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Effect of Angeli's salt on the glutamate/glutamine cycle activity and on glutamate excitotoxicity in the hamster retina

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ABSTRACT

Glutamate is the main excitatory neurotransmitter in the retina, but it is toxic when present in excessive amounts. It is well known that NO is involved in glutamate excitotoxicity, but information regarding the possibility that NO-related species could reciprocally affect glutamate synaptic levels was not previously provided. The dependence of glutamatergic neurons upon glia via the glutamate/glutamine cycle to provide the precursor for neurotransmitter glutamate is well established. The aim of the present work was to comparatively analyze the effect of nitroxyl and NO on the retinal glutamate/glutamine cycle *in vitro* activity. For this purpose, Angeli's salt (AS) and diethylamine NONOate (DEA/NO) were used as nitroxyl and NO donor, respectively. AS and DEA/NO significantly decreased retinal L-glutamate uptake and glutamine synthetase activity, but only AS decreased L-glutamine influx. Dithiothreitol prevented all the effects of AS and DEA/NO. The intravitreal injection of DEA/NO (but not AS) or a supraphysiological concentration of glutamate induced retinal histological alterations. Although AS could increase glutamate synaptic concentration *in vitro*, the histological alterations induced by glutamate were abrogated by AS. These results suggest that nitroxyl could regulate the hamster retinal glutamatergic pathway by acting through differential mechanisms at pre- and postsynaptic level.

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1. Introduction

There is a growing body of evidence on the role of nitric oxide (NO) in retinal pathophysiology (Becquet et al., 1997; Goldstein et al., 1996; Roth, 1997; Toda and Nakanishi-Toda, 2007). More recently, interest has been developed in the functional role of an alternative redox form of NO, namely nitroxyl (HNO). HNO chemical features, biological actions and pharmacology were thoroughly reviewed by a experts forum in 2011 (Bullen et al., 2011; Choe et al., 2011; Flores-Santana et al., 2011; Fukuto and Carrington, 2011; Kemp-Harper, 2011; Tocchetti et al., 2011). HNO is formed by a number of diverse biological reactions, such as the decomposition of S-nitrosothiols in the presence of thiols (Arnelle and Stamler, 1995; Wong et al., 1998), manganese superoxide dismutase (Murphy and Sies, 1991), reduction of NO by mitochondrial cytochrome c (Sharpe and Cooper, 1998), hemoglobin (Gow and Stamler, 1998), or xanthine oxidase (Saleem and Ohshima, 2004). Moreover, several authors have suggested that NOS produces nitroxyl rather than NO under certain conditions, particularly at low cofactor concentrations (Adak et al., 2000; Schmidt et al., 1996; Woodward et al., 2010), although this hypothesis has been challenged (Xia and Zweier, 1997). Despite the fact that HNO is a highly unstable species, the introduction of nitroxyl donors such as Angeli's salt (AS, sodium trioxodinitrate) (Bonner and Ravid, 1975; Miranda et al., 2005) has facilitated the elucidation of its biological actions. Indeed, considerable amount of evidence strongly support that AS is a HNO (but not a NO) releasing compound which releases nitroxyl at high rate under physiological conditions ($t_{1/2}$, 37 °C = 2.8 min) (Hughes and Cammack, 1999).

Nitroxyl has been proposed as a regulator of vascular tone (Favaloro and Kemp-Harper, 2007; Irvine et al., 2003), and heart contractility (Paolocci et al., 2003), it inhibits human platelet aggregation (Bermejo et al., 2005), breast tumor growth and angiogenesis (Norris et al., 2008). However, the effect of HNO in the central nervous system is still under debate (Choe et al., 2011). In that sense, it has been shown that exposure to AS provokes a time- and concentration-dependent increase in neural cell death in primary murine mixed cortical cell cultures (Hewett et al.,

Abbreviations: AS, Angeli's salt or sodium trioxodinitrate; DEA/NO, diethylamine NONOate; HNO, nitroxyl; IBMX, 3-isobutyl-1-methylxanthine; NO, nitric oxide; DTT, dithiothreitol; GS, glutamine synthetase; 8-Br-cGMP, 8-bromoguanosine 3′,5′-cyclic monophosphate; GCL, ganglion cell layer; IPL, inner plexiform layer; INL, inner nuclear layer; OPL, outer plexiform layer; ONL, outer nuclear layer; OS, photoreceptor outer segment.

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2005), but it was also demonstrated that HNO induces neuroprotection by attenuating *N*-methyl-D-aspartate (NMDA) receptor activity in cortical neuronal cultures (Colton et al., 2001). Moreover, it was demonstrated that AS increases cerebral ischemic damage in mice (Choe et al., 2009). In a previous report, we have shown that HNO and DEA/NO decrease retinal NOS activity, and that HNO decreases L-arginine uptake (Sáenz et al., 2007).

Glutamate is the main excitatory neurotransmitter in the retina but it is toxic when present in excessive amounts. Elevated levels of extracellular glutamate have been implicated in the pathophysiology of neuronal loss in ophthalmic disorders such as glaucoma, ischemia, diabetes, and inherited photoreceptor degeneration (Brooks et al., 1997; Delyfer et al., 2005; Dkhissi et al., 1999; Lieth et al., 1998; Martin et al., 2002; Moreno et al., 2005). Therefore, an appropriate clearance of synaptic glutamate is required for the normal function of retinal excitatory synapses and for prevention of neurotoxicity. Glial cells, mainly astrocytes and Müller glia, surround glutamatergic synapses, and express glutamate transporters and the glutamate-metabolizing enzyme, glutamine synthetase (GS). Glutamate is transported into glial cells and amidated by GS to the non-toxic amino acid glutamine. Glutamine is then released by glial cells and taken up by neurons, where it is hydrolyzed by glutaminase to form glutamate again, completing the retinal glutamate/glutamine cycle (reviewed by Bringmann et al., 2009). In this way, the neurotransmitter pool is replenished and glutamate neurotoxicity is prevented. Excitotoxic damage is mediated by over activation of the NMDA-type glutamate receptors, which results in excessive Ca^{2+} influx that may lead to a pathological generation of NO (Adachi et al., 1998; Vorwerk et al., 1997). Moreover, Kashii et al. (1996) demonstrate that calcium influx resulting from stimulation of NMDA receptor and elevated NO concentration, can become toxic and mediate glutamate-induced neurotoxicity in cultured retinal neurons. Despite the involvement of the nitridergic pathway in glutamate excitotoxicity, no information regarding the possibility that NO-related species could reciprocally affect glutamate synaptic levels was previously provided. Therefore, the aim of the present work was to comparatively analyze the effect of nitroxyl and NO on the retinal mechanisms that regulate glutamate clearance and recycling in the golden hamster retina. In addition, the effect of nitroxyl on retinal glutamate neurotoxicity was examined.

2. Materials and methods

2.1. Reagents and drugs

Angeli's salt (AS) and DEA/NO were used a nitroxyl and NO donor, respectively. Solutions of AS in NaOH 0.1 M were freshly prepared as previously described (Hughes and Cammack, 1999). The final concentration was determined by the absorbance of the intact trioxodinitrate anion at 242 nm ($\varepsilon = 8300\ M^{-1}\ cm^{-1}$). DEA/NO was obtained from Cayman Chemical Co. (Ann Arbor, MI, USA). L-[^{3}H]-glutamine (specific activity 44.0 Ci/mmol) and L-[^{3}H]-glutamate (specific activity 50.6 Ci/mmol) were obtained from New England Nuclear Corp. (Boston, MA, USA). Dowex resin (AG1-X2, 200–400 mesh chloride form) was purchased from Bio-Rad Laboratories, Richmond, CA, USA. All other reagents were analytical grade and were obtained from Sigma Chemical Co. (St. Louis, MO, USA).

2.2. Animals and tissues

Male golden hamsters (average weight 120 ± 20 g), derived from a stock supplied by Charles River Breeding Laboratories (Wilmington, MA, USA), were purchased from a local dealer. The animals were kept under a photoperiod of 14 h of light-10 h of darkness (lights on at 06.00 h), with free access to food and water. Animals were sacrificed at midday by decapitation Eyeballs were quickly enucleated after death and corneas were removed; the lens and vitreous were dissected under a surgical microscope and the retinas were detached by blunt dissection. The retinas were examined to eliminate possible choroidal tissues. Immediately after dissecting, retinas were used as described below for each protocol. All experiments were performed in ambient light under standard fluorescent room lamps (300 lux). All animal use procedures were in strict accordance with the NIH Guide for Care and Use of Laboratory Animals. All efforts were made to minimize the number of animals used for the studies.

2.3. L-^{3}H-glutamate and L-^{3}H-glutamine uptake assessment

L-Amino acid uptake was assessed in a crude synaptosomal fraction of hamster retinas. Retinas were homogenized (1:9 w/v) in 0.32 M sucrose containing 1 mM $MgCl_2$ and centrifuged at 900*g* for 10 min at 4 °C. Nuclei-free homogenates were further centrifuged at 30,000*g* for 20 min. The pellet was immediately resuspended in buffer Tris–HCl, containing 140 mM NaCl, 5 mM KCl, 2.5 mM $CaCl_2$, 1 mM $MgCl_2$, 10 mM Tris, 10 mM glucose, (adjusted to pH 7.4 with HCl) and aliquots (100–300 μg protein/100 μl) were incubated for 10 min at 37 °C in the absence or presence of AS or DEA/NO (0.2–200 μM), before the addition of L-[^{3}H]-amino acids. At the end of the preincubation period, 100 μl of L-[^{3}H]-glutamine or L-[^{3}H]-glutamate (500,000–800,000 dpm/tube) were added. Synaptosomal fractions were preincubated without or with 10 mM dithiothreitol (DTT) or 1 mM 3-isobutyl-1-methylxanthine (IBMX) for 30 min before adding AS or DEA/NO. After 4 min for L-[^{3}H]-glutamine or 10 min for L-[^{3}H]-glutamate uptake was terminated by adding 4 ml of ice cold Tris–HCl buffer. The mixture was immediately poured onto Whatman GF/B filters under vacuum. The filters were washed twice with 4 ml-aliquots of ice-cold buffer and the radioactivity on the filters was counted in a liquid scintillation counter. Non-specific uptake of L-[^{3}H]-amino acid into synaptosomes was assessed by adding an excess of L-glutamine or L-glutamate (10 mM).

2.4. Glutamine synthetase assessment

Retinas were homogenized (1:10 w/v) in 10 mM sodium phosphate buffer adjusted to pH 7.2. Homogenates were incubated in buffer Tris–HCl for 30 min at 37 °C, with or without AS or DEA/NO (0.2–20 μM). Retinal homogenates were preincubated without or with 10 mM DTT or 1 mM IBMX for 30 min before adding AS or DEA/NO. GS activity was assessed as previously described (Sáenz et al., 2004). Reaction mixtures contained 150 μl of retinal homogenates and equal volume of a stock solution (100 mM imidazole–HCl buffer, 40 mM $MgCl_2$, 50 mM β-mercaptoethanol, 20 mM ATP, 100 mM glutamate and 200 mM hydroxylamine, adjusted to pH 7.2). Tubes were incubated at 37 °C for 15 min. The reaction was stopped by adding 0.6 ml of ferric chloride reagent (0.37 M $FeCl_3$, 0.67 M HCl and 0.20 M trichloroacetic acid). Samples were placed for 5 min on ice. Precipitated proteins were removed by centrifugation, and the absorbance of the supernatants was read at 535 nm against a reagent blank. Under these conditions, 1 μmol of γ-glutamylhydroxamic acid gives an absorbance of 0.340. GS specific activity was expressed as micromoles of γ-glutamylhydroxamate per hour per milligram of protein.

2.5. L-[^{3}H]-glutamine and L-[^{3}H]-glutamate release

L-Amino acid release was examined in a crude synaptosomal fraction of hamster retinas, as previously described (Sáenz et al., 2004). The synaptosomal fraction were incubated for 30 min at

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37 °C with [^{3}H]-amino acids (1000,000–1500,000 dpm/retina) in 500 μl of Tris–HCl buffer. According to Deitmer et al. (2003) this pre-loading period is a compromise between equilibration of intracellular amino acids pools and avoiding metabolism of glutamine or glutamato inside the tissue. The retinal synaptosomal fractions were washed several times in fresh buffer in order to remove the excess of [^{3}H]-amino acids, and then incubated for 10 min in the presence or absence of AS or DEA/NO, with gentle shaking with same buffer or a high K^+ (50 mM) buffer in which osmolarity was conserved by equimolar reduction of Na^+ concentration. Synaptosomes were centrifuged, pellets were digested with hyamine hydroxide and radioactivity in the medium and that incorporated into the tissue were determined in a scintillation counter. Fractional release was calculated as the ratio: radioactivity released/total radioactivity uptaked by the tissue. Greater than 80% of the released radioactivity was identified as authentic glutamine or glutamate by paper chromatography.

2.6. Glutaminase activity assessment

Retinas were homogenized in 40 μl of 0.1% Triton X-100 in 7.5 mM Tris–HCl, pH 8.8. Homogenates were incubated in buffer Tris–HCl for 30 min at 37 °C, with or without AS or DEA/NO (0.2–200 μM). Glutaminase activity was assesses as previously described (Sáenz et al., 2004). The assay mixture contained 30 μl of retinal homogenate (200–400 μg of protein), 50 mM glutamine, 0.2–0.5 μCi L-[^{3}H]-glutamine, and 63 mM potassium phosphate, pH 8.2, in a total volume of 100 μl. Tubes were incubated for 1 h at 30 °C, with gentle agitation. The reaction was stopped by adding 1 ml of cold 20 mM imidazole, pH 7.0. Samples were briefly centrifuged, and the supernatants were applied to 0.6 × 3.5 cm beds of anion exchange resin (Dowex, AG1-X2, 200–400 mesh chloride form, Bio-Rad Laboratories) previously charged with 1 M HCl and washed with water. The reaction substrate was removed with 6 ml of imidazole buffer, which were discarded and the reaction product was eluted with 3 ml of 0.1 M HCl. Aliquots of this fraction were mixed with scintillation cocktail for measurement of radioactivity. Blank were determined from samples lacking homogenates. Glutaminase specific activity was expressed as μmol glutamate per mg protein per hour.

2.7. Intravitreal injection

Hamsters were anesthetized with ketamine hydrochloride (150 mg/kg) and xylazine hydrochloride (0.5 mg/kg) administered intraperitoneally. A drop of proparacaine (2%) was administered in each eye for local anaesthesia. With a Hamilton syringe and a 30-gauge needle, 2 μl of 1.8 M AS or DEA/NO or 0.5 M glutamate in sterile pyrogen-free saline) were injected into one eye of anesthetized hamsters and an equal volume of vehicle (sterile pyrogen-free saline solution) was injected in the fellow (control) eye. A group of animals received an intravitreal injection of AS (3.6 μmol) or vehicle and 10 min later an injection of glutamate (1 μmol) or vehicle. Injections were applied at 1 mm near the limbus, and the needle was left in the eye for 60 s to allow aqueous humor to flow out; this small volume prevented the increase of intraocular pressure and the volume loss.

2.8. Histological evaluation

Hamsters were euthanized 7 days after intravitreal injections. Eyes were immediately enucleated and stored in phosphate-buffered saline 0.1 M (pH = 7.4) with 4% formaldehyde for 24 h. Eyecups were then dehydrated, embedded in paraffin, sectioned with a microtome at 5-μm thickness. Each section was cut along the vertical meridian of the eye through the optic nerve head. Microscopic images were digitally captured with a Nikon Eclipse E400 microscope (illumination: 6-V halogen lamp, 20 W, equipped with a stabilized light source) via a Nikon Coolpix s10 camera (Nikon, Abingdon, VA, USA). Sections were stained with hematoxylin and eosin and analyzed by masked observers. The total retinal, inner plexiform layer (IPL), and inner nuclear layer (INL), outer plexiform layer (OPL), outer nuclear layer (OPL) and photoreceptors layers (OS) thickness (in μm) was measured for each eye and the number of cells in the ganglion cell layer (GCL) was calculated by determining linear cell density for each section (cells/100 μm). No attempt was made to distinguish cell types in the GCL for enumeration of cell number. Measurements were obtained at 1 mm dorsal and ventral from the optic disk. For each eye, results obtained from tree separate sections were averaged and the mean of four to six eyes was recorded as the representative value for each group.

2.9. Protein assessment

Protein content was determined by the method of Lowry et al. (1951), using bovine serum albumin as the standard.

2.10. Statistical analysis

Statistical analysis of results was made by a two-way ANOVA followed by a Dunnett's or Tukey's test, as stated.

3. Results

3.1. In vitro effect of AS and DEA/NO on the retinal glutamate/glutamine cycle activity

Fig. 1 depicts the effect of AS and DEA/NO (0.2–200 μM) on retinal synaptosomal fraction L-[^{3}H]-glutamate uptake. Although AS was more effective than DEA/NO, both compounds significantly decreased this parameter with a threshold concentration of 20 and 200 μM, respectively. The preincubation of synaptosomal fractions with 10 mM DTT significantly prevented the effect of AS and DEA/NO (200 μM) on glutamate uptake. DTT *per se* did not affect this parameter. The effect of AS and DEA/NO on GS activity is shown in Fig. 2. At all concentrations examined (0.2–200 μM), AS and DEA/NO significantly decreased this enzymatic activity. DTT, which showed no effect *per se*, prevented the effect of both donors (200 μM) on GS activity. Table 1 shows the effect of AS and DEA/NO on retinal L-[^{3}H]-glutamine and L-[^{3}H]-glutamate release. In control conditions, ~50% of the pre-loaded radioactivity was released from retinal synaptosomal fractions during the efflux period, for both glutamate and glutamine. AS and DEA/NO did not affect basal or high K^+ induced retinal L-[^{3}H]-glutamine and L-[^{3}H]-glutamate release.

As shown in Fig. 3, only AS (but not DEA/NO) significantly decreased L-[^{3}H]-glutamine uptake in retinal synaptosomal fractions at 200 μM, but not at lower concentrations. The effect of 200 μM AS on glutamine influx was prevented by DTT which was ineffective *per se*.

When a solution of 200 μM AS was allowed to be decomposed at 37 °C (pH 7.4) for 60 min, and then added to tissue samples, or when tissue samples were incubated in the presence of an equivalent concentration of sodium nitrite, no effect on glutamate uptake, GS activity, and glutamine influx were observed (data not shown).

The effect of AS and DEA/NO on retinal glutaminase activity is shown in Fig. 4. The conversion of glutamine to glutamate was not modified by neither of these donors at all the concentrations tested. Table 2 shows the effect of 8-bromoguanosine 3′,5′-cyclic monophosphate (8-Br-cGMP, a permeable cGMP analogue) and

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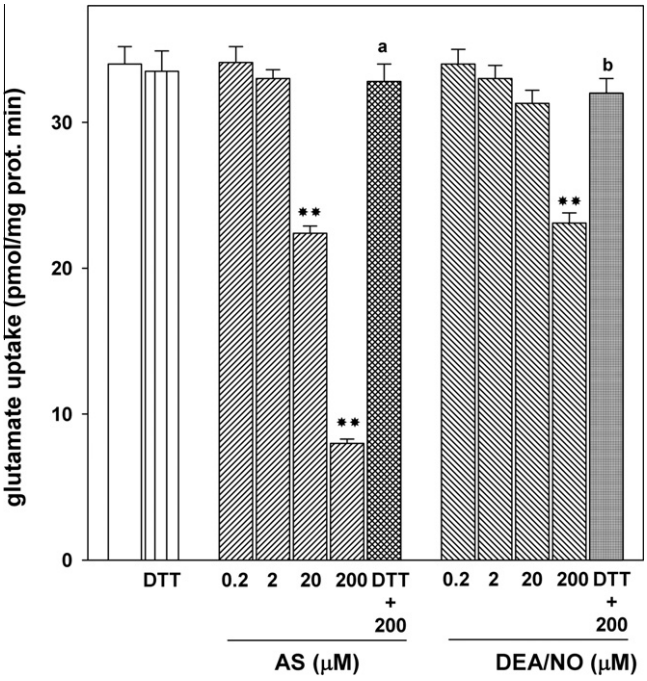

Fig. 1. Effect of AS and DEA/NO on L-[^{3}H]-glutamate uptake in retinal synaptosomal fraction. The synaptosomal fractions were preincubated for 30 min without or with DTT, and then for 10 min in the presence or absence of AS or DEA/NO at different concentrations (0.2–200 μM). AS and DEA/NO significantly decreased L-glutamate influx with a threshold concentration of 20 and 200 μM, respectively. DTT which was ineffective *per se*, significantly prevented the effect of AS and DEA/NO (200 μM). Data are the mean ± SEM of three independent experiments, performed in triplicate. $^{**}p < 0.01$ vs. control, a: $p < 0.01$ vs. AS in the absence of DTT, b: $p < 0.01$ vs. DEA/NO in the absence of DTT, by Tukey's test.

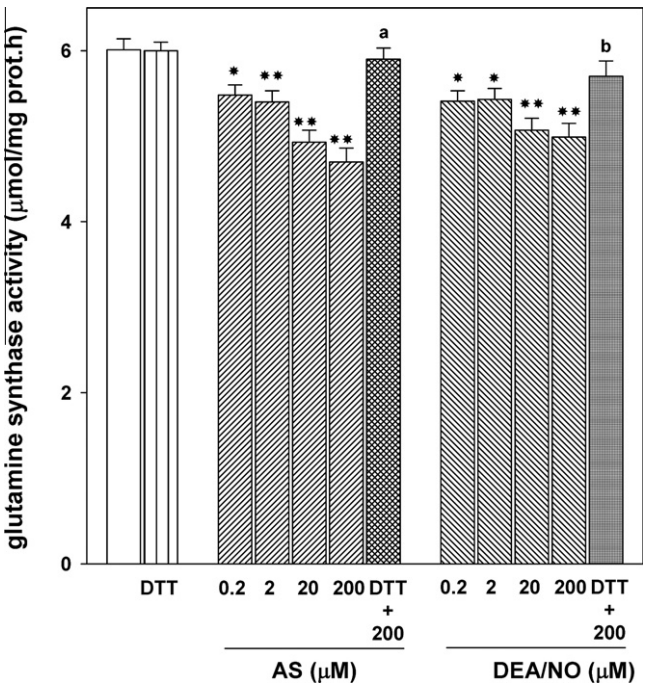

Fig. 2. Effect of AS and DEA/NO on retinal GS activity. Retinal homogenates were preincubated for 30 min without or with DTT, and then, for 30 min in the presence or absence of AS or DEA/NO (0.2–20 μM). AS and DEA/NO significantly decreased retinal GS activity at any concentration tested. Data are mean ± SEM ($n = 8$ retinas per group). $^{*}p < 0.05$, $^{**}p < 0.01$ vs. control, a: $p < 0.01$ vs. AS in the absence of DTT, b: $p < 0.01$ vs. DEA/NO in the absence of DTT, by Tukey's test.

Table 1
Effect of AS and DEA/NO on [^{3}H]-glutamine and [^{3}H]-glutamate fractional release in the golden hamster retina.

	Glutamate release		Glutamine release	
	Basal	High K^+	Basal	High K^+
Control	0.54 ± 0.03	0.66 ± 0.02**	0.56 ± 0.03	0.70 ± 0.02**
AS	0.56 ± 0.02	0.66 ± 0.01**	0.60 ± 0.02	0.69 ± 0.03**
DEA/NO	0.55 ± 0.01	0.65 ± 0.02**	0.59 ± 0.02	0.68 ± 0.02**

The retinal synaptosomal fractions were incubated with or without AS or DEA/NO (200 μM). High K^+ (50 mM) induced a significant [^{3}H]-glutamine or [^{3}H]-glutamate release. AS and DEA/NO were ineffective in the presence or absence of high K^+. Shown are means ± SEM of three independent experiments, performed in triplicate. $^{**}p < 0.01$ when compared basal with K^+-treated samples, by Tukey's test.

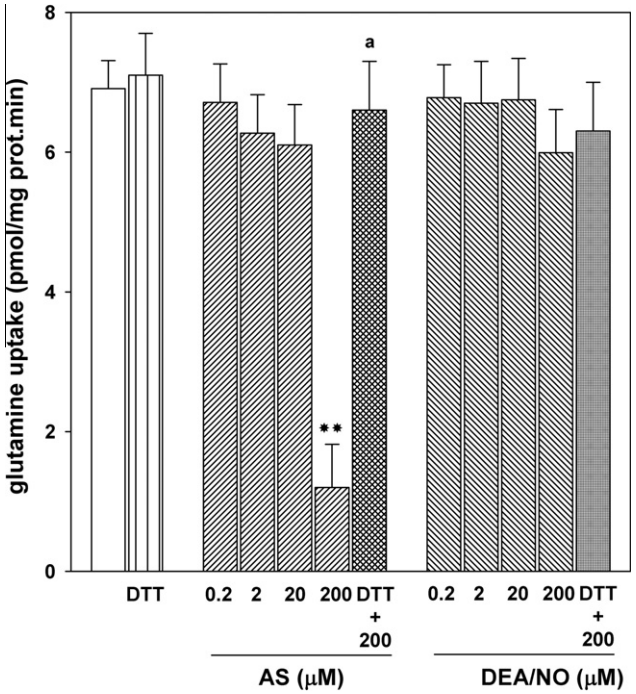

Fig. 3. Effect of AS and DEA/NO on L-[^{3}H]-glutamine uptake in hamster retinal synaptosomal fractions. AS significantly decreased this parameter at 200 μM. Data are mean ± SEM of three independent experiments, performed in triplicate. $^{**}p < 0.01$ vs. control, a: $p < 0.01$ vs. AS in the absence of DTT, by Tukey's test.

IBMX, a phosphodiesterase inhibitor, on glutamate and glutamine uptake, and GS and glutaminase activities. The nucleotide analogue significantly increased glutamate uptake and slightly (but significantly) decreased GS activity and it did not affect glutamine uptake nor glutaminase activity. Similar results were obtained with 2′-*O*-dibutyrylguanosine 3′,5′-cyclic monophosphate (data not shown). IBMX alone did not affect any of these parameters. Moreover, IBMX did not modify the effect of AS or DEA/NO on glutamate uptake and GS activity (data not shown).

3.2. Effect of intravitreal injection of AS or DEA/NO on hamster retinal histology

A single intravitreal injection of AS (3.6 μmol) (Fig. 5B) did not induce obvious alterations of retinal morphology as compared with vehicle-treated eyes (Fig. 5A). In contrast, an intravitreal injection of 3.6 μmol DEA/NO (Fig. 5C) caused a significant decrease in total retinal, OS, and ONL thickness.

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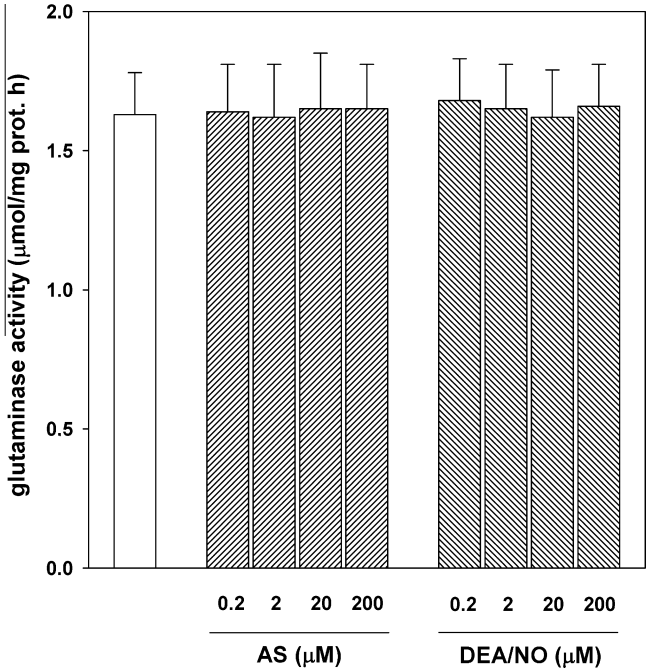

Fig. 4. Effect of AS and DEA/NO on glutaminase activity in golden hamster retina. This enzymatic activity was assessed as described in Materials and Methods. AS and DEA/NO did not change this enzymatic activity at any of concentrations tested. Data are mean ± SEM values (n = 7 retinas per group).

Table 2
In vitro effect of 8-Br-cGMP and IBMX on the retinal glutamate/glutamine cycle activity.

	Control	8-Br-cGMP (500 μM)	IBMX (1 mM)
L-[^{3}H]-Glutamate uptake (pmol/mg prot. min)	32.5 ± 1.5	37.2 ± 1.2*	31.8 ± 1.3
Glutamine synthetase activity (γ-glutamylhydroxamate/mg prot. h)	6.16 ± 0.05	5.79 ± 0.04**	6.09 ± 0.06
L-[^{3}H]-Glutamine uptake (pmol/mg prot. min)	6.3 ± 0.9	6.5 ± 0.7	6.1 ± 0.6
Glutaminase activity (μmol glutamate /mg prot. h)	1.7 ± 0.2	1.6 ± 0.1	1.5 ± 0.2

The cGMP analog (8-Br-cGMP) and IBMX were incubated for 30 min prior to the determination of amino acids uptake or enzymatic activities in the conditions described in Section 2. Data are the mean ± SEM (n = 6 retinas per group). *p < 0.05, **p < 0.01 vs. control, by Dunnett's t-test.

3.3. Effect of intravitreal injection of glutamate in the presence or absence of AS on hamster retinal histology

An intravitreal injection of 1 μmol of glutamate induced a significant reduction in the total retinal, and inner nuclear (INL) and inner plexiform layer (IPL) thickness, and a significant decrease in the GCL cell number (Fig. 6C), as compared with retinas from vehicle-injected eyes at 7 days post-injection (Fig. 6A). When 3.6 μmol AS were intravitreally injected 10 min before 1 μmol glutamate i.v. injection, a complete preservation of the retinal histological structure was observed (Fig. 6D), while AS *per se* did not affect retinal structure (Fig. 6B). Total retinal and retinal layer thickness are shown in Fig. 6E and F, respectively, for all the experimental groups.

4. Discussion

The foregoing results suggest that nitroxyl and NO could be implicated in the regulation of the *in vitro* retinal glutamate/glutamine cycle activity in the hamster. AS and DEA/NO significantly decreased glutamate uptake and GS activity but only AS significantly decreased the retinal influx of glutamine. As already mentioned, glutamate is the main excitatory neurotransmitter in the retina, but it is neurotoxic when present in excessive amounts. Thus, a tight control of glutamate concentrations in the synaptic cleft is crucial for neurotoxicity prevention. Since no enzymes exist extracellularly that degrade glutamate, glutamate transporters are responsible for maintaining low extracellular glutamate concentrations. The present results indicate that HNO from AS, and NO from DEA/NO significantly decreased glutamate influx, suggesting that HNO and NO could provoke an increase in retinal synaptic glutamate concentrations. Some observations support that HNO, and not intact AS nor nitrite from AS, is the chemical entity promoting the regulation of the glutamate/glutamine cycle activity: (1) the effect of AS on glutamate and glutamine uptake was evident after 10 min of incubation which is compatible with its half life as a HNO donor and (2) after a 60 min-period of incubation, decomposed AS did not modify these parameters. Moreover, decomposed AS did not reproduce the effect of freshly prepared AS on GS activity.

The synaptically released glutamate is taken up into glial cells, where it is converted into glutamine by GS activity. The present results indicate that AS and DEA/NO significantly decreased the conversion of glutamate to glutamine. As the assay of GS activity involves the incubation of retinal homogenates in the presence of controlled concentrations of L-glutamate, one can assume that the lower GS activity observed in the presence of AS or DEA/NO were not merely a consequence of a decrease in L-glutamate uptake. Furthermore, the effect of 0.2 μM AS and DEA/NO on GS activity was evident, whereas glutamate uptake was not affected by this concentration of both donors. In this way, both donors could contribute to an increase in glutamate synaptic levels, through a possibly redundant mechanism. Although it was demonstrated a common transcriptional regulation of the most abundant retinal glutamate transporter (GLAST1) and GS in cultured retinal Müller glial cells (Rauen and Wiessner, 2000), the fact that AS and DEA/NO effect on both parameters was evident after a short period of incubation, makes it unlikely the involvement of transcriptional and/or translational mechanisms. Thus, although the effective concentrations of AS and DEA/NO for modulating these parameters were different, it seems possible that not only the expression but also the activity of the proteins mediating retinal uptake and degradation of synaptically released glutamate may be coordinately regulated. In agreement, it was demonstrated that GS activity influences the retinal clearance of extracellular glutamate and that the inhibition of GS causes a decline in glutamate uptake (Shaked et al., 2002). The precise mechanisms responsible for the effects of HNO and NO on the glutamate/glutamine cycle activity remain to be established. In a previous report, we showed that AS and DEA/NO significantly increases cGMP accumulation in the hamster retina (Sáenz et al., 2007). In order to analyze the involvement of this cyclic nucleotide in the effect of AS and DEA/NO on glutamate/glutamine cycle activity, the effect of two cGMP analogs and a phosphodiesterase inhibitor (IBMX) on glutamate and glutamine uptake, GS and glutaminase activities was examined. These nucleotide analogs significantly increased glutamate uptake and decreased GS activity, whereas IBMX did not affect these parameters. In addition, IBMX did not modify the effect of AS or DEA/NO on glutamate/glutamine cycle activity, suggesting that the effect of nitroxyl and NO on glutamate clearance involves other mechanisms than the increase in cGMP levels. Tocchetti et al. (2007) show that nitroxyl effect in myocyte sarcoplasmic reticular Ca^{2+} uptake and release improvement that is independent of cGMP/PKG pathway but, rather, related to thiol modification in cysteines.

Many lines of evidence support the involvement of thiol groups in HNO from AS and NO from DEA/NO biochemical outcomes

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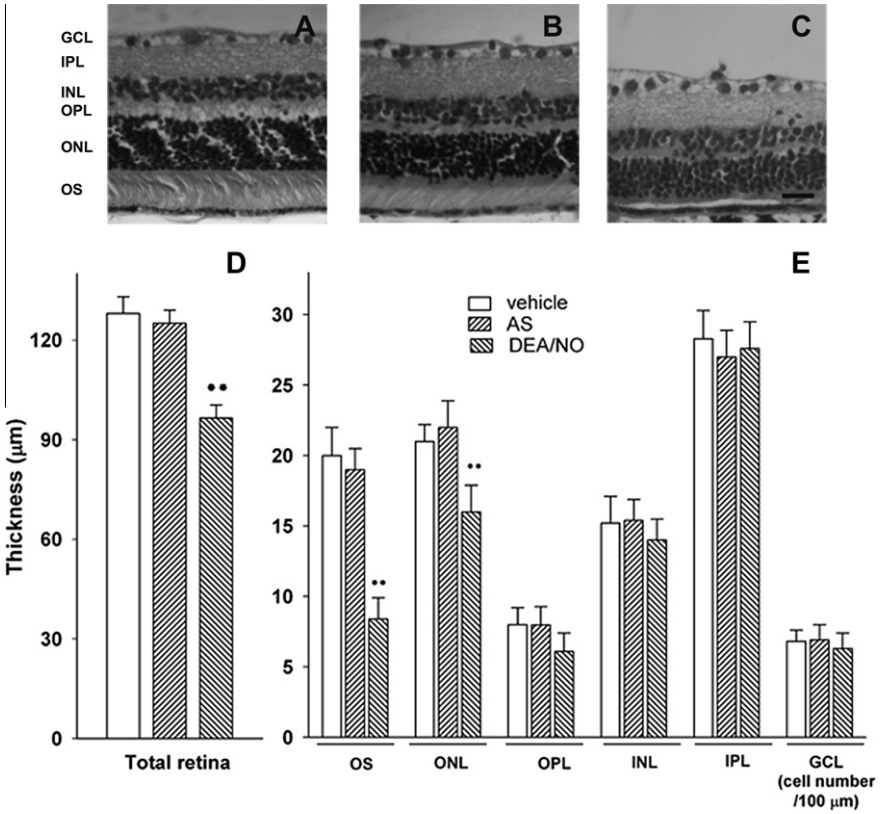

Fig. 5. Morphometric analysis of the effect of AS and DEA/NO on retinal histology, 7 days after an intravitreal injection. Light micrographs of representative retinal sections from eyes that received an injection of vehicle (A), AS (B) and DEA/NO (C). Scale bar = 20 μm. Intravitreal injection of DEA/NO decreased total retina, OS, and ONL thickness (D and E). Data are the mean ± SEM (n = 4–6 retinas per group). $^{**}p < 0.01$, by Dunnett's test.

(Fernhoff et al., 2009; Flores-Santana et al., 2009; Fukuto and Carrington, 2011; Sáenz et al., 2007; Wong et al., 1998). Earlier evidence demonstrating that HNO targeting highly reactive cysteines leads to protein modifications that have well defined functional effects was provided by Froehlich et al. (2008). In this vein, as shown herein, all the effects induced by AS and DEA/NO were prevented by the presence of a thiol-reducing reagent (DTT), supporting the involvement of sulfhydryl groups in their *in vitro* modulation of the glutamate/glutamine cycle activity. In agreement, the presence of functional thiol groups in the main retinal glutamate transporter (GLAST) and in GS was reported (Li and Puro, 2002; Mutkus et al., 2005; Mysona et al., 2009; Rao et al., 1973). Furthermore, it was demonstrated that neural and glial glutamate transporters are post-translationally modified at the level of critical cysteines (Trotti et al., 1997a,b). We have previously shown that AS and DEA/NO significantly decrease retinal protein thiol levels and increase S-nitrosothiol levels. However, HNO is more effective than NO on protein thiol levels, while NO is more effective than nitroxyl on S-nitrosothiol levels (Sáenz et al., 2007), suggesting that differences in the chemical reactivity of these species occur in the presence of sulfhydryl groups. Taken together, these results suggest that HNO and NO could induce a significant increase in glutamate synaptic concentrations. Since it is well established that supraphysiological concentrations of glutamate provoke retinal ganglion cell (RGC) death (Chiu et al., 2005; Fernandez et al., 2009), and based on the results discussed above, it seemed possible that AS and DEA/NO can be toxic to retinal cells by increasing endogenous synaptic levels of glutamate. In that sense, it has been shown that AS induces neurotoxicity in dopaminergic neurons *in vivo* and *in vitro* (Väänänen et al., 2003) and that HNO increases cerebral injury induced by ischemia/reperfusion (Choe et al., 2009). Moreover, in some retinal processes which involve increased synaptic glutamate concentrations such as glaucoma (Belforte et al., 2007) and ischemia/reperfusion injury (Kaur et al., 2008), NO overproduction has been implicated in neuronal death. A significant decrease in OS and ONL thickness was induced by an intravitreal injection of DEA/NO, whereas no major retinal morphological changes were observed after the intravitreal injection of AS, at least at the dose and time examined. Although there is no clear explanation for this discrepancy, a differential chemical behavior of HNO and NO towards thiol groups can account for their differential effects on retinal histology. In agreement with the effect of DEA/NO shown herein, it has been demonstrated that an intravitreal injection of sodium nitroprusside (another NO donor) (Ju et al., 2001; Osborne and Wood, 2004) causes significant damage to photoreceptors while the rest of the retina remains relatively unaffected. Since the main target for retinal glutamate excitotoxicity is the inner retina (Chiu et al., 2005; Fernandez et al., 2009), the effect of DEA/NO on retinal histology cannot be merely attributed to an increase in glutamate synaptic concentrations.

Despite the lack of effect of AS *per se* on retinal histology, and the finding that AS inhibits the uptake of glutamate at higher doses, while it significantly reduced the activity of the GS in a broader range of concentrations, it was still plausible that HNO exposure could increase the neurotoxic effect of exogenous glutamate by inhibiting its uptake and its conversion to glutamine. However, this prediction was not experimentally verified. Paradoxically, when AS was intravitreally injected before glutamate, it prevented the effect of glutamate on the total retina, IPL, INL layer thickness, and GCL cell number. In that sense, despite the key role of GS in glutamate recycling and is high sensitivity to the effect of AS, it should be taken into account the demonstration that the transport of circulating glutamate to the brain normally plays only

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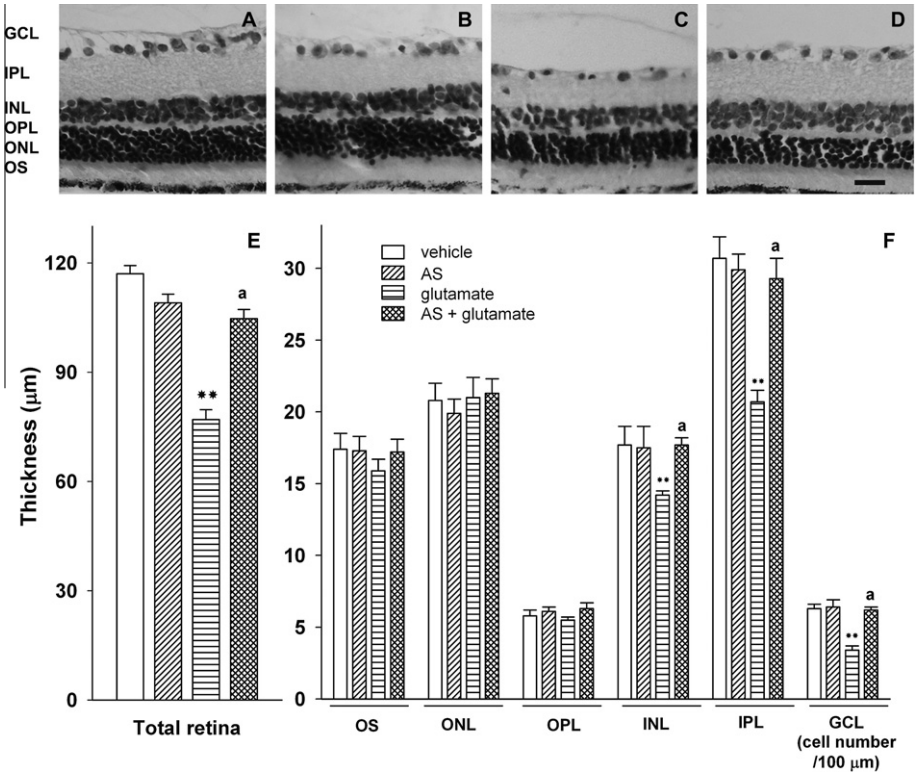

Fig. 6. Upper panel: representative retinal photomicrographs showing histological appearance of retinas at 7 days after two intravitreal injections, separated by 10 min of each other of: vehicle + vehicle (A), AS + vehicle (B), vehicle + glutamate (C), AS + glutamate (D). Glutamate induced significant retinal alterations, whereas when AS was injected before glutamate, the retinal structure was notably preserved. Scale bar = 20 μm. Lower panel: total and retinal layer thickness, and GCL cell count in retinas from eyes injected as above. AS significantly prevented the decrease in total retina (E), IPL and INL layers thickness and in GCL cell number (F) induced by glutamate. Data are the mean ± SEM (n = 6 retinas per group). $^{**}p < 0.01$ vs. control, a: $p < 0.01$ vs. glutamate, by Tukey's test.

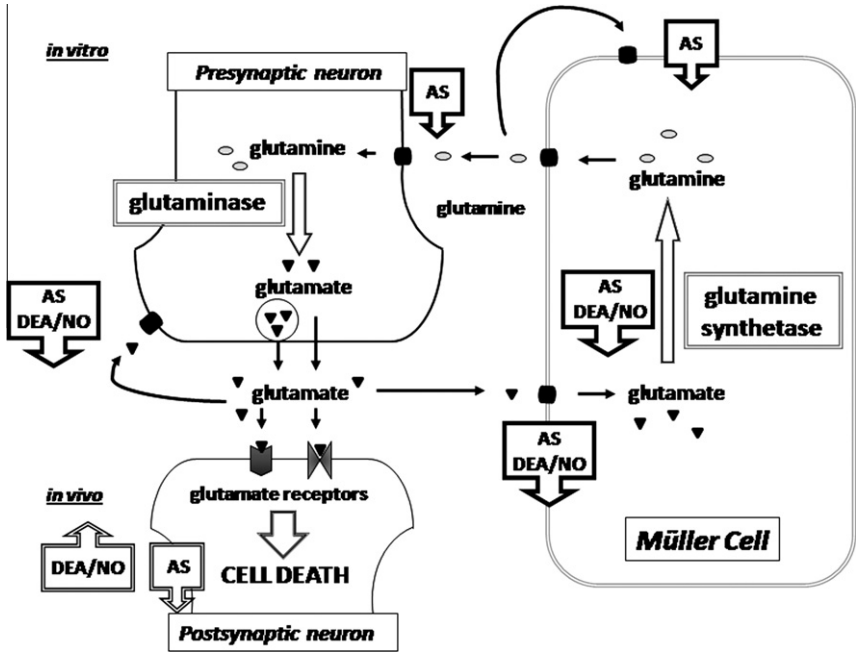

Fig. 7. Schematic representation of the retinal glutamate/glutamine cycle and its modulation by AS and DEA/NO. AS and DEA/NO decreased glutamate uptake and glutamine synthetase activity, but only AS decreased glutamine uptake. The intravitreal injection of DEA/NO provoked histological alterations, whereas AS did not affect the retinal structure. A supraphysiological concentration of glutamate induced a significant retinal damage which was prevented by AS. The upward and downward arrows indicate positive and negative modulations, respectively.

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a minor role in regulating the levels of brain glutamate (Cooper et al., 2003).

It was shown that glutamate neurotoxicity in cultured spinal motor neurons is attenuated by NOS inhibitors (Kume et al., 2005). Since AS significantly decreases retinal NO production (Sáenz et al., 2007), the inhibition of the retinal nitridergic pathway activity by HNO could account for its protective effect against glutamate-induced neurotoxicity. In addition, other mechanism(s) could be involved in HNO-induced neuroprotection, such as the HNO reaction with sulphydryl groups of the NMDA receptor (Kim et al., 1999), which control glutamate-induced neurotoxicity by down-regulating the receptor activity.

In summary, the present results (summarized in Fig. 7) indicate that HNO could differentially regulate the retinal glutamatergic pre- and post-synapsis (i.e. by increasing glutamate synaptic levels and decreasing its postsynaptic toxic effects). It remains unknown whether the HNO signaling observed in the present study can be operative with the endogenous pool. Very recent publications pointing to the detection of HNO *in vivo* are expected to assist the interpretation of its biochemistry (Samuni et al., 2010; Boron et al., 2011). Regardless of whether endogenous synthesis is ultimately confirmed, the present results suggest that HNO significantly interacts with retinal glutamate, supporting its involvement in the regulation of retinal pathophysiology.

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