Inhibition of protein synthesis by activation of NMDA receptors in cultured retinal cells: a new mechanism for the regulation of nitric oxide production

Marcelo Cossenza, Daniel V. Cadilhe, Rodrigo N. Coutinho and Roberto Paes-de-Carvalho

Program of Neuroimmunology, Institute of Biology, Federal Fluminense University, Niterói, RJ, Brazil

Abstract

The synthesis of nitric oxide (NO) is limited by the intracellular availability of L-arginine. Here we show that stimulation of NMDA receptors promotes an increase of intracellular L-arginine which supports an increase in the production of NO. Although L-[3H]arginine uptake measured in cultured chick retina cells incubated in the presence of cycloheximide (CHX, a protein synthesis inhibitor) was inhibited approximately 75% at equilibrium, quantitative thin-layer chromatography analysis showed that free intracellular L-[3H]arginine was six times higher in CHX-treated than in control cultures. Extracellular L-[3H]citrulline levels increased threefold in CHX-treated groups, an effect blocked by N G-nitro-L-arginine, a NO synthase (NOS) inhibitor. NMDA promoted a 40% increase of free intracellular L-[3H]arginine in control cultures, an effect blocked by the NMDA antagonist 2-amino 5-phosphonovaleric acid. In parallel, NMDA promoted a reduction of 40–50% in the incorporation of 35S[methionine or L-[3H]arginine into proteins. Western blot analysis revealed that NMDA stimulates the phosphorylation of eukaryotic elongation factor 2 (eEF2, a factor involved in protein translation), an effect inhibited by (+)-5-methyl-10,11-dihydro-5H-dibenzo[a,d]cyclohepten-5,10-imine maleate (MK801). In conclusion, we have shown that the stimulation of NMDA receptors promotes an inhibition of protein synthesis and a consequent increase of an intracellular L-arginine pool available for the synthesis of NO. This effect seems to be mediated by activation of eEF2 kinase, a calcium/calmodulin-dependent enzyme which specifically phosphorylates and blocks eEF2. The results raise the possibility that NMDA receptor activation stimulates two different calmodulin-dependent enzymes (eEF2 kinase and NOS) reinforcing local NO production by increasing precursor availability together with NOS catalytic activity.

Keywords: glutamate receptor, intracellular pools, L-arginine, L-citrulline, metabolism, retina.


In the last few years the biochemistry and metabolism of L-arginine (L-Arg) have been refocused due to the great importance attached to nitric oxide (NO) physiology (Wiesinger 2001). L-Arg is required for the synthesis of several molecules including polyamines, creatine, agmatine and NO and is a major constituent of proteins, and the metabolic overlapping of these reactions remains to be explored. In all cases the production of these compounds depends on the intracellular concentration of L-Arg. Two pathways have been described: (i) synthesis of L-Arg from different substrates and/or (ii) transport of L-Arg to intracellular compartments.

The transport of cationic amino acids across cell membranes is mediated by a group of well-described transport systems (y+, y'L, b0,+ and B0,+) that take up L-Arg with different properties (sodium dependence, affinity and capacity). The transport is mediated predominantly by the ubiquitous y+ system, which promotes high-affinity L-Arg uptake in a sodium-independent manner (MacLeod and Kakuda 1996; Palacín et al. 1998; Cossenza and Paes-de-Carvalho 2000; Pow 2001; Wiesinger 2001). Recently, the
proteins that mimic y+ activity were discovered and called cationic amino acid transporters (CAFs) (Kakuda and MacLeod 1994; MacLeod et al. 1994; MacLeod and Kakuda 1996; Palacin et al. 1998). The y+L and b0,+ systems also mediate L-Arg transport in a sodium-independent manner and two glycoproteins, rBAT and 4F2hc, were associated with these activities (Kakuda and MacLeod 1994; MacLeod et al. 1994; MacLeod and Kakuda 1996). Finally, the only sodium-dependent system for L-Arg uptake, B0,+ as yet has no protein described to be associated with its activity, although apparently it has been found in some preparations (Hosoya et al. 1997; Cossenza and Paes-de-Carvalho 2000).

In the CNS the urea cycle enzymes carbamoylphosphate synthetase and ornithine transcarbamylase are not expressed, and intracellular L-Arg synthesis is dependent on the synthesis of L-citrulline (L-Cit) by NO synthase (NOS) and its recycling by argininosuccinate synthetase and argininosuccinate lyase (Wiesinger 2001). However, l-Arg levels do not depend only on the L-Arg/NO/L-Cit pathway. Other reactions where L-Arg is consumed, such as synthesis of arginine, catalysed by L-Arg decarboxylase, and protein synthesis, may contribute to the regulation of intracellular L-Arg pools.

Marin et al. (1997) and Scheetz et al. (1997, 2000) showed a decrease in neuronal protein synthesis after NMDA receptor activation. This effect is mediated by phosphorylation of eukaryotic elongation factor 2 (eEF2) by eEF2 kinase (eEF2K), a calcium/calmodulin-dependent enzyme (also described as CAMKIII) (Ryzanov 2002). Phosphorylation of eEF2 has been implicated in the inhibition of protein polypeptide elongation (Palfrey and Nairn 1995; Proud 2002). The role of local protein synthesis in neurons seems to be important in events such as synapse formation, plasticity and neuronal cell death (Marin et al. 1997; Scheetz et al. 1997, 2000; Kandel 2001; Steward and Schuman 2001, 2003; Mendez and Wells 2002; Richter and Lorenz 2002; Tang and Schuman 2002).

Nitric oxide has been involved in physiological functions in the CNS such as learning and memory, long-term potentiation, neurotransmitter release (Bredt and Snyder 1992; Kandel et al. 1997; Wells and Fallon 2000; Blackshaw et al. 2003; Bon and Garthwaite 2003; Stanton et al. 2003; Williams et al. 2003) and in several events during development such as regulation of cell survival, proliferation and differentiation (Peunova and Enikolopov 1995; Yoshioka et al. 2003; Chen et al. 2004). NMDA receptors are involved in many of these events, participating in the generation of NO after activation of the calcium/calmodulin pathway. The relationship between NMDA receptors and NO synthesis has been discussed in the literature (Boehning and Snyder 2003).

The relationship between NO and protein synthesis has not been well studied. As L-Arg is an important structural amino acid and is present in almost all proteins, we decided to refocus the regulation of cytoplasmic L-Arg supply. Our previous work showed the presence of a high affinity and capacity L-Arg uptake in avian retina cultures (Cossenza and Paes-de-Carvalho 2000). In the present study we found a high L-Arg incorporation into proteins. As L-Arg is a key element for several intracellular reactions, we decided to investigate whether protein synthesis could be related to the pathway of NO synthesis after stimulation of NMDA receptors. We found that activation of NMDA receptors promotes inhibition of protein synthesis and L-Arg incorporation in proteins, increasing the availability of this amino acid for the synthesis of NO. Hence we propose that the rate of protein synthesis arises as a new mechanism for the control of intracellular L-Arg availability and its regulation by membrane receptors such as NMDA receptors, and could participate in several events involving neurotransmission or neuromodulation.

**Experimental procedures**

**Preparation of cultures**

Cultures were performed as described by de Mello (1978). Briefly, retinas from 8-day-old chick embryos were dissected from surrounding tissues and treated with 0.1% trypsin in calcium- and magnesium-free saline solution (NaCl, 131 mM; KCl, 4.09 mM; NaH2PO4·7H2O, 0.92 mM; KH2PO4, 0.45 mM; glucose·H2O, 12.2 mM; NaHCO3, 9.4 mM) for 20 min at 37°C. Cells were suspended in basal medium of Eagle supplemented with 5% fetal calf serum, penicillin (100 U/mL) and streptomycin (100 µg/mL), mechanically dissociated and plated at a density of 2 × 104 cells/mm2. Cells were maintained at 37°C in a humid atmosphere containing 5% CO2 and 95% air and were used after 6 days in culture. The medium was replaced every other day. Under these conditions, cultures consist of a mixed population of neurons growing over a layer of flat glial cells (Cossenza and Paes-de-Carvalho 2000).

**L-[3H]Arginine uptake**

For uptake studies, cells were incubated with L-[3H]Arg (5.8 Ci/mmol, 5 µCi/mL; Amersham Biosciences, Piscataway, NJ, USA) in incubation saline (NaCl, 140 mM; KCl, 5 mM; HEPES, 20 mM; glucose, 4 mM; MgCl2, 1 mM; CaCl2·H2O, 2 mM) at 37°C several times in the absence or presence of 355.4 µM (0.1 mg/mL) cycloheximide (CHX; Sigma, St Louis, MO, USA). Cells were washed three times with IS, lysed with water and frozen overnight at −20°C. After thawing, the radioactivity was determined by scintillation counting. For determination of free intracellular amino acids, proteins were precipitated with 5% trichloroacetic acid, centrifuged and the supernatant neutralized with NaOH was analysed by thin-layer chromatography (TLC).

**Measurement of intracellular and released L-[3H]amino acids**

Cultures were incubated for 10 min in IS in the absence or presence of CHX (355.4 µM) and L-[3H]Arg (10 µCi/mL) was added and cultures further incubated for 30 min. Cells were then washed four times with IS (3 min each) and further incubated for 15 min in the absence or presence of different drugs. After this time, IS was removed and cells lysed with water. Intracellular and extracellular radioactivity were determined by scintillation counting. For separ-
ation and measurement of intracellular and released amino acids, the procedure was the same as described above.

**Experiments on L-[3H]arginine availability**

After previous incubation in the presence or absence of CHX for 10 min, and L-[3H]Arg uptake for 30 min, cells were washed four times with IS and stimulated for 15 min with NMDA (1 mM) or NMDA + 2-amino-5-phosphonovaleric acid (100 μM, added in the two previous washing periods and together with NMDA). All solutions containing NMDA were magnesium-free. To evaluate the time course of the effect of CHX, cultures were incubated with this inhibitor during the 15-min period of stimulation or several times in control or NMDA-treated cultures. Cells were then lysed, proteins precipitated and supernatants analysed by TLC.

**Measurement of L-[3H]citrulline formation**

Nitric oxide synthase activity was measured by conversion of L-[3H]Arg to L-[3H]Cit. Cells were loaded with L-[3H]Arg in the absence or presence of CHX during all periods of treatment. In some cases, N^G-nitro-L-arginine (L-NA, 500 μM; Sigma) was added during the washing period after loading with L-[3H]Arg to avoid any effect on the uptake process, as previously described (Cossenza and Paes-de-Carvalho 2000). Stimulation was performed in a period of 15 min after washing and in the absence or presence of CHX. Intracellular and extracellular L-[3H]Cit were measured by TLC after protein precipitation.

**Thin-layer chromatography analysis**

Aliquots from IS and cell lysates previously centrifuged to eliminate proteins were added to silica gel plates (60 F254; Merck Biosciences, Darmstadt, Germany) with a standard solution containing L-Arg, L-ornithine and L-Cit (5 mm). The eluent was a mixture of chloroform, methanol and amonium hydroxide (2 : 3 : 2, v/v/v). After staining with ninhydrin (0.1% ethanol/acetic acid, 5 : 1, v/v), retention factors (Rfs) were determined (L-Arg, 0.20; L-ornithine, 0.40; L-Cit, 0.69), amino acids removed from plates and radioactivity incorporated in proteins in relation to total radioactivity taken up by cells.

**Measurement of L-[3H]arginine or [35S]methionine incorporation**

 Cultures were washed three times and incubated for 10 min in IS with or without 355.4 μCi/mL CHX. L-[3H]Arg (5 μCi/mL) or [35S]Met (1000 Ci/mmol, 0.5 μCi/mL; Amersham Biosciences) was then added and cultures further incubated for different periods of time at 37°C. When NMDA or (+)-5-methyl-10,11-dihydro-5H-dibenz[a,d]cyclohepten-5,10-imine maleate (MK801) (Sigma) was used, the IS was magnesium-free. After incubation, cells were washed, lysed with 5% trichloroacetic acid and intracellular radioactivity determined. Cells were then removed from dishes and centrifuged at 30 000 g for 20 min at 4°C. The pellet was washed, resuspended in 1 N NaOH and the radioactivity determined. Results were then expressed as percentage of radioactivity incorporated in proteins in relation to total radioactivity taken up by cells.

**Western blot analysis**

For detection of eEF2 phosphorylation, cultures were pre-incubated for 10 min, treated for 15 min with different agents, washed, cells scraped off the dishes in sample buffer and the material boiled for 6 min. Samples containing 20 μg protein were submitted to sodium dodecyl sulfate-polyacrylamide gel electrophoresis and proteins transferred to polyvinylidene difluoride membranes which were incubated overnight with an antibody that specifically recognizes eEF2 phosphorylated at Thr56 (1 : 1500 dilution; Cell Signaling, Danvers, MA, USA) or anti-β-actin (1 : 200 dilution; Santa Cruz Biotechnology, Santa Cruz, CA, USA), washed, incubated with peroxidase-conjugated secondary antibody anti-rabbit (1 : 3000; Amersham Biosciences) and revealed by enhanced chemiluminescence (ECL). The total amount of protein in each sample was determined using the Bradford reagent, with bovine serum albumin as standard. Quantitative analysis of blots was performed by scanning images and using the computer program Scion Image (Scion Corporation, Frederick, MD, USA).

**Statistical analysis**

Statistical analysis was performed by ANOVA, Student’s t-test or the post-test of Bonferroni using the program Graph Pad Prism III.

**Results**

**Relation between L-[3H]Arg uptake and release with protein synthesis**

Our previous work showed a high-affinity L-[3H]Arg uptake in avian retinal cells in culture. Autoradiographic studies showed the presence of grains over glial cells as well as neurons (Cossenza and Paes-de-Carvalho 2000). As L-Arg is a structural amino acid present in almost all proteins, it is possible that inhibition of protein synthesis is able to interfere with L-[3H]Arg uptake. Indeed, addition of the protein synthesis inhibitor CHX (355.4 μM) dramatically reduced this uptake by around 60% (2118.4 ± 175.0–856.6 ± 168.6 fmol/mg protein) after 15 min and 73% (4203.0 ± 315.1–1136.0 ± 171.5 fmol/mg protein) at equilibrium (Fig. 1a). This effect of CHX was concentration-dependent, attaining maximal inhibition at concentrations around 70 μM (Fig. 1b). In subsequent experiments we used a concentration of 355.4 μM (0.1 mg/mL) and this concentration of CHX did not show any effect on cell viability as determined by LDH measurements (Fig. 1d). The radioactivity resident in the protein fraction after trichloroacetic acid precipitation was 1664.5 ± 138.0 fmol/mg protein (60% of total uptake after 15 min). Incubation with CHX decreased the incorporation to 13.9 ± 2.6 fmol/mg protein (1.6% of total uptake after 15 min in the presence of CHX), representing a decrease of 92% in L-[3H]Arg incorporation after 15 min of incubation (Fig. 1c).
The decrease of L-[3H]Arg uptake and weak incorporation after inhibition of protein synthesis indicate that this process is an important driving force in the recruitment of amino acids. To determine the variations in free L-Arg concentrations, we decided to quantify free L-[3H]Arg in cell lysates. Interestingly, we found, despite the decrease of L-[3H]Arg uptake, an increase of intracellular free L-[3H]Arg of around 600% (130.0 ± 7.0–756.3 ± 12.1 fmol/mg protein) after CHX treatment (Fig. 1c).

L-[3H]citrulline formation after L-[3H]arginine loading when protein synthesis is blocked

In another series of experiments, we compared the concentrations of free intracellular L-Arg in cultures incubated in the absence or presence of CHX. Cultures were pre-treated with IS in the absence or presence of CHX for 15 min, and then L-[3H]Arg was added. After 30 min, cells were washed four times and incubated again in IS in the absence or presence of CHX for 15 min. Intracellular and extracellular solutions were then both analysed by TLC. As shown in Fig. 2(a), both intracellular and extracellular levels of free L-[3H]Arg increased when cultures were treated with CHX (460.0 ± 42.2–1014.6 ± 36.5 fmol/mg protein, representing 233.5% of intracellular control and 131.8 ± 7.2–505.3 ± 23.2 fmol/mg protein, representing 375% of extracellular control, respectively).

The presence and function of NOS have been described in chick embryo retina cultures (de Faria et al. 1995; Paes-de-Carvalho and Mattos 1996; Paes-de-Carvalho et al. 1996; Ientile et al. 1999; Cossenza and Paes-de-Carvalho 2000). We decided to verify whether NO production could be modified by increasing the intracellular concentrations of L-Arg when protein synthesis was inhibited before loading with L-[3H]Arg. When L-[3H]Cit formation was measured in both intracellular and extracellular compartments, we verified that production of L-[3H]Cit increased in both salines (89.6 ± 2.4–250.9 ± 9.4 fmol/mg protein, an increase of around 280% for the extracellular compartment and 337.8 ± 19.1–675.7 ± 111.8 fmol/mg protein, an increase of around 200% for the intracellular compartment) when treated with CHX (Figs 2b and c). This effect was partially blocked by the NOS inhibitor L-NA (89.6 ± 2.4–170.2 ± 13.2 fmol/mg protein for the extracellular compartment and 337.8 ± 19.1–434.6 ± 1.2 fmol/mg protein for the amino acid were measured. In control conditions, the radioactivity resident in the protein fraction was 1664.5 ± 138.0 fmol/mg protein (60% of total uptake after 15 min). The incubation with CHX decreased the incorporation to 13.9 ± 2.6 fmol/mg protein (1.6% of total uptake in the presence of CHX), representing a decrease of 92% in L-[3H]Arg incorporation after 15 min of incubation. A large increase of intracellular free L-[3H]Arg (approximately 600%) (130.0 ± 7.0–756.3 ± 12.1 fmol/mg protein) was observed after CHX treatment. (d) Measurement of intracellular lactate dehydrogenase (LDH) after incubation of cultures for 30 min with 355.4 μM CHX. The points represent the mean of three (a and b) or four (c and d) experiments ± SEM. The bars in (b) were smaller than the symbol size. The asterisks denote statistical significance (*p < 0.05; **p < 0.01; ***p < 0.001).

Fig. 1 (a) Time course of L-[3H]arginine (L-[3H]Arg) uptake in the absence or presence of cycloheximide (CHX). Cultures were washed and incubated for different times in incubation saline containing L-[3H]Arg in the absence or presence of CHX (355.4 μM) and then processed for measurement of intracellular radioactivity. ○, uptake in control cultures; ●, uptake in the presence of CHX (355.4 μM). Addition of CHX promoted a 60% reduction in uptake after 15 min and 73% at equilibrium. Maximal uptake levels (100%) were 4203.0 ± 315.1 fmol/mg protein. (b) Effect of increasing concentrations of CHX on the total uptake of L-[3H]Arg. (c) Comparison of CHX effects on L-[3H]Arg total uptake, incorporation in proteins and free intracellular concentrations. Cultures were incubated for 15 min with L-[3H]Arg in the presence or absence of CHX (355.4 μM) and the intracellular levels of the amino acid were measured. In control conditions, the radioactivity resident in the protein fraction was 1664.5 ± 138.0 fmol/mg protein (60% of total uptake after 15 min). The incubation with CHX decreased the incorporation to 13.9 ± 2.6 fmol/mg protein (1.6% of total uptake in the presence of CHX), representing a decrease of 92% in L-[3H]Arg incorporation after 15 min of incubation. A large increase of intracellular free L-[3H]Arg (approximately 600%) (130.0 ± 7.0–756.3 ± 12.1 fmol/mg protein) was observed after CHX treatment. (d) Measurement of intracellular lactate dehydrogenase (LDH) after incubation of cultures for 30 min with 355.4 μM CHX. The points represent the mean of three (a and b) or four (c and d) experiments ± SEM. The bars in (b) were smaller than the symbol size. The asterisks denote statistical significance (*p < 0.05; **p < 0.01; ***p < 0.001).
intracellular compartment). This partial blockade could perhaps be explained by the fact that the addition of L-NA was performed 30 min after L-[3H]Arg loading. However, no significant effect of L-NA was observed in control cultures (Figs 2b and c).

These results raised the possibility that the increased intracellular concentrations of L-Arg observed after inhibition of protein synthesis are available as substrate for the synthesis of NO.

Inhibition of protein synthesis by stimulation of NMDA receptors
Marin et al. (1997) and Scheetz et al. (1997, 2000) showed that stimulation of NMDA receptors promotes inhibition of protein synthesis by activation of eEF2K, also called calcium/calcmodulin-dependent kinase type III (CAMKIII), that promotes phosphorylation of eEF2, interrupting the ribosome translocation through the mRNA chain. Using the [35S]Met incorporation technique, we verified a 40% reduction in the incorporation of this amino acid into protein when cultures were treated with NMDA (Fig. 3a). The inhibitory effect was observed as early as 15 min after treatment onset and also at 30 or 60 min of incubation when the steady state was attained (10 990.1 ± 1169.0 fmol/mg protein for control and 7093.2 ± 1055.1 fmol/mg protein for NMDA treatment) (Fig. 3a). The effect of NMDA was concentration-dependent with an IC50 of approximately 50 μM (Fig. 3e). Interestingly, a simple removal of Mg2+ did promote a decrease to 66% of control (2196.8 ± 12.0–1443.1 ± 180.0 fmol/mg protein) in [35S]Met incorporation (Fig. 3b), probably reflecting an activation of glutamate receptors by endogenous glutamate. Addition of MK801 (100 μM) was able to block both the effect of NMDA and Mg2+ removal (Fig. 3b). As stimulation with NMDA was efficient in reducing protein synthesis, incorporation of L-[3H]Arg into protein must be compromised in this situation. To evaluate this hypothesis, we performed the same experimental sequences using L-[3H]Arg as a tracer. Unpublished data from our laboratory show that L-[3H]Arg could be released upon NMDA stimulation. To confirm that the decrease in L-[3H]Arg and [35S]Met signal present in proteins is due to an inhibition of incorporation and not by release of amino acids, experiments were performed to measure the radioactivity of radiolabeled amino acids incorporated in proteins and relating to the total intracellular radioactivity. Results were expressed as incorporation of radiolabeled amino acids in percentage of intracellular radioactivity. Addition of NMDA was able to inhibit 50% of the L-[3H]Arg incorporation with a maximal effect observed after 15 min, in a way very similar to that observed with [35S]Met (1952.9 ± 150.8 for control to 1073.4 ± 341.2 fmol/mg protein for NMDA treatment at the equilibrium state) (Fig. 3c). Removal of Mg2+ was able to inhibit L-[3H]Arg incorporation to around 80% of control (1537.8 ± 14.2–1202.2 ± 160.3 fmol/mg protein) and the
effects of NMDA and Mg$^{2+}$ removal were blocked by MK801 (Fig. 3d).

Intracellular l-[3H]arginine availability after NMDA receptor activation

As shown in Figs 1(c) and 2(a), incubation with CHX promotes an increase in free l-[3H]Arg concentration during uptake and efflux assays. In accordance with the data showing NMDA-induced inhibition of protein synthesis and inhibition of l-[3H]Arg incorporation, we decided to evaluate whether activation of NMDA receptors is also able to increase l-[3H]Arg intracellular pools. Cells were then loaded for 30 min with l-[3H]Arg, washed and incubated for increasing periods of time with 1 mM NMDA. We found that NMDA raised the concentrations of free intracellular l-[3H]Arg by approximately 40% (Fig. 4). The effect was observed as early as 5 min after addition of NMDA and remained approximately constant up to 30 min (Fig. 4a). The effect of NMDA was concentration-dependent with maximal stimulation obtained with a concentration of 500 μM and (100 μM) on [35S]Met (b) and l-[3H]Arg (d) incorporation. Control incorporation levels correspond to 2196.8 ± 12.0 and 1537.8 ± 14.2 fmol/mg protein, respectively. In (a) and (c) the difference between each ○ and ● was significant (p < 0.05). In (b), (d) and (e) the asterisks denote statistical significance (*p < 0.05; **p < 0.01; ***p < 0.001 as compared with control).

Fig. 3 Effect of NMDA on [35S]methionine ([35S]Met) and l-[3H]arginine (l-[3H]Arg) incorporation into proteins. Cultures were incubated for different times with incubation saline containing [35S]Met or l-[3H]Arg and the proteins were precipitated using 5% trichloroacetic acid. Time course of [35S]Met (a) or l-[3H]Arg (c) incorporation in the absence (○) or presence (●) of NMDA (1 mM). Maximal incorporation levels (100%) for [35S]Met and l-[3H]Arg were 10 990.1 ± 1169.0 and 1525.9 ± 150.8 fmol/mg protein, respectively. Maximal inhibition by NMDA was approximately 35% for [35S]Met and 55% for l-[3H]Arg. Effect of magnesium removal, NMDA (1 mM) and MK801 (MK)
EC$_{50}$ around 250 µM (Fig. 4b). The next experiments were then performed using an incubation time of 15 min. As shown in Fig. 4(c), CHX (gray bar) and Mg$^{2+}$ removal were also able to produce the effect (approximately 80 and 30%, respectively). The presence of the NMDA antagonist 2-amino-5-phosphonovaleric acid (ADV; 100 µM) abolished the effect of both NMDA and Mg$^{2+}$ removal, decreasing intracellular L-[3H]Arg concentrations to control levels. To ensure that inhibition of protein synthesis is responsible for the increase of intracellular L-[3H]Arg, the same experiment was performed in the presence of CHX during the complete incubation period. As expected, the presence of CHX raised intracellular L-[3H]Arg concentrations by approximately 120% at all treatment conditions but no additional increase by NMDA was observed (Fig. 4c). It was clearly observed that the effect of NMDA was not present when the protein synthesis machinery was inhibited.

**Relationship between L-[3H]citrulline formation and L-[3H]arginine availability**

The activation of NOS by NMDA has been described in the literature for several systems (Southam and Garthwaite 1991; Schuman and Madison 1994; Snyder and Ferris 2000; Stanton et al. 2003). In this study, using chick embryo retinal cultures, we found an increase of around 50% in extracellular and intracellular L-[3H]Cit production when these cultures were previously loaded with L-[3H]Arg and then stimulated for 15 min with NMDA (89.6 ± 2.4–131.8 ± 7.2 fmol/mg protein for the extracellular and 337.8 ± 19.0–505.3 ± 23.1 fmol/mg protein for the intracellular production). Mg$^{2+}$ removal was also capable of raising L-[3H]Cit production by 18% for extracellular (Fig. 5) and 30% for intracellular production (Fig. 6). In all cases, the NOS inhibitor L-NA completely blocked these effects and the NMDA antagonist APV promoted a partial inhibitory effect. When cells were incubated with CHX only during the 15-min period of stimulation after L-[3H]Arg loading, we observed an increase of around 50% in extracellular (Fig. 5, gray bars) and 30% in intracellular (Fig. 6, gray bars) L-[3H]Cit formation that could also be completely blocked by L-NA, strongly suggesting a relationship between inhibition of protein synthesis and NO production. When experiments were performed in the presence of CHX during the whole incubation period, including the period of L-[3H]Arg loading, we found the expected increase of L-[3H]Cit of

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**Fig. 4 Assay of intracellular L-[3H]arginine (L-[3H]Arg) after stimulation with NMDA or cycloheximide (CHX).** (a) Cultures were incubated several times with L-[3H]Arg and then incubated for different times in Mg$^{2+}$-free incubation saline in the absence or presence of NMDA (1 mM). Control intracellular L-[3H]Arg levels were 183.1 ± 8.9 fmol/mg protein. (b) Effect of increasing concentrations of NMDA on intracellular free L-[3H]Arg. The EC$_{50}$ was approximately 250 µM. (c) Effect of absence of magnesium, NMDA (1 mM) and NMDA + (APV) (100 µM) (○), 15 min of incubation with CHX (355.4 µM) (■) or cultures being incubated with CHX since 10 min before pre-loading with L-[3H]Arg and during the whole experiment (▲). Control intracellular L-[3H]Arg levels (absence of CHX) were 460.0 ± 42.2 fmol/mg protein. Results are expressed as the mean ± SEM of three (a) or four (c) separate experiments. The results in (b) are from one experiment performed in duplicate. In (a) the difference between each time point with NMDA and control was significant (p < 0.05). The asterisks denote statistical significance (**p < 0.001) as compared with control. No significant difference was observed between control and other conditions in cultures incubated with CHX (▲).
approximately three times for extracellular and two times for intracellular production (Figs 5a and 6a, black bars). However, under these conditions, the stimulation of L-[3H]Cit production by NMDA was only about 20% for extracellular (Fig. 5a) and intracellular (Fig. 6a) production. Figures 5(b) and 6(b) clearly show this decreased stimulation by NMDA when controls in the absence (white bars) or presence (black bars) of CHX were normalized to 100%.

Eukaryotic elongation factor 2 phosphorylation by NMDA

To test the hypothesis that the effect of NMDA must involve a decrease of protein synthesis by coupling with activation of the eEF2K pathway, we performed experiments to stimulate cultures with NMDA in the presence or absence of MK801 and analyse the phosphorylation of eEF2 by western blotting using an antibody against phospho-eEF2. As can be observed in Fig. 7, stimulation with NMDA did promote an increase of approximately 30% in the amount of phospho-eEF2, an effect inhibited by MK801.

Discussion

The regulation of NO synthesis is of pivotal importance in CNS physiology. One limiting point is the control of availability of the NO precursor L-Arg. Cytoplasmic L-Arg levels are classically regulated in two ways: (i) synthesis of L-Arg through a complex coupled enzymatic machinery and/or (ii) L-Arg uptake through different transport systems. In the present work we propose a third mechanism for enhancement of L-Arg availability, which depends on the regulation of protein synthesis.

Fig. 5 Assay of extracellular L-[3H]citrulline (L-[3H]Cit) in different conditions. (a) Cultures were incubated for 30 min with L-[3H]arginine (L-[3H]Arg) in the presence or absence of cycloheximide (CHX) (355.4 μM) and further incubated for an additional period of 15 min in the absence of CHX (□ and ■) and stimulated for 15 min with CHX or CHX plus Nω-nitro-L-arginine (L-NA) (500 μM) (■) or NMDA (1 mM), L-NA (500 μM), NMDA + APV (100 μM) or NMDA + L-NA (□). Other cultures (■) were incubated for 30 min with L-[3H]Arg in the presence of CHX and stimulated for 15 min with NMDA and/or APV or L-NA still in the presence of CHX. (b) Results in (a) were normalized by their respective controls. Control uptake levels were 89.6 ± 2.4 and 250.8 ± 9.4 fmol/mg protein, respectively, in the absence or presence of CHX. Incubation saline did not contain magnesium in all conditions in which NMDA was used. Results are expressed as the mean ± SEM of three separate experiments. The letters denote statistical significance (b as compared with control without CHX; c as compared with control with CHX; a as compared with the indicated pairs) (one letter, \( p < 0.05 \); two letters, \( p < 0.01 \); three letters, \( p < 0.001 \)).
Relation between protein synthesis and L-arginine biochemistry

Several studies have proposed a direct relationship between L-Arg transport and NO production (Wiesinger 2001), such as a fine tuning between constitutive transporters, inducible expression under special conditions and a photic regulation in the retina (Kakuda et al. 1998; Nicholson et al. 2001; Saenz et al. 2002). Many authors searched for a good explanation for the ‘arginine paradox’ which states that the production of NO is increased by extracellular L-Arg even when intracellular L-Arg concentrations are high. Kurz and Harrison (1997) suggested that intracellular L-Arg could be sequestered in one or more pools that are poorly accessible to NOS, whereas extracellular L-Arg transported into cells is preferentially delivered to NO biosynthesis. It was demonstrated that L-Arg derived from cells that usually do not express NOS, like glial cells, can be recruited after glutamatergic stimulation or depolarization and gives support to NO production in neurons (Grima et al. 1997, 1998; Vega-Agapito et al. 1999, 2002; Cossenza and Paes-de-Carvalho 2000). However, the question of how L-Arg pools are regulated is still obscure. McDonald et al. (1997) elegantly demonstrated the important role of caveolas on the compartmentalization of CAT-1 (a constitutive transporter for L-Arg) and the membrane-bound endothelial NOS (or NOS-III), providing direct evidence for a physical coupling between L-Arg influx and synthesis of NO. Lee et al. (2003) showed that a decreased availability of L-Arg blocks the induction of inducible NOS (or NOS-II) in cytokine-stimulated astrocytes. Inhibition of inducible NOS protein expression was due to

![Diagram](image1.png)

**Fig. 6** Assay of intracellular L-[³H]citrulline (L-[³H]Cit) in different conditions. (a) Cultures were incubated for 30 min with L-[³H]arginine (L-[³H]Arg) in the presence or absence of cycloheximide (CHX) (355.4 μM) and further incubated for an additional period of 15 min in the absence of CHX (■ and □) and stimulated for 15 min with CHX or CHX plus N⁵-nitro-L-arginine (L-NA) (500 μM) (□) or NMDA (1 mM), L-NA (500 μM), NMDA + APV (100 μM) or NMDA + L-NA (■). Other cultures (■) were incubated for 30 min with L-[³H]Arg in the presence of CHX and stimulated for 15 min with NMDA and/or APV or L-NA still in the presence of CHX. (b) Results in (a) were normalized by their respective controls. Control uptake levels were 337.8 ± 19.0 and 675.7 ± 111.8 fmol/mg protein, respectively, in the absence or presence of CHX. Incubation saline did not contain magnesium in all conditions in which NMDA was used. Results are expressed as the mean ± SEM of three separate experiments. The letters denote statistical significance (b as compared with control without CHX; c as compared with control with CHX; a as compared with the indicated pairs) (one letter, *p* < 0.05; two letters, *p* < 0.01; three letters, *p* < 0.001).
intracellular free L-Arg increase. The availability of these when this incorporation is blocked by CHX the pools of protein synthesis may arise as an intermediate step between L-Arg pools working in the storage of this amino acid. Therefore, represents an important driving force for L-Arg uptake. More an explanation for the ‘arginine paradox’. status of the eukaryotic initiation factor 2 alpha, suggesting uptake of L-Arg negatively regulates the phosphorylation inhibition of translation of inducible NOS mRNA and the production and release was detected in efflux experiments. Phosphorylation of the eEF2 results in its inactiva-

tion and may therefore represent an inhibition of protein synthesis at the elongation stage (Ryazanov 2002). This phosphorylation is catalysed by a very specific eEF2K, a calcium/calmodulin-dependent enzyme (also called CAMKIII). It was suggested that intracellular calcium regulates the rate of protein synthesis by eEF2 inactivation. Recently, it was discovered that activation of ionotropic glutamate receptors promotes inhibition of protein synthesis through the influx of calcium and stimulation of eEF2K (Marin et al. 1997; Scheetz et al. 1997, 2000). In this study we found an inhibition of protein synthesis by NMDA, either using a [35S]Met or an L-[3H]Arg incorporation assay (Fig. 3), probably mediated by calcium influx and activation of eEF2K. As evidence that this mechanism is present in our system we also showed that stimulation with NMDA increases phospho-eEF2 levels, an effect blocked by MK801 (Fig. 7). Curiously, together with this effect, NMDA also increases intracellular L-Arg concentration (Fig. 4). We also observed a large increase of intracellular L-Arg when protein synthesis was inhibited by CHX and no further increase by NMDA was detected under this condition (Fig. 4b), strongly suggesting that the increase of intracel-

ular L-Arg mediated by NMDA is dependent on the inhibition of protein synthesis.

Fig. 7 Effect of NMDA on the phosphorylation of eukaryotic elongation factor 2 (eEF2). Cultures were treated in the absence or presence of MK801 (MK) for 10 min and further incubated with NMDA (1 mM) for an additional 15 min before extraction and preparation for sodium dodecyl sulfate–polyacrylamide gel electrophoresis and western blotting. (a) Representative blot for phospho-eEF2 (P-eEF2) and corresponding control blotting with an antibody against β-actin. (b) Quantitative analysis of the results. Data were normalized to percentage of control and are the mean ± SEM of five separate experiments. Asterisks denote statistical significance: *p < 0.05 compared with control and **p < 0.01 compared with NMDA.

inhibition of translation of inducible NOS mRNA and the uptake of L-Arg negatively regulates the phosphorylation status of the eukaryotic initiation factor 2 alpha, suggesting an explanation for the ‘arginine paradox’.

In the present study we demonstrated that protein synthesis represents an important driving force for L-Arg uptake. More than 70% of intracellular L-Arg is incorporated in proteins and when this incorporation is blocked by CHX the pools of intracellular free L-Arg increase. The availability of these pools was confirmed when a considerable increase of L-Cit production and release was detected in efflux experiments (Figs 2, 5 and 6). Hence, according to our results, proteins could play an active role in the compartmentalization of L-Arg pools working in the storage of this amino acid. Therefore, protein synthesis may arise as an intermediate step between L-Arg influx and the control of its intracellular pools available for the synthesis of signaling molecules in cellular compartments.

Regulation of protein synthesis and L-arginine availability by NMDA receptors

Several translation factors participate in the process of protein synthesis at the initiation, elongation and ending phases. Phosphorylation of the eEF2 results in its inactiva-
tion and may therefore represent an inhibition of protein synthesis at the elongation stage (Ryazanov 2002). This phosphorylation is catalysed by a very specific eEF2K, a calcium/calmodulin-dependent enzyme (also called CAMKIII). It was suggested that intracellular calcium regulates the rate of protein synthesis by eEF2 inactivation. Recently, it was discovered that activation of ionotropic glutamate receptors promotes inhibition of protein synthesis through the influx of calcium and stimulation of eEF2K (Marin et al. 1997; Scheetz et al. 1997, 2000). In this study we found an inhibition of protein synthesis by NMDA, either using a [35S]Met or an L-[3H]Arg incorporation assay (Fig. 3), probably mediated by calcium influx and activation of eEF2K. As evidence that this mechanism is present in our system we also showed that stimulation with NMDA increases phospho-eEF2 levels, an effect blocked by MK801 (Fig. 7). Curiously, together with this effect, NMDA also increases intracellular L-Arg concentration (Fig. 4). We also observed a large increase of intracellular L-Arg when protein synthesis was inhibited by CHX and no further increase by NMDA was detected under this condition (Fig. 4b), strongly suggesting that the increase of intracel-

ular L-Arg mediated by NMDA is dependent on the inhibition of protein synthesis.

1-L-Arginine availability mediated by NMDA receptors and L-citrulline production

As discussed above, protein synthesis appears to be related to the regulation of free L-Arg intracellular pools. Therefore, it is possible that increases in such pools may serve for NOS to support the synthesis of NO. This hypothesis was confirmed when incubation with CHX promoted the formation of L-Cit, in a process which was completely blocked by L-NA (Fig. 5). We have also shown that intracellular pools of L-Arg could be mobilized by glutamate signaling, probably through an inhibition of protein synthesis. Hence, we propose that NMDA that classically promotes NOS activation through calcium–calmodulin complex formation also promotes NOS stimulation by increasing its substrate availability. This hypothesis was strengthened by the finding that the increase of L-Cit production by NMDA was smaller when cultures were incubated with CHX (Figs 5b and 6b). This could be explained by the existence of two different mechanisms: (i) mobilization of substrate for NOS through inhibition of protein synthesis mediated by NMDA receptors and (ii) activation of NOS by NMDA receptors after calcium influx and formation of calcium/calmodulin complex. In CHX-treated cultures we did not observe an NMDA-stimulated increase of L-Arg concentration as protein synthesis was not occurring and levels of free L-Arg were maximal.

1-L-Arginine levels and the regulation of nitric oxide synthase activity

Our present results indicate that an increase of intracellular L-Arg levels is able to promote an increase in the production
of L-Cit. We believe that the increase of L-Arg promoted by CHX or NMDA increases the maximal velocity (V\text{max}) of NOS. This is possible because the L-Arg concentration is far below the K\text{m} and the enzyme kinetics are in the linear phase. Any increase of L-Arg would then promote an increase in V\text{max}. In a different way, activation of NMDA receptors, influx of calcium, formation of calcium/calmodulin complex and binding of this complex to the enzyme are able to activate the enzyme by lowering the K\text{m}, i.e. increasing the affinity of L-Arg to its binding site in the enzyme. As a consequence, lower concentrations of L-Arg would be able to increase enzyme maximal velocities.

We were unable to measure control and stimulated levels of NO in our cultures by the Griess method, suggesting that these levels are low as shown in other areas of the CNS. On the other hand, TLC was shown to be a very reliable method of measuring the production of L-Cit in our cultures. Lentile et al. (1999) have shown that NMDA produces an increase of cGMP levels in the same cultures but their methods were unable to detect variations of intracellular and extracellular L-Arg and L-Cit concentrations as shown in the present study. Moreover, the relatively low levels of L-Cit observed in the present study could reflect levels of NO able to stimulate soluble guanylyl-cyclase and to produce large amounts of cGMP. Indeed, recent results from our laboratory (Mejia-Garcia, Cossenza and Paes-de-Carvalho, unpublished observations) showed that the NO donor S-nitroso-N-acetyl penicillamine (SNAP), as well as exogenously added L-Arg or 3(5′-hydroxymethyl-2′-furyl)-1-benzylindazole (YC-1), a soluble guanylyl-cyclase stimulator, increased the phosphorylation of 3-phosphoinositide dependent protein kinase-1 (AKT), a phenomenon mimicked by inhibition of protein synthesis by CHX. These results reinforce the idea that inhibition of protein synthesis and the consequent increase of intracellular L-Arg levels are able to produce increased NO levels which can promote biologically relevant responses such as accumulation of intracellular cGMP and activation of AKT.

**Localization in retinal neurons**

One important question concerning the mechanism proposed in this study is its localization in neurons or glial cells. Preliminary experiments using purified cultures of retinal neurons grown in the absence of glial cells showed that incubation with CHX promotes increases of 70% of extracellular L-Arg and around 30% of extracellular L-Cit. NMDA also promoted an increase of approximately 40% of extracellular L-Cit. These results strongly suggest that the mechanism of increase of NO production regulated by inhibition of protein synthesis induced by NMDA and increase of L-Arg availability is present in retinal neurons. However, more experiments are necessary to exclude the participation of glial cells in this process.

**Local protein synthesis and nitric oxide production**

Several lines of evidence indicate that many important proteins are synthesized locally in synaptic regions. Some of these proteins, such as CAMKII, are involved in phenomena such as long-term potentiation, long-term depression and synapse formation (Ouyang et al. 1999; Scheetz et al. 2000; Aakalu et al. 2001; Steward and Schuman 2001; Mendez and Wells 2002; Richter and Lorenz 2002; Steward and Worley 2002; Tang and Schuman 2002; Murray et al. 2003). Indeed, regulation of local protein synthesis by NMDA receptors could have important consequences during embryonic development. NO also has important effects during CNS development on events of cell proliferation and survival (Peunova and Enikolopov 1995) and also on the synaptic physiology of mature neurons as, for example, in the regulation of neurotransmitter release (Barcellos et al. 2000; Blackshaw et al. 2003; Stanton et al. 2003). Our present findings could have important consequences in these phenomena, establishing a new relationship between local regulation of protein synthesis and NO production.

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