

## Effects of L-NOARG, a nitric oxide synthase inhibitor, on Ag-NOR activity in striatum of mice

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Nitric oxide (NO) is an unconventional intercellular messenger in the brain synthesised from L-arginine by a family of enzymes called NO synthases (NOS). Recent studies have demonstrated that NO plays a role in the control of dopamine release in rat striatum. Presumably, NOS inhibitors could decrease locomotor activity by interfering with striatal dopamine. The aim of this study was to assess the effect of L-NOARG (90 mg/kg), a potent NOS inhibitor, on neuronal protein synthesis activity in striatum of mice after subchronic administration for 4 consecutive days. Neuronal protein synthesis activity was analyzed by quantifying nuclear areas and number of silver-stained nucleolar organizer regions (Ag-NORs) per nucleus. These Ag-NORs may represent the transcriptional activity of the cell. The sections of striatum examined were silver stained according to the method described by Ploton et al. (1986). The results showed that mean number of Ag-NOR per nucleus significantly increased in the striatum of mice, as compared with the control group ( $p < 0.05$ ). These findings indicate the existence of an increase in transcriptional activity after L-NOARG treatment, suggesting that the neostriatal dopaminergic innervation quickly develops tolerance to the interruption of dopamine transmission by L-NOARG.

*Efectos del L-NOARG, un inhibidor de la síntesis del óxido nítrico, sobre la actividad AgNOR en el estriado de ratones.* El óxido nítrico (ON) es un mensajero intercelular atípico sintetizado a partir de la L-arginina por una familia de enzimas denominadas óxido nítrico sintasas (NOS). Recientes estudios han demostrado que el ON está implicado en el control de la liberación de dopamina en el estriado de la rata. Presumiblemente, los inhibidores de la NOS podrían reducir la actividad locomotora bloqueando a la dopamina estriatal. El objetivo de este trabajo es evaluar el efecto de la administración subcrónica (durante 4 días consecutivos) de L-NOARG (90 mg/Kg), un potente inhibidor de la NOS, sobre la actividad de síntesis de proteína neuronal en el estriado de ratones. Dicha actividad fue analizada mediante la cuantificación del área nuclear y el número de regiones organizadoras nucleolares teñidas con plata (Ag-NORs) por núcleo. Estos Ag-NORs pueden representar la actividad transcripcional de la célula. Las secciones del estriado examinadas fueron teñidas con plata de acuerdo con el método descrito por Ploton y cols. (1986). Los resultados mostraron un incremento significativo del número medio de Ag-NORs por núcleo en el estriado de los ratones, en comparación con el grupo control ( $p < 0.05$ ). Dichos resultados indican la existencia de un aumento de la actividad transcripcional tras el tratamiento con L-NOARG, sugiriendo que la innervación dopaminérgica neostriatal desarrolla rápidamente tolerancia al bloqueo de la transmisión dopaminérgica inducido por L-NOARG.

It is a well-known fact that striatal dopamine plays a crucial role in the control of motor behaviour. Pharmacological studies have mainly implicated to the family of D2-like receptors in extrapyramidal side-effects of antipsychotic drugs. Likewise, behavioural studies have demonstrated that inhibition of dopamine release induces catalepsy, hypokinesia, sedation and loss of the righting reflex (Schmidt et al., 1991; Navarro et al., 1997a, b; 1998).

Neuronal plasticity represents an important part of the compensatory processes by which the central nervous system (CNS)

adapts to pathological insult, repeated exposure to drugs or neuronal loss (Pedigo, 1994). In this sense, the striatal dopamine system has been extensively used as a CNS model of super and subsensitivity (Starr et al., 1995). Thus, agents that interrupt the dopaminergic neurotransmission produce an enhanced responsiveness to the transmitter when are administered repeatedly.

Nitric oxide is an unconventional intercellular messenger in the brain synthesised from L-arginine by a family of enzymes called nitric oxide synthases (NOS) (Cavas, Pedraza and Navarro, 1999). Recent studies have demonstrated that nitric oxide plays a role in the control of dopamine release in rat striatum. Presumably, NOS inhibitors could decrease locomotor activity by interfering with striatal dopamine (Marras et al., 1995; Navarro et al., 1997c). In the striatum, NADPH-diaphorase staining has identified a population of 1-2% of aspiny interneurons that contain nitric oxide synthase (NOS), the enzyme responsible for nitric oxide production.

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Striatal NOS interneurons have been shown to receive afferent inputs from dopaminergic nigrostriatal terminals and corticostriatal inputs. As these nitric oxide producing cells are evenly distributed throughout the striatum and have a dense plexus of axon collaterals, they are in position to modulate the activity of multiple striatal cell types and afferent inputs (Fujiyama and Masuko, 1996; Casas and Navarro, 2002).

The shape, number and size of the nucleolus may correlate with cellular activity and, especially, with protein synthesis intensity (Crespo et al., 1988). The nucleolar organizer regions (NORs) are stained chromatin regions around which, at the end of telophase, nucleoli are reformed after their disappearance during the mitotic phase of the cell (Derenzini, 2000). The argentic impregnation technique selectively labels the argyrophilic proteins associated to the NORs and the result of this stained structure is called Ag-NORs (García-Moreno et al., 1993; 2001; Vargas et al., 2000).

The aim of this study was to assess the effect of L-NOARG (90 mg/kg), a potent NOS inhibitor, or vehicle, on AgNOR activity in striatum of mice after subchronic administration for 4 consecutive days.

## Materials and methods

### Animals

20 albino female mice of the OF.1 strain weighing 25-30 g were obtained from CRIFFA (Barcelona, Spain). Animals arrived in the laboratory at 42 days of age and were housed in groups of five in transparent plastic cages (24x13.5x13 cm) under standardized lighting conditions (white lights on: 20:00-8:00), a constant temperature (20±2°C) and food and tap water available «ad libitum».

### Drug administration

L-NOARG (Sigma Laboratories) was added to 5mL of 0.1 N HCl and heated gently to dissolve. Then, the volume was adjusted to 10 mL using distilled water. The dose used was 90 mg/kg. Control animals were treated with distilled water plus the same proportion of the solvent used (HCl).

### Silver staining of the NORs and morphometric analysis

30 min after drug administration, animals were anaesthetised with ethylic ether (Panreac) prior to the vascular perfusion with 10% formaldehyde in phosphate buffer (0.1 M, pH 7.4). Therefore, their brains were quickly removed and the striatum dissected with coronal cuts. After this, the pieces were embedded in paraffin and cut into 20 µm-thick sections in series. The sections were silver stained according to the method of Ploton et al. (1986). Slides were immersed in a solution of one volume of 2% gelatine (pH 2.8) in 1% aqueous formic acid and two volumes of 50% silver nitrate at room temperature (24°C). The sections were incubated in the dark for 18 min. After staining, the slides were washed in several baths of distilled water, dehydrated in xylene, and mounted in a synthetic balsam.

Neuronal protein synthesis activity was analyzed by quantifying nuclear areas and number of silver-stained nucleolar organizer regions (Ag-NORs) per nucleus. The stained slides were

quantified with a computer-aided image analysis system (Visi-Long 5.0) and a microscope with a x100 oil-immersion objective lens. This quantification was blind since the people who carried out the quantification did not know from which animal the sections had been taken. 100 neurons were selected from each of the animals.

### Statistical analysis

The results are expressed in mean±S.E.M. The statistical analysis was performed using nonparametric Mann-Whitney U-tests, since the criteria for parametric statistics were not met by the data. The level of significance was accepted at  $P < .05$ .

## Results and discussion

Nucleolar organizer regions (NORs) are defined as nucleolar components containing a set of argyrophilic proteins, which are selectively stained by silver methods. After silver-staining, the NORs can be easily identified as black dots exclusively localized throughout the nucleolar area, and are called "AgNORs". Each silver-stained dot corresponds, at the ultrastructural level, to a fibrillar centre with a closely associated dense fibrillar component (Trerè, 2000). As Table 1 shows, the mean number of Ag-NOR per nucleus significantly increased in the striatum of mice, as compared with the control group ( $P < 0.05$ ). Therefore, our results show that the quantification of the parameter number of Ag-NORs per nucleus as an index of synthetic activity of the cells is sensitive to the application of a subchronic treatment with L-NOARG.

Groups	Area	Number of AgNORs
Vehicle	97.49 (±21.4)	2.09 (±0.9)
L-NOARG (90 mg/kg)	95.98 (±17.7)	2.45 (±0.9) *
* $P < .05$ , as compared with the vehicle group		

Neuronal plasticity constitutes an important compensatory mechanism by which the central nervous system adapts to repeated exposure to a given drug (Pedigo, 1994). The chronic exposure to drugs may induce tolerance to their pharmacological effects. This phenomenon has been especially observed after the repeated administration of compounds with a D2 antagonist profile (Navarro et al., 1997a; Navarro and Manzaneque, 1997).

Nitric oxide plays a crucial role in the control of dopamine releasing in rat striatum by activation of the enzyme guanil cyclase. In this way, the administration of L-NOARG, a potent nitric oxide synthase inhibitor, could decrease dopaminergic activity by interfering with striatal dopamine release (Marras et al., 1995; Navarro et al., 1997c). Our findings indicate the existence of an increase in transcriptional activity after L-NOARG treatment, suggesting that the neostriatal dopaminergic innervation quickly develops tolerance to the interruption of dopamine transmission by L-NOARG. This tolerance could be explained by an upregulating of striatal dopaminergic receptors.

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