Proc. Natl. Acad. Sci. USA
Vol. 90, pp. 9191–9194, October 1993
Neurobiology

Altered synaptic plasticity and memory formation in nitric oxide synthase inhibitor-treated rats

(long-term potentiation/brain slices/behavior)

GEORG ANDREES BÖHME*, CHRISTELLE BON, MARTINE LEMAIRE, MICHEL REIBAUD, ODILE PIOT, JEAN-MARIE STUTZMANN, ADAM DOBLE, AND JEAN-CHARLES BLANCHARD

Rhône-Poulenc Rorer S.A., Centre de Recherches de Vitry-Alfortville, 94403 Vitry-Sur-Seine cedex, France

Communicated by Eric R. Kandel, June 23, 1993

ABSTRACT **Nitric oxide (NO) is a messenger molecule that is produced in the brain from the metabolism of L-arginine to L-citrulline. Growing evidence suggests a physiological role for NO in long-term potentiation (LTP). Since LTP is a form of synaptic plasticity thought to be involved in learning and memory, we have tested whether inhibition of endogenous NO production affects memory capacities of rats. We found that the NO synthase [L-arginine, NADPH:oxygen oxidoreductase (nitric oxide-forming), EC 1.14.13.39] inhibitor N^{ω}-nitro-L-arginine, at doses blocking LTP in hippocampal slices, impairs spatial learning in a radial arm maze and olfactory memory in a social recognition test. In contrast, N^{ω}-nitro-L-arginine left shock-avoidance learning unaffected. These results indicate that NO is involved in some but not all forms of memory and further support the existence of a causal link between LTP and spatial learning.**

Nitric oxide (NO) is a diffusible molecule endowed with intercellular messenger properties in several biological systems including the brain (1, 2). NO mediates the stimulation of soluble guanylate cyclase upon activation of *N*-methyl-D-aspartate (NMDA) receptors (3) and serves as its own negative feedback effector by blocking NMDA-evoked responses (4, 5). This messenger is produced from the enzymatic conversion of L-arginine to L-citrulline by a constitutive NO synthase [NOS; L-arginine, NADPH:oxygen oxidoreductase (nitric oxide-forming), EC 1.14.13.39] which can readily be blocked by arginine analogs, such as N^{ω}-nitro-L-arginine [Arg(NO_2); also called N^{G}-nitro-L-arginine, where G refers to the guanidino-carbon] (6, 7).

Long-term potentiation (LTP) is a persistent increase, which can last for days or weeks, in the synaptic efficacy of pathways produced by brief periods of high-frequency stimulations (HFS) (8). This phenomenon, best characterized in the hippocampus, is thought to be a cellular event involved in the acquisition, storage, or retrieval of information in the brain (9–12). We and others reported recently that NOS inhibitors and NO scavengers block hippocampal LTP in rat brain slices (13–16). This led us to test whether a systemic treatment with the NOS inhibitor Arg(NO_2) affects LTP and the memory capacities of rats (17).

MATERIALS AND METHODS

Drug Administration. To ensure proper brain permeation, Arg(NO_2) (Sigma) or, on occasion, its inactive enantiomer D-Arg(NO_2) (SENN Chemicals, Switzerland), suspended in saline containing 0.3% polysorbate, was administered intraperitoneally (i.p.) twice daily for the 4 days preceding the experiments. A similar pretreatment with Arg(NO_2) is known to irreversibly block brain NOS enzymatic activity (7). Separate groups of animals were used for each experiment. Vehicle-treated animals were used as controls.

Electrophysiology. Sixteen hours after the last injection, transverse hippocampal slices (0.5-mm thick) were prepared from Sprague–Dawley rats (150–200 g) pretreated with Arg(NO_2) (25–100 mg/kg of body weight i.p.) or vehicle. Slices were maintained in a submersion-type recording chamber under superfusion (2.5–3 ml/min) with gassed (95% O_2/5% CO_2) medium containing 124 mM NaCl, 5 mM KCl, 2 mM $MgSO_4$, 2 mM $CaCl_2$, 26 mM $NaHCO_3$, 1.25 mM KH_2PO_4, and 10 mM glucose at 32°C as described (18). Stimulation and recording electrodes were positioned in the CA1-stratum radiatum, and field excitatory postsynaptic potentials (EPSPs) were evoked every 5 s. The stimulus strength (0.1-ms duration at 2–20 V) was adjusted to evoke EPSPs of at least 0.3-mV amplitude without producing a population spike. HFS to induce LTP consisted of two trains of stimuli (1 s at 100 Hz) at twice the baseline voltage. Synaptic efficacy was monitored for 60 min after HFS by measuring the negative slope of the extracellular field EPSP.

Radial Eight-Arm Maze (RAM). The effects of the NOS inhibitor on learning tasks requiring spatial memory formation were assessed by using a RAM (19). Sprague–Dawley rats (150–200 g) were kept on a restricted diet of standard laboratory chow (*ca.* 20 g per day) with water available ad lib. After having been pretreated with Arg(NO_2) (25 or 100 mg/kg i.p.) as indicated above, the animals were trained every day for 12 days to explore the RAM environment to collect bait placed at the arm extremities. Treatments were maintained once daily 90 min before each session. The maze consisted of eight arms (50 × 10 cm) extending radially from a central area (30 cm in diameter), with the height of the sidewalls evenly declining from the central area (20 cm) to the extremities (6 cm). The apparatus was placed 80 cm above the floor.

Two days before the actual training started, animals were allowed to visit the apparatus with their cage mates in groups of four to encourage exploration. For the following 12 daily sessions of the training *per se*, each animal was placed individually in the center of the maze and allowed to collect food until all arms were visited or 10 min had elapsed. The maze was baited only once per session with two pellets of laboratory chow placed in small cups near the end of each arm. An arm entry was counted when all four limbs of the rat were within a given arm. A reentry in an already visited arm was regarded as an error. For each daily session, the total number of errors and the time to explore all arms were recorded.

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Abbreviations: HFS, high-frequency stimulation; LTP, long-term potentiation; NOS, nitric oxide synthase; Arg(NO_2), N^{ω}-nitro-L-arginine; EPSP, excitatory postsynaptic potential; RAM, radial 8-arm maze; RID, ratio of investigation duration.

*To whom reprint requests should be addressed.

Social Memory. The effect of Arg(NO_2) on olfactory memory formation was assessed by using a test of social recognition based on the fact that rats explore familiar juveniles less than unfamiliar ones. The formation of an olfactory memory trace can be measured as the decrease in the length of time a male adult rat spends investigating a male juvenile during the second of two brief encounters separated by less than about 1 hr (20, 21). Male 6-month-old adult Wistar rats (400–450 g) were housed in isolation and male 1-month-old juveniles of the same strain (90–120 g) were housed in groups of five with food and water available ad lib. The experiments were performed after the adults had been isolated for 2 weeks. On the day of testing, a given juvenile was placed in the home cage of an adult for 5 min and then again 30 min later. The adult rats were pretreated with 25 or 100 mg of Arg(NO_2) per kg (see above) and once again 90 min before testing. The behavioral items defining social investigation (sniffing, grooming, nosing, and close-following) were rated blindly. The time the adult rat spent investigating the juvenile was recorded during each exposure, and a ratio of investigation duration (RID) was calculated (duration of second exposure/duration of first exposure).

Shock-Avoidance Learning. The capacity of rats to learn shock avoidance was measured as the latency to reenter a dark compartment in which the animals had experienced an electric footshock 24 hr previously. The avoidance apparatus consisted of a lit compartment (40 cm × 40 cm × 40 cm) and a smaller dark compartment (25 cm × 25 cm × 20 cm) with a grid floor. A sliding door separated the two parts of the apparatus. Sprague–Dawley rats (150–200 g), pretreated with 100 or 300 mg of Arg(NO_2) per kg and given an additional dose 90 min before testing, were placed individually in the lit compartment. The door to the dark compartment was opened, and the latency before entering was recorded. When the animal had stepped through, the door was closed and a footshock (0.8 mA for 20 s) was delivered. Twenty-four hours later, each rat was placed again in the lit compartment and the step-through latency was recorded until 300 s had elapsed.

Statistical Analysis. All data were expressed as means ± SEM and analyzed by using parametric statistics, except for RID values in the social memory test and escape latencies in the shock-avoidance test, which were expressed as medians and interquartile range and were analyzed by using nonparametric statistics because of the non-Gaussian distribution of these data. A value of $P < 0.05$ was considered to be statistically significant.

RESULTS

Arg(NO_2) Blocks LTP in Hippocampal Slices. Arg(NO_2) blocked LTP dose-dependently in hippocampal slices prepared from rats pretreated with the inhibitor. The lowest tested dose of Arg(NO_2) (25 mg/kg i.p.; $n = 6$) was inactive (Fig. 1). Identical results were obtained from the slices prepared 16 hr after the last Arg(NO_2) injection and when an additional 25 mg of Arg(NO_2) per kg was given 90 min before sacrifice. A dose of 50 mg/kg of the inhibitor only partially blocked LTP ($n = 9$), and doses of 100 mg/kg were necessary to block plasticity. In the latter case, HFS produced only a short-lived increase in synaptic efficacy. Indeed, after 45 min there was no significant difference between slope values before (0.174 ± 0.006 mV/ms, mean ± SEM; $n = 7$) and after (0.191 ± 0.011 mV/ms; $P > 0.5$, paired t test) HFS treatment.

Arg(NO_2) Impairs Spatial Learning. While the mean number of reentries in already visited arms diminished within all groups, suggesting that all rats learned something about the spatial environment of the RAM over the 2-week training period, the animals treated with 100 mg of Arg(NO_2) per kg (Fig. 2*B*), but not those treated with 25 mg/kg (Fig. 2*A*), learned more slowly than the controls. Consistent with this interpretation, a two-way analysis of variance (ANOVA) on the number of errors made by animals treated with Arg(NO_2) at 100 mg/kg ($n = 8$) revealed a significant main effect of group [$F(1,154) = 24.2$; $P < 0.0003$], a significant main effect of days [$F(11,154) = 10.8$; $P < 0.0001$], and a significant group × day interaction [$F(11,154) = 3.01$; $P < 0.002$]. Spatial learning was delayed by the treatment, as indicated by a post hoc Dunnett's multiple t test showing that the number of errors was significantly higher ($P < 0.05$) for the rats treated with Arg(NO_2) at 100 mg/kg on days 3–10, except on day 9. On days 11 and 12, these animals performed as well as the controls.

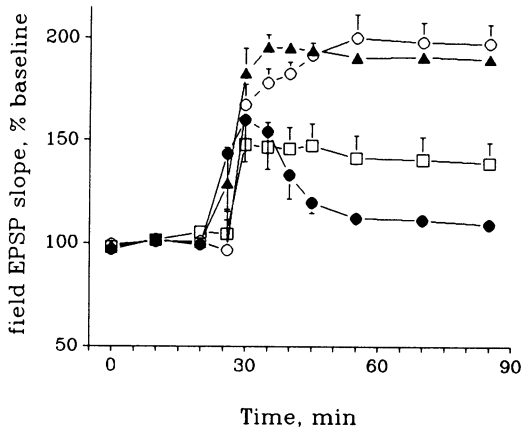

FIG. 1. Blockade of LTP recorded in rat hippocampal slices after repeated Arg(NO_2) administration to the intact animal. The time course is shown of changes in synaptic efficacy at the level of synapses between Schaffer collateral–commissural fibers and CA1 cells after HFS was delivered at $t = 25$ min. The means ± SEM of field EPSP slope values are normalized with respect to pre-HFS values. ○, Controls; ▲, Arg(NO_2) at 25 mg/kg i.p.; □, Arg(NO_2) at 50 mg/kg; ●, Arg(NO_2) at 100 mg/kg.

Total exploration time was not decreased by any tested dose of Arg(NO_2), suggesting that the NOS inhibitor did not impair locomotor performance and that the 100 mg/kg-treated rats compensated for time lost in exploring already visited arms by running faster (Fig. 2 *D* and *E*). Motivation was not affected, since all of the arms were visited and all of the bait was consumed during each session. Arg(NO_2) (100 mg/kg i.p.) also impaired radial maze learning when water was used as the positive reinforcing stimulus (data not shown). Repeated treatment with D-Arg(NO_2) (100 mg/kg i.p.), which does not inhibit NOS activity, did not affect RAM performance (Fig. 2 *C* and *F*).

Arg(NO_2) Impairs Social Memory Formation. Pretreatment with Arg(NO_2) at 25 mg/kg affected significantly neither the way adult rats explored unfamiliar juveniles nor the way adult rats recognized familiar juveniles. The duration of social investigation at the first contact was 97 ± 13 s and 72 ± 12 s (mean ± SEM; $n = 10$) for vehicle- and 25 mg/kg-treated animals, respectively ($P > 0.15$, unpaired t test) (Fig. 3*A*). A marked reduction in the duration of exploration of the juveniles at the second contact was observed with adults from both groups, suggesting that this dose of Arg(NO_2) did not impair olfactory recognition. The median and interquartile range of the RID values for controls and treated animals were 0.48 (0.41–0.65) and 0.46 (0.37–0.58), respectively ($P > 0.7$, Mann–Whitney) (Fig. 3*B*).

Pretreatment with Arg(NO_2) at 100 mg/kg did not alter the duration of the initial investigation significantly (101 ± 6 s and 79 ± 10 s for controls and 100 mg/kg-treated rats, respectively; $n = 9$, $P > 0.09$) (Fig. 3*A*), suggesting that the animals' sense of smell was not affected. However, this dose of the inhibitor strongly hindered subsequent recognition. The median RID value for the two encounters was close to unity

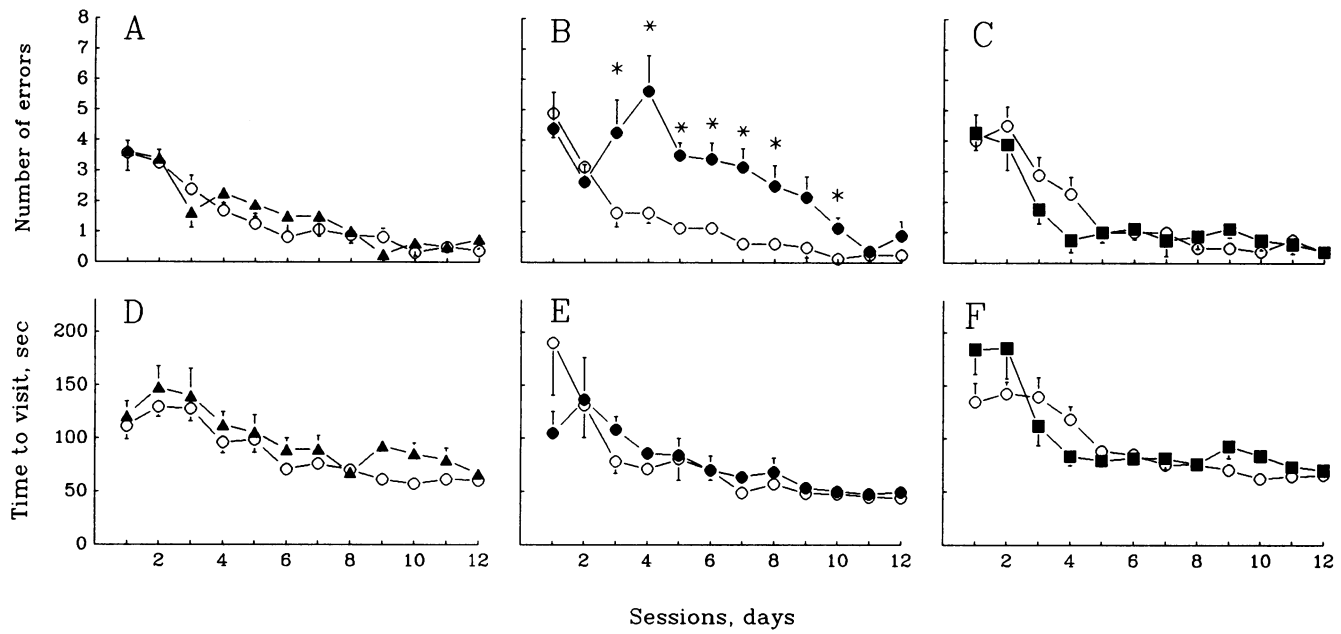

FIG. 2. Effects of Arg(NO_2) on spatial learning in a RAM. Animals were trained to visit the apparatus in consecutive daily sessions for 2 weeks. Spatial learning was measured as the decline in the number of reentries in already visited arms (exploration errors). The means ± SEM of the number of errors (*Upper*) and of the time spent to visit the maze (*Lower*) are shown for each day of learning. Results from three separate experiments are shown. ○, Controls; ▲, Arg(NO_2) at 25 mg/kg; ●, Arg(NO_2) at 100 mg/kg; ■, D-Arg(NO_2) at 100 mg/kg. Significant differences from controls are indicated by asterisks ($P < 0.05$).

(1.11, interquartile range: 0.84–1.17) and, as such, was twice as high for the 100 mg/kg-treated rats as for the vehicle-treated controls (0.51, 0.44–0.63; $P < 0.05$, Mann–Whitney) (Fig. 3*B*).

Arg(NO_2) Does Not Affect Shock Avoidance Learning. Neither acquisition nor retention of the passive avoidance behavior, tested 24 hr after the footshock, was impaired by pretreatment with Arg(NO_2) at doses of 100 and 300 mg/kg i.p. The median latencies to enter the dark compartment on the day of acquisition were 30 s (interquartile range: 15–46), 27 s (19–59), and 21 s (15–35) for controls, 100 mg/kg-, and 300 mg/kg-treated animals, respectively ($n = 15$; $P > 0.3$, Kruskal–Wallis analysis of variance), indicating again the absence of locomotor impairment despite the high doses injected (Fig. 4*A*).

Twenty-four hours after acquisition, only 1 rat of 15 in both the 100 mg/kg- and 300 mg/kg-treated groups entered the dark box within the 300-s cutoff time, and none did so in the control group. Median latencies and interquartile range were identical in all groups (median: 300 s, range: 300–300), indicating that Arg(NO_2) induced no deficit in retention of the passive avoidance task (Fig. 4*B*). In contrast, acute treatment with scopolamine at 1 mg/kg i.p., a known amnesic drug, significantly reduced retention latency (median: 146 s, range: 10–300 s; $P < 0.05$, Mann–Whitney).

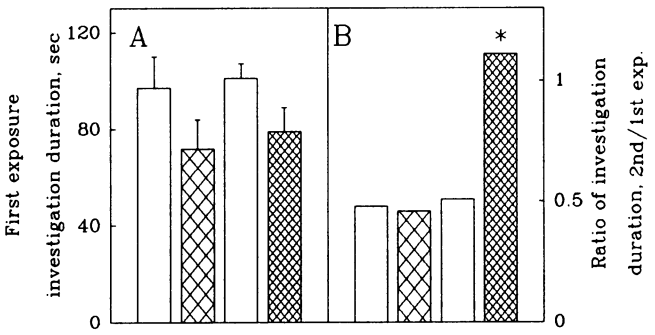

FIG. 3. Effects of Arg(NO_2) on olfactory recognition in the social memory model. Social interaction between male adult and male juvenile rats of the same strain was recorded during two brief encounters 30 min apart. Results from two separate experiments are shown. (*A*) Means ± SEM for the duration of investigation of juveniles by adults during the first encounter. (*B*) Medians for RID values (see text for interquartile ranges). Open bars, controls; wide crosshatched bars, Arg(NO_2) at 25 mg/kg; narrow crosshatched bars, Arg(NO_2) at 100 mg/kg. A significant difference from controls is indicated by an asterisk ($P < 0.05$).

DISCUSSION

The present findings indicate that the NOS inhibitor Arg(NO_2) given systemically is able to penetrate the brain to block hippocampal LTP, slow down spatial learning, and impair olfactory recognition in the social memory test but not shock-avoidance learning. Other investigators reported recently that systemic treatment with NOS inhibitors impairs conditioned eyeblink learning in rabbits (22) and taste avoidance learning in chicks (23).

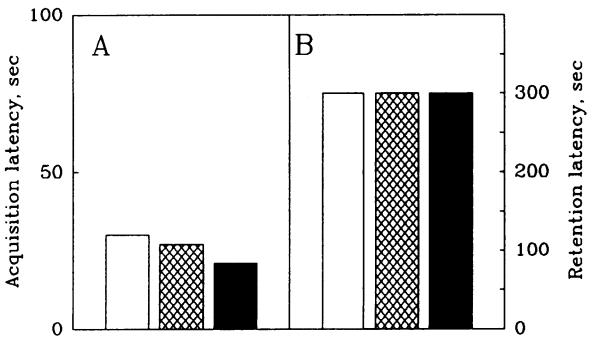

FIG. 4. Effect of Arg(NO_2) on shock-avoidance learning. Latencies to enter a dark compartment in which a footshock was delivered were recorded on the day of acquisition (*A*) and 24 hr later (*B*) with a 300-s cutoff time. Values are given as medians (see text for interquartile ranges). Open bars, controls; crosshatched bars, Arg(NO_2) at 100 mg/kg; filled bars, Arg(NO_2) at 300 mg/kg.

Because the inhibitor given systemically would be expected to exert its effects at multiple sites, the learning impairments could have resulted from a nonspecific performance deficit. In another series of experiments not included here, we observed that the repeated treatment with Arg(NO_2) at 100 mg/kg i.p. caused an elevation in blood pressure in unanaesthetized rats of ≈45 mmHg. However, it is unlikely that this hypertension alone accounted for the performance deficits, since Arg(NO_2) apparently did not impair activity in the RAM test or the shock-avoidance procedure or social interaction at first contact in the olfactory recognition model.

NO production appears to be involved, therefore, in some but not all forms of memory. This suggests that distinct brain processes underlie spatial and olfactory memory formation on the one hand and shock-avoidance learning on the other hand. A pharmacological dissociation between these forms of memory has been reported previously on the basis of experiments with protease and protein synthesis inhibitors (24–26). Recent work suggests that another form of learning, the conditioned eyeblink reflex, is localized in the cerebellum (27).

Since doses of the inhibitor effective (and ineffective) in electrophysiological tests and behavioral tests correlated, the present results also support the hypothesis that hippocampal LTP is a physiological substrate of spatial and olfactory learning. Previous studies have shown a similar correlation between the concentrations of *N*-methyl-D-aspartate antagonists infused into the brain that block LTP and those that impair spatial learning in the Morris water maze (9, 10). Conversely, *N*-methyl-D-aspartate or glycine-site agonists have been shown to facilitate hippocampus-dependent learning (28). Further evidence for the existence of a causal link between LTP and certain forms of memory is suggested by the possibility of transiently impairing spatial learning by saturating hippocampal LTP (12). Moreover, transgenic alterations of the expression of key proteins in the LTP process, such as Ca^{2+}/calmodulin-dependent kinase II (29) or fyn tyrosine kinase (30), also lead to concomitant spatial learning deficits and impairment of LTP.

Induction of LTP in the CA1 area of the hippocampus depends on an increase of postsynaptic Ca^{2+} concentrations following a transient alleviation of the Mg^{2+} block of *N*-methyl-D-aspartate receptor channels (31–33). Whether the maintenance of LTP depends on a sustained increase in presynaptic transmitter release or on a change in postsynaptic responses remains, however, a matter of debate (34). The presynaptic hypothesis has led to the suggestion that a retrograde messenger is emitted by the postsynaptic elements and acts on presynaptic terminals to maintain LTP (35, 36). NO seems to adequately fulfill this role, since there is evidence that exogenous NO mimicks LTP (13, 18, 37) and induces neurotransmitter release from brain slices (38, 39).

We thank P. Ascher, P. Barnéoud, S. Laroche, and R. G. M. Morris for helpful discussions and critical reading of earlier versions of the manuscript; D. Gandon for assistance in the statistical analysis of the data; and K. Pepper for stylistic revision.

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