelated and, in a similar way, mimicked using a calcium ionophore. Furthermore, Src phosphorylation in our cultured cells paradigm was strictly regulated by CP-AMPARs. These results suggest that the main source for NO generation specifically in cultured retinal neurons comes from the activation of AMPA receptors that signal to activate Src in a PKG-dependent manner.

**AMPA-induced NO Production Regulates Src, ERK, and Retinal Cell Death**—It has been extensively reported that Src activation depends on the phosphorylation status of two tyrosine residues, namely Tyr-416 and Tyr-527 (13). In this regard we showed here that CP-AMPARs regulate both Src Tyr-416 phosphorylation and Tyr-527 dephosphorylation. Moreover, Src activation is up-regulated by direct AMPA receptor activity and not as a consequence of NMDA receptors activation. Additionally, using FAK Tyr-925 phosphorylation as a functional index of Src activity (33), we also demonstrated that AMPA regulated the phosphorylation of this residue in cultured retinal cells. To further ascertain a physiological role of AMPA-regulated Src activation, we injected AMPA directly into the vitreous body of adult rat eyes. As expected, we could also observe an AMPA-triggered Src Tyr-416 and FAK Tyr-925 phosphorylation. Therefore, it is likely that AMPA receptors positively regulate Src kinase activity in the processes of retinal neurons. Accordingly, Mishra et al. (18) have demonstrated that Src activation induced by hypoxia in the cerebral cortex of newborn piglets required the activity of nNOS. However, in that particular case, excitatory neurotransmission was not directly correlated with their effect. From our knowledge, here we present the first clear evidence that AMPA-mediated neurotransmission directly couples to Src activation in retinal neuronal cells.

Moreover, in cultured retinal neurons AMPA-induced Src phosphorylation was achieved in a NO-dependent manner. We were able to demonstrate that AMPA effect required NOS activity (likely nNOS) as well as the recruitment of the canonical NO signaling pathway. Moreover, double-labeling immunocytochemistry strongly indicates that the AMPA/NO/PKG pathway regulates Src activation in cultured retinal neurons but not in cultured Muller glia. These data are corroborated by previous findings, which indicate that (i) both nNOS and l-arginine transport system are expressed in cultured retinal cells (6) and that (ii) in retinal mixed cultures, neurons, but not glial cells, are set to produce NO in response to glutamate stimulation (10).

Regardless, there are conclusive reports demonstrating that NO regulates Src activation in other cellular systems. For instance, it has been demonstrated that NO is capable of S-nitrosylating c-Src at cysteine 498, and this accounts for β-estradiol-enhanced Src activation in MCF7 cells (33). Herein, our results support the notion that NO-induced Src activation might be independent of S-nitrosylated Src as the blockade of either sGC or PKG completely abrogated the activation of Src induced by AMPA or SNAP. However, additional experiments need to be performed to securely rule out that Src is not S-nitrosylated in retinal neurons, especially by nitrosothiol-related NO donors such as SNAP. Nonetheless, Leung et al. (34) demonstrated that PKG-Iα regulates c-Src activity in human ovarian cancer cells in a NO- and sGC-dependent manner. Likewise, Rangaswami et al. (35) have elegantly demonstrated that cGMP/PKG-II regulate SH2 domain-containing tyrosine phosphatase 1 activity, a signaling pathway that plays a strict role in Src activation in osteoblasts that accounts for cGMP-regulated Src activation in these cells. Hence, the data presented here are in accordance with previous reports as we demonstrated that Src activity in cultured retinal neurons is modulated by cGMP and PKG. In line with this, we ruled out a down-regulation of CSK activation as the responsible mechanism for CP-AMPARs to promote Src activation. These data are important if one considers that endogenous repression of Src is regulated by CSK and that AMPA activates Src in a PKG-dependent manner, sug-
suggesting that AMPA-activated PKG does not target CSK to regulate Src activation. Therefore, mechanisms of AMPA-regulated Src activation could be mediated by SH2 domain-containing tyrosine phosphatase 1 or 2 in our system, but we cannot discard that PKG may directly interact with Src to regulate its activity as suggested by Leung et al. (34). However, the mechanism by which PKG regulates Src function in cultured retinal cells requires further investigation.

Moreover, we have previously reported that glutamate, through AMPA/kainate receptors, rapidly stimulated ERK phosphorylation leading to CREB transcription factor activation in retinal cells (10). Here, we further clarify this intricate signaling pathway showing that CP-AMPARs activation modulates ERK phosphorylation in retinal neurons in a NO- and Src-dependent manner, which suggests that NO, PKG, and Src are concatenated upstream regulators of ERK activation in neuronal cells. These results were further supported by genetic tools that fine-tuned regulated the activity of Src in cultured retinal cells, namely (i) CSK knockdown coupled to selective Src inhibition with SKI-1, (ii) usage of phospho mimetic and phospho defective Src constructs, and (iii) Src knockdown.

Specifically in the retina, CP-AMPARs mediate several modulatory functions under physiological conditions (2). For instance, they regulate the process of neurite outgrowth in cultured retinal cells (22). Accordingly, here we demonstrated that CP-AMPARs also increased apoptosis in cultured retinal cells. Furthermore, NOS and Src activation displayed a clear participation in CP-AMPARs-induced apoptosis in our paradigm. In line with our data, it has been demonstrated that in cultured cortical neurons glutamate-mediated excitotoxicity is completely dependent on Src and SH2 domain-containing tyrosine phosphatase 1 (36). In addition, our data suggest that Src is activated by CP-AMPARs in a NOS-dependent and CSK-independent manner to promote retinal cell death. Moreover, glutamate-induced cell death displays an nNOS-dependent component in cortical neurons (29). However, in cultured retinal cells NO has also been claimed to be a neuroprotective agent (37), although several lines of evidence correlate NO with toxic effects in the CNS. For instance, NO may regulate cortical nerve cell loss by a mechanism involving poly(ADP-ribose) synthetase activation and DNA damage (38). Therefore, it seems that NO plays a dual role regarding cell survival/death in retinal cells.

Overall, here we presented evidence that signaling mediated by CP-AMPARs activation is tightly coupled to neuronal NOS activation and NO production. Still, NO engages to its canonical intracellular pathway and activates Src-tyrosine kinase. Long term AMPA stimulation led to increased retinal cell death, an effect that involves both NOS and Src activity (Fig. 8C). Because both NO and Src are pivotal for the normal functioning of several CNS regions, we suggest that this concatenated signaling, in turn, may regulate signaling events associated with the survival of developing retinal neurons.

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