

# Concentration dynamics of nitric oxide in rat hippocampal subregions evoked by stimulation of the NMDA glutamate receptor

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Nitric oxide (\*NO) production in response to stimulation of the NMDA glutamate receptor is implicated not only in the synaptic plasticity in hippocampus but may also participate in excitotoxic cell death. Using \*NO-selective microensors inserted into the diffusional field of \*NO in acute hippocampal slices, we describe the \*NO concentration dynamics evoked by NMDA receptor activation and report profound differences along the trisynaptic loop of the hippocampus. We measured the oxygen gradient across the slice thickness and conclude that \*NO measurements were performed at cell layers experiencing physiological oxygen tensions. Recordings performed at increasing distances from the point of NMDA receptor stimulation resulted in a progressive decrease of \*NO signals, reaching undetectable levels for distances >400  $\mu\text{m}$ , supporting the notion of a wide diffusional spread of endogenously generated \*NO in the hippocampus. Neither a picoinjection nor a continuous perfusion of NMDA resulted in high steady-state \*NO levels; rather all signals were transient, suggesting that cells are able to efficiently respond to high \*NO concentrations (typically 200–400 nM) bringing it to very low nM levels; the claimed high micromolar \*NO range achieved by excessive stimulation of NMDA receptor may have to be reevaluated. The distinct responses to NMDA receptor stimulation along the trisynaptic loop suggest a differential \*NO activity and/or regulation among the hippocampal subregions. These findings may be relevant for the understanding of the role of \*NO in physiologic mechanisms in the hippocampus and the differential sensitivity of the hippocampal subregions to NMDA receptor-dependent neurodegeneration.

carbon fiber microelectrode | NO diffusional spread | hippocampus

**A** neural role of nitric oxide (\*NO) as an intercellular signaling molecule in the nervous system was first suggested by Garthwaite and Boulton (1), who related the activation of glutamate NMDA receptor and \*NO synthesis in brain slices. This notion has been confirmed by subsequent studies (for review see ref. 2). Conversely to conventional messenger molecules in the brain, because of its low molecular weight, hydrophobic nature, and very high diffusion constant, \*NO diffuses isotropically from its site of synthesis regardless of overruling cellular or membrane structures (3, 4). Thus, the current understanding of \*NO bioactivity in the brain implies the formation of a “sphere of influence” of \*NO affecting a volume of tissue containing many neurons irrespective of functional connections through synapses; i.e., \*NO operates as a diffusible intercellular messenger. A critical tenet of \*NO bioactivity establishes that its actions at any location are determined by the local \*NO concentration (5). Yet, the evaluation of \*NO in terms of concentration dynamics has been limited because of the difficulties in measuring \*NO with spatiotemporal resolution and appropriate sensitivity and selectivity. Although it has been shown that \*NO produced postsynaptically exerts actions at the presynaptic level (6–9), the quantification of the diffusional spread of endogenously generated \*NO in the brain lacks experimental evidence. Theoretical modeling predicted that \*NO produced from

a single physiologic source for 1–10 s diffuses within a 200- $\mu\text{m}$ -diameter sphere (4).

The hippocampus is a structure of the brain medial temporal lobe implicated in declarative memory formation (10) affected during aging and Alzheimer’s disease (11). At glutamatergic synapses in hippocampus (12), the link between NMDA receptor activation and the production of \*NO by neuronal NO synthase (nNOS) is  $\text{Ca}^{2+}$  entry through the activated receptor channel, leading to the formation of the  $\text{Ca}^{2+}$ /calmodulin complex, which in turn is capable of activating intracellular nNOS (1).

The measurement of \*NO dynamics during functionally induced changes in the hippocampus by activation of the NMDA receptor has generated much interest inasmuch as the activation of this receptor is essential to signaling pathways that underlie neuronal plasticity, but also triggers neurodegeneration that occurs in senescence and disease, suggesting a role for \*NO in these processes (13). For instance, in the CA1 and dentate gyrus (DG) subregions of the hippocampus, long-term potentiation (LTP) is triggered by the activation of the NMDA receptor as a result of heavy stimulation of the presynaptic terminal (14). In this context, \*NO has been suggested to be the diffusible retrograde messenger required for LTP (15, 16).

Excessive activation of the NMDA receptor mediates neurodegeneration in neurological disorders, inducing Alzheimer’s disease, Parkinson’s disease, multiple sclerosis, and AIDS dementia (13, 17). \*NO has been implicated in such excitotoxic phenomenon in a variety of cellular model systems (13, 18).

All of this information leads to the notion of \*NO as a crucial mediator of pathophysiological pathways in the hippocampus. Yet, knowledge regarding the concentration dynamics of endogenously produced \*NO is scarce and, clearly, this knowledge is imperative in understanding its role in signaling pathways. In this study, we use microensors developed to monitor the dynamics of \*NO production and decay in the different subregions of acute rat hippocampal slices upon stimulation of the NMDA receptor. First, we determined the spread of endogenously generated \*NO and, then, inserting the sensors within the diffusional field of \*NO, we studied its concentration dynamics and provided evidence for a heterogeneous distribution and transient nature of \*NO signals as a function of the hippocampal subregion.

## Materials and Methods

**Chemicals and Solutions.** NMDA, D-(-)-2-amino-5-phosphonopentanoic acid (D-AP5), and  $N_G$ -nitro-L-arginine (L-NNA) were ob-

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Abbreviations: nNOS, neuronal NO synthase; DG, dentate gyrus; LTP, long-term potentiation; D-AP5, D-(-)-2-amino-5-phosphonopentanoic acid; L-NNA,  $N_G$ -nitro-L-arginine; AA, ascorbate; aCSF, artificial cerebrospinal fluid.

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tained from Tocris Cookson (Avonmouth, U.K.). Ascorbate (AA), dopamine, and *o*-phenylenediamine were from Fluka. 5-Hydroxytryptamine, glutathione, diethylenetriaminepentacetic acid, and diethylenetriamine/nitric oxide adduct were from Sigma. Nafion was from Aldrich, and nitrite was from Merck. All other reagents were reagent grade.

Buffer used for microsensor testing and calibrations was PBS with the following composition: 140 mM NaCl, 2.7 mM KCl, 8.1 mM NaHPO<sub>4</sub>, 1.8 mM KH<sub>2</sub>PO<sub>4</sub>, and 0.1 mM diethylenetriaminepentacetic acid, pH 7.4.

Media for hippocampal slice experiments were artificial cerebrospinal fluid (aCSF) composed of 124 mM NaCl, 2 mM KCl, 25 mM NaHCO<sub>3</sub>, 1.25 mM KH<sub>2</sub>PO<sub>4</sub>, 1.5 mM CaCl<sub>2</sub>, and 10 mM D-glucose. For dissection and recovery modified aCSF was used to increase viability. Composition of this aCSF was 124 mM NaCl, 2 mM KCl, 25 mM NaHCO<sub>3</sub>, 1.25 mM KH<sub>2</sub>PO<sub>4</sub>, 0.5 mM CaCl<sub>2</sub>, 10 mM MgSO<sub>4</sub>, 0.2 mM AA, 1 mM glutathione, and 10 mM D-glucose. In both cases, aCSF was continuously bubbled with humidified carbox (95% O<sub>2</sub>/5% CO<sub>2</sub>) for pH buffering (pH 7.4) and oxygenation.

**Electrochemical Instrumentation.** Fast cyclic voltammetry was carried out on an EI-400 potentiostat (Ensmann Instruments, Bloomington, IN), and signals were monitored on a digital storage oscilloscope (Tektronix TDS 220).

Amperometric currents from microsensor modification and calibration and oxygen measurements in tissue were recorded on a PGSAT 12 potentiostat (EcoChimie, Utrecht, The Netherlands) with low current module, controlled by GPES software, version 4.9. Amperometric currents recordings in slices were performed on the inNO model T electrochemical detection system (Innovative Instruments, Tampa, FL).

In hippocampal recordings of both \*NO and O<sub>2</sub>, a two-electrode circuit was used, with a Ag/AgCl pellet as a reference electrode and the microsensor as a working electrode. For other experiments, a three-electrode cell with a Pt-wire auxiliary electrode, a Ag/AgCl (3M) reference electrode, and the microsensor as a working electrode was used.

The working electrode was held at a constant potential of either +0.9 or -0.8 V for \*NO or O<sub>2</sub> measurements, respectively.

**\*NO Microsensor Construction and Surface Modification.** Microsensors were fabricated as described (19–21). Briefly, single carbon fibers (8 μm i.d.; Courtaulds, London) were inserted into borosilicate glass capillaries (1.16 mm i.d. × 2.0 mm o.d.; Harvard Apparatus), cleaned with acetone, and pulled on a vertical puller (Harvard Apparatus). The protruding carbon fibers were cut to tip length of ≈100 μm. The electrical contact between the carbon fiber and the copper wire was provided by conductive silver paint (RS, Northants, U.K.).

Microsensors were first coated with Nafion by dipping the fiber into a Nafion solution at room temperature for 30 s and drying for 10 min at 170°C in an oven. Microsensors were then modified by electropolymerization of *o*-phenylenediamine (*o*-PD) as described (22). A 5-mM *o*-PD solution in PBS supplemented with 0.1 mM AA was made fresh each day and used immediately. Electropolymerization on the carbon surface was performed by amperometry at constant potential of +0.9 V vs. Ag/AgCl for 15 min.

**Microsensor Testing Procedures.** Each microsensor was tested for general recording characteristics in PBS by using fast cyclic voltammetry at a scan rate of 200 V/s between -0.4 and +1.6 V. This potential range provides an electrical pretreatment of the carbon fiber that improves sensitivity. A stable background current and sharp transients at reversal potentials indicated suitable recording properties of the microsensor.

The microsensors for \*NO were calibrated by a single stream flow injection analysis system by using a homemade flow cell with PBS

as a carrier solution at a flow rate of 2.0 ml/min. Transient oxidation currents were measured in response to 100 μl of diethylenetriamine/NO adduct solution in deaerated PBS injected repeatedly with a four-valve port.

The microelectrodes for O<sub>2</sub> measurement were calibrated in a 2-ml cell with PBS as a support electrolyte. The reduction current was measured for three distinct O<sub>2</sub> tensions (0, 156, and 700 torr) achieved by bubbling the PBS with argon, allowing it to achieve atmospheric PO<sub>2</sub> and bubbling it with Carbox, respectively.

**Rat Hippocampal Slices.** Male Wistar rats (100–150 g) were killed by cervical displacement according to approved guidelines. The brain was rapidly removed and placed in ice-cold modified aCSF. The hippocampi were dissected and placed on the stage of a McIlwain tissue chopper (Campden Instruments, London), and 400-μm-thick sections were obtained. The slices were separated and transferred to a preincubation chamber (BSC-PC, Harvard Apparatus) containing modified aCSF at room temperature, continuously bubbled with Carbox. Slices were recovered under these conditions for at least 1 h before recordings.

**Recording \*NO Concentration Dynamics.** Individual slices were placed in a recording chamber (BSC-BU with BSC-ZT top, Harvard Apparatus) and perfused with normal aCSF at 32°C (temperature controller model TC-202A, Harvard Apparatus) continuously bubbled with humidified Carbox at a flow rate of 2 ml/min. A microsensor was placed in the desired subregion of the hippocampal slice (for CA1 and CA3 subregions the microsensor was placed at the level of the pyramidal cell layer and in the DG at the granular cell layer) 100–200 μm into the tissue. These sites are known to be concentrated in nNOS (23, 24) and were easy to identify, enabling precise reproduction of microsensor insertion.

For slice stimulation, a pressure ejection protocol was applied, using a Picospritzer II (General Valve, Fairfield, NJ). A glass pipette capillary with an inner tip diameter of 8 μm was filled with stimulation solution (5 mM NMDA in 0.1 M phosphate buffer containing 154 mM NaCl, pH 7.4) and placed at the slice surface ≈50 μm away (or as otherwise indicated) from the point of insertion of the microsensor. A 3-s pulse was applied at a pressure of 10 psi. In experiments where antagonists or inhibitors were used these drugs were added to the perfusion media.

**Measurement of PO<sub>2</sub> Across Slice Thickness.** The oxygen tension (PO<sub>2</sub>) across the hippocampal slice was measured as described (25). Bare carbon fiber microelectrodes were prepared as described above for the \*NO microsensor, with no surface modification. The exposed tip was cut at 10–20 μm to increase spatial resolution in recordings.

The microelectrode was placed at different depths (surface, 100, 200, 300, or 400 μm) of the tissue with the help of a micromanipulator.

**Data Analysis.** Data are expressed as the mean ± SEM and were analyzed for statistical significance defined as *P* < 0.05 using Student's *t* test. Total charge was calculated as the time integral of the amperometric current.

The individual \*NO recordings obtained in the different subregions of hippocampal slices challenged with NMDA were divided into two phases: ascendant and descendent. The ascending phase was fitted to a sigmoid function, which allowed the *T*<sub>80</sub> for this phase to be calculated. The descending phase was fitted to an exponential first-order decay function and the time constant was calculated for each recording.

All analysis was performed with commercially available software.

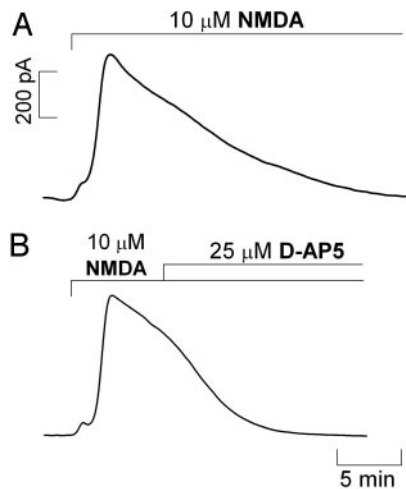
## Results

**Determination of \*NO Diffusion Field in Hippocampal Slices.** Experiments illustrated in Fig. 1 were aimed at determining the diffusional









**Fig. 6.**  $^*NO$  production evoked by continuous perfusion of  $10\ \mu M$  NMDA. Recordings were performed in the CA1 pyramidal cell layer of rat brain slices. (A) A continuous stimulation with NMDA. (B) A continuous stimulation with addition of  $25\ \mu M$  D-AP5 during the decay of the signal. Typical recording is representative of several done.

gion for the two working potentials,  $+0.9$  and  $+0.4$  V, shows that at  $+0.9$  V total charge of recorded signals was  $23 \pm 4$  nC ( $n = 35$ ) and at  $+0.4$  V total charge was  $1.1 \pm 0.6$  nC ( $n = 7$ ). Moreover, a differential pulse amperometry recording in the CA1 subregion followed similar kinetics to the amperometric recordings and  $10\ \mu M$  of dopamine, 5-hydroxytryptamine, and AA were not significantly detected, as would be expected because only electroactive species oxidized between  $+0.7$  and  $+0.9$  V are detected. Finally,  $^*NO$  dynamics were measured in the cell layers experiencing an  $O_2$  tension similar to that found *in vivo* (Fig. 2), thus excluding a reaction with  $O_2$  as a major route for  $^*NO$  decay.

There are a number of possible cellular sources for constitutive NOS activity in the hippocampus, including nNOS from neurons and interneurons, endothelial NOS from endothelial cells in blood vessels (33–38), and constitutive NOS activity deriving from astrocytes (39). The use of NMDA as the test stimuli imparts specificity to the production of  $^*NO$  via the NMDA glutamate receptor-nNOS pathway.

Specifically, the major findings can be listed as follows: (i) the diffusional field of  $^*NO$  upon stimulation of multiple NOSs within a radius of  $100\ \mu m$  is  $\approx 400$  microns in the CA1 subregion; (ii)  $^*NO$  signals are transient even under conditions of continuous stimulation of the NMDA receptor; (iii)  $^*NO$  is produced in all subregions in response to NMDA receptor stimulation; (iv)  $^*NO$  concentration dynamics (rate and pattern of production and decay) is heterogeneous along the trisynaptic loop in the cell body layers of the CA1, CA3, and DG subregions; (v) a steep gradient of  $O_2$  is operative in the slice cell layers, being physiological at the core of the tissue; (vi) cells efficiently bring  $^*NO$  to low nM levels, preventing high steady concentrations; and (vii) at variance with current dogma, the NMDA receptor does not suffer a feedback blockage by  $^*NO$ .

Experimental evidence and theoretical models for  $^*NO$  diffusion in biological settings support the notion that  $^*NO$  is highly diffusible (diffusing more rapidly than it reacts) and spreads randomly in all directions driven by a spatial concentration gradient from its local of synthesis (3–5, 26, 40, 41). In addition to its diffusibility, the amount and rate at which it is generated, the duration of release from a source cell, and the type and number of targets in the vicinity as well as the rate (and compartmentalization) of chemical reactions shape the  $^*NO$  concentration-time profile. The experimental approach used in this study implies that multiple  $^*NO$  sources within a tissue volume are simultaneously activated by the localized ejection of NMDA. Under these conditions, encompassing the

diffusion of NMDA in the tissue and a perfusion flow of  $2\ ml/min$ ,  $^*NO$  diffused at least  $>400$  microns from the NMDA ejection site. Of note, within this tissue volume,  $^*NO$  signals are transient. The transitory nature of  $^*NO$  signals was also observed under conditions where the slices were continuously perfused with NMDA (Fig. 6A). However, in this case, the decay was linear and slower compared with the ejection approach, which is characterized by an exponential decay (e.g., Fig. 3A), and D-AP5 brings  $^*NO$  to basal levels, thus suggesting that NMDA receptor-dependent  $^*NO$  synthesis is operative (Fig. 6B). The perfusion system is unlikely to determine the transitory nature of the signals as perfusion of a  $400\ nM$  solution of  $^*NO$  prepared from  $^*NO$  gas induced a constant signal (data not shown). These observations suggest that, at variance with what is conventionally expected, maintaining a continuous and simultaneous activation of multiple cellular  $^*NO$  sources does not result in a monotonous rise of  $^*NO$  or high steady-state concentrations, rather the concentration of  $^*NO$  rises transiently. The decay of  $^*NO$  observed under continuous perfusion is not likely to be accounted for by a negative feedback of NOS (42, 43) or the NMDA receptor, known to occur through S-nitrosylation of specific SH residues (44–49), for D-AP5 would have induced no inhibition of  $^*NO$  decay kinetics. Thus, the results suggest the occurrence of mechanisms for the prevention of high steady-state  $^*NO$  concentrations. In agreement with this notion, it has been recently proposed that brain cells possess powerful  $^*NO$  inactivation mechanisms that shape  $^*NO$  signals (50, 51). At variance with the synthesis of  $^*NO$  by NOS, which is a highly regulated process (52), its decay/consumption is thought to be an unregulated process, depending largely on the local availability of potential targets (hemoglobin, soluble guanylate cyclase, cytochrome oxidase, superoxide anion, thiol groups... ). The results shown here are consistent with the view whereby brain cells are able to efficiently respond to a high  $^*NO$  concentration bringing it to very low nM levels.

The subregional differences in  $^*NO$  signals evoked with NMDA raise at least two important questions: what are the underlying mechanisms and how do these differences translate into tissue physiological activity?

Regarding the latter, it may be noted that the strength of  $^*NO$  responses in CA1 pyramidal neurons accomplishes an essential requirement posed by the notion of  $^*NO$  as a messenger in synaptic plasticity mechanisms linked to memory and learning: NMDA-dependent LTP (53) is seen mainly in the CA1 subregion of the hippocampus; in CA3 a different type of potentiation is observed (54). Moreover, in humans, a regional-specific neuron loss in CA1 region is associated with the cognitive decline in Alzheimer's disease (55). Thus, one might expect to see differences in  $^*NO$  concentration dynamics in CA1 relative to CA3 and DG.

The elucidation of the mechanisms that support the subregional differences of  $^*NO$  signals is an open question. It is currently conceived that the neuronal isoform of NOS is not confined to small specific areas, but is distributed in a small population of neurons throughout brain areas, including hippocampus (36). Assuming a homogeneous distribution of nNOS and NMDA receptor, the heterogeneous concentration dynamics of  $^*NO$  among the subregions of hippocampus evoked by stimulation of NMDA receptors suggests a region-specific regulation of  $^*NO$  bioactivity. However, despite species and development variations in hippocampal nNOS (37), there may also be a gradient of expression of nNOS in the subregions of the hippocampus. A recent report showed a higher level of nNOS expression in the CA1 subregion of the rat hippocampus, but these observations were made in the total extract of each region in a manner that cannot distinguish between nNOS that is coupled to the NMDA receptor and nNOS present in other cellular compartments (38). Also, the differences reported are not as large as the ones shown in this study for  $^*NO$  production.

It may be argued that the differential level of expression of the NMDA receptor in the subregions of the hippocampus may account for the differential NMDA-dependent  $^*NO$  signals observed.

This type of glutamate receptor is more highly expressed in the CA1 subregion (56). In agreement with this hypothesis, reports on the production of \*NO in hippocampal subregions not dependent on NMDA receptors showed less significant differences of \*NO production as compared with those in this study. Smith *et al.* (57), using nicotine as the stimulus for \*NO production, reported that CA3 and DG peak amplitudes were  $\approx 70\%$  of peak amplitude in CA1 area. More recently, a bioimaging approach with diaminofluorescein derivatives indicated that \*NO is produced mainly in the CA1 area in hippocampal slices under ischemic conditions (58).

Finally, the NMDA receptor and nNOS may be modulated at different levels in the different subregions by mechanisms that may range from the organization of the NMDA receptor–postsynaptic density-95 protein–nNOS molecular complexes through protein–protein interactions to the action of superoxide anion ( $O_2^{\cdot-}$ ). For instance, PIN, the protein inhibitor of nNOS expressed in the hippocampus, inhibits nNOS by binding to monomers and thus blocking dimerization or even by dissociating already formed dimers (59, 60).

Likewise,  $O_2^{\cdot-}$  produced by NMDA receptor activation appears to work in conjunction with \*NO during induction of LTP in hippocampus (61). It is noteworthy that in rat brain the localization of Cu/Zn-SOD overlaps with that of NOS in the pyramidal cell layers of CA1, CA3, and DG subregions of hippocampus (62). Also, a regional vulnerability to  $O_2^{\cdot-}$  has been reported in organotypic hippocampal slice cultures, being CA1 pyramidal neurons selectively affected (63), a pattern of damage similar to that observed after hypoxia/ischaemia (64). Considering the very fast reaction of both superoxide dismutase (SOD) [ $K = 2.3 \times 10^9 \text{ M}^{-1}\text{s}^{-1}$  (65)] and \*NO [ $K = 1.9 \times 10^{10} \text{ M}^{-1}\text{s}^{-1}$  (66)] with  $O_2^{\cdot-}$ , the latter yielding cytotoxic peroxynitrite, the production of  $O_2^{\cdot-}$  accompanying the synthesis of \*NO requires

fine-tuning between the competition of SOD and \*NO for  $O_2^{\cdot-}$ . A disturbance in the balance between the levels of \*NO and  $O_2^{\cdot-}$  would determine differently the availability of \*NO for transcellular diffusion in hippocampal subregions, which, accordingly, would be reflected in effective \*NO concentration.

The implications of this study for cell function are as follows: the \*NO production in the dependency of NMDA receptor activation has been shown to participate in LTP as a retrograde messenger but also in excitotoxic cell death. In the former, \*NO is assumed to be at low nM concentration, whereas the latter is associated with excessive stimulation of NMDA receptor and, therefore, it is alleged a high production of possibly pathologic \*NO concentration at the micromolar level. A selective vulnerability of the CA1 subregion to NMDA and glutamate stimuli has been reported (67). In this study, subregions of hippocampus respond differently in terms of \*NO production. In the CA1 pyramidal cell layer, \*NO concentration reached the highest levels (typically  $\approx 250 \text{ nM}$ ) but, even under conditions of continuous NMDA stimulation, \*NO rose only transiently. Assuming that data obtained with isolated brain slices reflect the concentrations *in vivo*, the claimed high concentration of \*NO at micromolar range in excitotoxicity achieved by excessive stimulation of NMDA receptor may have to be reevaluated. Moreover, it may have implications for the \*NO-dependent excitotoxic pathways in the different regions after NMDA receptor activation. Finally, the differential concentration dynamics may reflect distinct regulatory pathways and biological activities for \*NO in hippocampal subregions.

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