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ABSTRACT

D-serine is an endogenous ligand for *N*-methyl D-aspartate (NMDA) receptors and alterations in its concentration have been related to several brain disorders, especially schizophrenia. It is therefore an important target neuromodulator for the pharmaceutical industry. To monitor D-serine levels *in vivo*, we have developed a microbiosensor based on cylindrical platinum microelectrodes, covered with a membrane of poly-phenylenediamine (PPD), and a layer of immobilized D-amino acid oxidase from the yeast *Rhodotorula gracilis* (RgDAAO). By detecting the hydrogen peroxide produced by enzymatic degradation of D-serine, this microbiosensor shows a detection limit of 16 nM and a mean response time of 2 s. Interferences by ascorbic acid, uric acid, L-cysteine and by biogenic amines and their metabolites are rejected at more than 97% by the PPD layer. Although several D-amino acids are potential substrates for RgDAAO, D-serine was the only endogenous substrate present in sufficient concentration to be detected by our microbiosensor in the central nervous system. When implanted in the cortex of anesthetized rats, this microbiosensor detected the increase in concentration of D-serine resulting from its diffusion across the blood brain barrier after an intraperitoneal injection. This new device will make it possible to investigate *in vivo* the variations in D-serine concentrations occurring under normal and pathological conditions, and to assess the pharmacological potency of new drugs designed to impact D-serine metabolism.

Alterations in brain concentrations of D-serine, the predominant D-amino acid in the mammalian central nervous system[1], have been recently related to several neurological and psychiatric diseases[2]. D-serine is synthesized in glial cells[3] and neurons[4, 5], through the racemization of L-serine by serine racemase[3]. It is released by astrocytes in response to chemical stimulation by glutamate, or glutamate receptor agonists such as α-amino-3-hydroxy-5-methylisoxazole-4-propionate (AMPA), kainic acid, or (1S,3R)-1-aminocyclopentane-1,3-dicarboxylic acid (t-ACPD)[6, 7]. D-serine is an endogenous agonist of the glycine site of the N-methyl-D-aspartate (NMDA) receptors[8], and plays important roles in synaptic plasticity[9, 10] and in several neurological and psychiatric disorders, such as schizophrenia[11, 12], ischemia[13], Alzheimer's disease[14] and amyotrophic lateral sclerosis[15]. In particular, D-serine concentration is decreased in the cerebrospinal fluid of schizophrenic patients[16, 17], and its administration enhances the therapeutic effects of known antipsychotic drugs[18, 19]. Notwithstanding the physiological importance of D-serine in these diseases, the variations in D-serine levels in the central nervous system (CNS) during behavior or pathological states remain largely unexplored. Also, the biochemical and cellular mechanisms controlling D-serine extracellular brain concentration are still elusive. To elucidate these important questions, the development of rapid and selective methods for monitoring D-serine dynamics in the brain is of paramount importance.

Detection of free D-serine in the CNS has been reported using microdialysis[20-23]. Despite its impressive analytical power, microdialysis is limited by the large size of the probes used to collect brain samples and by the slow diffusion of molecules through the dialysis membrane. In particular, implantation of microdialysis probes into the CNS often causes lesions that impair the

physiological processes taking place around the probe[24-26]. Moreover, microdialysis probes are too large to be inserted into brain slices or small structures of the rat or mouse CNS.

To overcome these technical limitations, we developed an enzymatic microbiosensor capable of detecting D-serine *in vivo*. Enzymatic biosensors have been used for monitoring extracellular levels of neurotransmitters like glutamate[27-31], adenosine[32], adenosine tri-phosphate[33], choline[34, 35], acetylcholine[36-38], as well as metabolic molecules like glucose[39] or lactate[40]. Moreover, D-amino acids (including D-serine) can be detected in food or beverages as markers of bacterial proliferation, using biosensors coated with the enzyme D-amino acid oxidase (DAAO)[41-44]. However, these biosensors are too large to be inserted into the CNS of laboratory animals, and do not reach a sufficient detection limit to monitor physiological D-serine concentrations in the low micromolar range[21]. In this study, we sought to optimize this principle to achieve successful electrochemical D-serine detection in the brain.

Our microbiosensor is based on a platinum microelectrode (25×150 µm) covered with a membrane of poly-m-phenylenediamine (PPD) for selectivity, and with a layer of D-amino acid oxidase purified from the yeast *Rhodotorula gracilis* (RgDAAO). This microbiosensor detects D-serine with an *in vitro* detection limit of 16 nM and a mean response time of 2.3 s. When used in the brain or in brain extracts, it is selective for D-serine over other endogenous amino acids or oxidizable molecules. Moreover, this microbiosensor can detect the basal D-serine extracellular concentration in the rat frontal cortex (3.1 µM) and D-serine diffusion through the blood brain barrier following an intraperitoneal injection. Parts of this study have been published in abstract form[45].

EXPERIMENTAL SECTION

Preparation of the enzyme

Recombinant *R. gracilis* D-amino acid oxidase (RgDAAO, EC 1.4.3.3) was overexpressed in *E. coli* and purified as reported by Molla et al.[46]. The final enzyme preparation had a specific activity of 55 U/mg protein on D-serine, and was concentrated up to 58 mg/ml of RgDAAO in a solution containing 25 mg/ml bovine serum albumin (BSA, Sigma, St-Quentin Fallavier, France) and glycerol (1%) in phosphate buffer (0.02 M, pH 8.5). Pig kidney DAAO was purchased from Sigma and prepared following the same protocol as RgDAAO.

Microbiosensor Preparation

Biosensors were constituted of a 25 µm diameter 90% Pt / 10% Ir wire (Goodfellow, Huntington, UK) glued to a 0.3 mm copper wire using electroconductive silver paint (Radiospares, Beauvais, France). It was then inserted into a pulled glass capillary (Harvard Apparatus, Edenbridge, UK) and the tip of the pipette was cut to let 150 µm of the platinum wire protrude out of the glass. The junction between the platinum wire and the glass micropipette was sealed with epoxy resin (Araldite®, Bostik, Paris, France). The electrodes were then washed for 30 min in KOH 0.5 M and 20 min in ethanol. A PPD layer was electrodeposited by dipping the electrodes 20 minutes in a solution containing 100 mM m-phenylenediamine in PBS 0.01 M at pH 7.4 under a constant potential of +700 mV *vs.* an Ag/AgCl reference electrode. The enzyme layer was deposited by dipping the Pt tip of the electrodes in the DAAO solution (see above). Control microbiosensors (i.e. without DAAO) were produced by dipping the electrode in a

solution of BSA (400 mg/ml) in phosphate buffered saline (PBS) 0.01 M, pH 7.4 and 1% glycerol. The enzyme was immobilized under saturated glutaraldehyde vapors for 120-135 s by placing the electrodes into a small chamber containing a few milliliters of a 50 % glutaraldehyde solution (under a fume hood because of the toxicity of glutaraldehyde vapors). Electrodes were then kept at -20°C in dry atmosphere for long-term storage.

All microbiosensors were tested for the detection of serotonin (5-HT, 20 µM in PBS), D-serine (1 µM in PBS) and H_2O_2 (1 µM in PBS) before use. Only the electrodes showing more than 7 pA/µM D-serine and less than 4 pA/20 µM 5-HT were included in this study. To determine this threshold, we tested a total of 30 electrodes: D-serine sensitivity was 9.2 ± 3.4 pA/µM and 16.7% were excluded using these criteria.

Microbiosensors used for *in vivo* experiments were covered with an additional Nafion membrane, to protect the enzyme layer during penetration into the brain. The tip of the electrode was dipped 5 times in Nafion 1% (5% commercial solution from Aldrich, Saint Quentin Fallavier, France, diluted in isopropanol) and allowed to dry for at least 10 minutes at room temperature[31, 34]. This additional membrane did not change the sensitivity or the response time of the electrodes.

Recordings

All recordings were made either with a patch-clamp amplifier Geneclamp GC500 (Molecular Devices, Sunnyvale, CA) or with an electrochemistry amplifier VA-10 (NPI Electronics, Tamm, Germany) used with a 2 electrode potentiostat. Data acquisition was performed with an ITC-18 acquisition board (Instrutech, Port Washington, NY) driven with a homemade software based on Igor Pro 5.0 procedures (Wavemetrics, Eugene, OR). The

oxidation current was sampled at 1 kHz and averaged over 1000 data points, yielding a final sampling frequency of 1 Hz.

In vitro calibrations were performed in standard solutions prepared with phosphate buffered saline (0.01 M, pH 7.4). The reference electrode was a chlorided silver wire placed directly into the recording chamber. Recordings were made in constant potential amperometry at 500 mV vs. the Ag/AgCl reference electrode. Calibration of the biosensors before and after *in vivo* experiments was performed in an artificial extracellular medium containing (in mM): NaCl 126, KCl 1.5, KH_2PO_4 1.25, $MgSO_4$ 1.5, $CaCl_2$ 2 and HEPES 10 (pH 7.4). All chemicals were purchased from Sigma-Aldrich (Saint-Quentin Fallavier, France).

Preparation of brain samples

Male Wistar rats (300-400 g) were decapitated under isofurane anesthesia, and the forebrain was removed and homogenized in 5 ml of 5% trichloroacetic acid (TCA) to precipitate macromolecules. The homogenate was then centrifuged at 20 000 g for 10 min. TCA was extracted 6 times from the supernatant using ether, before lyophilization and storage at -20°C.

High pressure liquid chromatography (HPLC) measurements

Lyophilized brain extracts (40 mg) were dissolved in 1 ml deionized water, and 50 µl aliquots were treated with 0.8 mg N-acetyl-cysteine and 0.25 mg *o*-phthaldialdehyde in a 0.1M borate buffer, pH 10.4, for derivatization of amino acids. HPLC measurements were performed using a Waters Alliance instrument (Waters Corporation, Guyancourt, France) with a Waters symmetry column (4.6×250mm). The column and sample compartments were kept at 30 and 8°C respectively. Flow rate was set at 1 ml/min and run time was 20 min for all analyses. L- and D-

serine were eluted with an isocratic method using phase A (990 ml of 0.1 M sodium acetate and 10 ml tetrahydrofurane, pH 6.2); the column was washed using phase B (500 ml of 0.1 M sodium acetate, 470 ml acetonitrile, 30 ml tetrahydrofurane, pH 6.2). Amino acid derivatives were detected using a Waters fluorescence detector (excitation 344 nm, emission 443 nm), and data were acquired using the Empower Pro software package (Waters Corporation, Guyancourt, France). Calibration of D-serine detection was performed using a 7-point standard curve.

In vivo experiments

Rats were anesthetized with a ketamine-xylazine mixture (42 mg/ml ketamine, 1,6 mg/ml xylazine, purchased from Centravet, Plancoët, France, and injected at 0.2 ml/100 g body weight) and placed in a stereotaxic apparatus (Stoelting Corporation, Wood Dale, IL). Body temperature was kept at 37°C with a homeothermic blanket (LSI Letica, Barcelona, Spain). An active RgDAAO biosensor was implanted in the frontal cortex (1 mm lateral from the midline, 3 mm anterior from the bregma, 1.5 mm ventral from the dura), side by side (0.5 mm) with a control biosensor covered with BSA. Both microbiosensors were covered with an additional Nafion layer. Before to start the recording, currents were allowed to stabilize for at least 30 min. D-serine 1g/kg body weight was injected intraperitoneally after an additional 30 min period of control recording. In order to determine their selectivity and sensitivity, both biosensors were calibrated using 5-HT 20 µM and D-serine 1 µM dissolved in an artificial extracellular medium (see above) before and after the *in vivo* experiment.

Statistics

Data are expressed as mean ± standard deviation (SD) except for *in vivo* or brain extracts measurements, where we used mean ± standard error of the mean (SEM). Comparisons between two data groups were performed using the Student's *t*-test for equal or unequal variances, as determined by the F-test (significance level was $p<0.05$). Statistics software was the analysis tool-pack of Excel (Microsoft Office XP).

RESULTS AND DISCUSSION

Principle of D-serine detection by the biosensor

D-serine detection was achieved by a two-step reaction using the enzyme DAAO: (1) DAAO converts D-amino acids into their corresponding α-ketoacids (hydroxypyruvate for D-serine) and generates equimolar amounts of H_2O_2; (2) H_2O_2 is oxidized at the surface of a platinum wire connected to a patch-clamp amplifier (Fig 1). The resulting H_2O_2 oxidation current corresponds to the D-serine concentration in the biosensor's microenvironment.

To optimize H_2O_2 detection by the microbiosensor, we generated cyclic voltammograms in a solution of H_2O_2 100 µM in PBS 0.01 M pH 7.4, using potential sweeps from 0 mV to 900 mV vs. Ag/AgCl reference electrode. As described by other authors[47, 48], holding potentials around 0 mV gave rise to electrochemical reduction of H_2O_2, whereas oxidation was achieved above +200 mV. Optimal H_2O_2 detection was performed at a holding potential of +500 mV vs. Ag/AgCl reference electrode, at which background currents were kept minimal. This value falls in the +500 mV and +700 mV range vs. Ag/AgCl reference electrode typically used for platinum wire enzymatic biosensors[27, 33, 49, 50].

Choice of the DAAO

The most commonly used and commercially available DAAO is the one isolated from pig kidney (pkDAAO). Several biosensors for D-amino acids detection in food samples have been successfully prepared using pkDAAO[41-44]. However, these biosensors achieved detection limits in the 10^{-5}-10^{-4} M range, which is not sufficient for detecting D-serine extracellular concentration

in the CNS, estimated at a few 10^{-6}M[20-22]. This low sensitivity to D-amino acids might be due to the low specific activity of pkDAAO used for these biosensors. However, the DAAO from the yeast *Rhodotorula gracilis* shows better properties for biotechnological applications. It binds the flavin adenine dinucleotide (FAD) cofactor with high affinity and shows a higher specific activity and stability compared to its mammalian counterpart[51].

We thus compared the sensitivity and selectivity of microbiosensors prepared with either pkDAAO or RgDAAO. Sensitivity for D-serine was 42% higher with RgDAAO microbiosensors than with pkDAAO ones: 9.2 ± 3.4 pA/µM (n=30) vs. 6.5 ± 2.0 pA/µM (n=4) respectively (Table 1). Moreover, RgDAAO microbiosensors were more selective for D-serine than pkDAAO ones. For example, RgDAAO microbiosensors showed much smaller electrochemical responses to D-aspartate (5.5% of the D-serine response vs. 38.6% for pkDAAO, n=4, Table 1) and were also slightly less sensitive to D-alanine (104 % of D-serine response vs. 165% for pkDAAO, n=4, Table 1). Because D-aspartate and D-alanine can also be found in the mammalian CNS, together with D-serine[52, 53], the lower electrochemical responses produced by the RgDAAO biosensor to these D-amino acids would contribute to a greater selectivity *in vivo*. For these reasons, biosensors were prepared using RgDAAO in the rest of this study.

In vitro detection of D-serine

We first calibrated the RgDAAO microbiosensors in D-serine standard solutions. The electrodes responded to changes in D-serine concentration by a step increase in the recorded oxidation current (Fig. 2A). The response time of the microbiosensors, defined as the duration of the rise between 10% and 90% of the response, was 2 ± 1 s (n=5, Fig. 2B).

D-serine oxidation current showed a linear relationship with concentration from 100 nM to 500 µM, with a sensitivity of 9.2 ± 3.4 pA/µM D-serine (n=25, Fig. 2C). Importantly, only the electrodes showing a sensitivity higher than 7 pA/µM D-serine were used for further studies (see Experimental section). When the recorded signal was averaged at 1 data point/s, the noise was about 0.05 pA, yielding a theoretical detection limit of 16 nM (signal/noise = 3). This detection limit is significantly lower than the estimated D-serine concentration in the CNS[20-22] and is therefore compatible with *in vivo* detection of this D-amino acid.

Oxygen dependence

D-amino acid oxidases require O_2 as a co-substrate to complete the catalytic cycle. In order to evaluate the dependence of D-serine detection on O_2 concentration, we performed similar calibration experiments using PBS in equilibrium with the ambient atmosphere, or saturated with O_2 or N_2 (Fig. 2C-D). Provided that O_2 is non-limiting, the relationship between the amplitude of the step in oxidation current detected by the biosensor and D-serine concentration can be approximated by a Michaelis-Menten equation:

$$(1) \qquad C = \frac{Cmax \times [D-serine]}{Km_{app} + [D-serine]}$$

where C is the oxidation current, and C_{max} and $K_{m,app}$ are analogous to the classical V_{max} and K_m kinetic parameters used for the equation of the free enzyme. In a O_2-saturated medium, the response of the microbiosensor followed a Michaelis-Menten kinetics at least up to 2.5 mM D-serine with a $K_{m,app}$ of 3.62 ± 0.38 mM, and a C_{max} of 54.2 ± 12.5 nA (n=4, Fig. 2D). $K_{m,app}$ was significantly lower than the K_m of the free enzyme in solution (13.75 mM[51]). Such a difference between free and immobilized RgDAAO was reported previously[54], and was attributed

to the conformation of the immobilized enzyme and to a higher concentration of D-serine in the hydrophobic enzymatic layer compared to the solution (partitioning effect). When calibrated in air-equilibrated buffer, the microbiosensor yielded very similar responses to those obtained in O_2 saturated medium (Fig. 2C-D). However, the calibration curve obtained in N_2-saturated buffer deviated significantly from the ones obtained in the presence of O_2 for D-serine concentrations greater than 30 µM (Fig. 2C-D). The oxidation current detected by the microbiosensor reached a plateau at about 8 nA, reflecting the decreased DAAO activity at low O_2 concentrations. The calibrations in the 0-30 µM D-serine range show a weak dependence on pO_2, like most biosensors based on the detection of enzymatically produced H_2O_2[27, 33, 55]. This weak dependence on pO_2 is probably due to O_2-regeneration during H_2O_2 oxidation on the Pt surface, that produces a concentration of O_2 at which the rate of reoxidation of reduced DAAO is not limiting[55]. Because physiological D-serine concentrations in the CNS are expected around 5-10 µM[20-22], D-serine detection at our microbiosensor should not depend on local pO_2, except, possibly, during pathological states like cerebral ischemia.

pH and temperature dependence

D-serine detection by the biosensor showed the same pH dependence as RgDAAO in solution[51, 56]. The response at 10 µM D-serine increased from pH 5.4 to 8.0, reached a maximum at pH 8.0-8.5, and decreased at higher values (n=4, Fig. 3A). The response at physiological pH (7.4) was 80.7% of the maximum response observed at pH 8.5.

The temperature dependence of D-serine detection was also similar to that of the free enzyme in solution[51]. The microbiosensor response to D-serine increased with temperature, from

15°C to 40°C (n=4, Fig 3B). Its sensitivity at 37°C (*in vivo* temperature) is 75% higher than that at 25°C (room temperature).

Operational and storage stability

The operational stability of the microbiosensors was estimated by placing the electrode in PBS and then adding 5 µM D-serine to produce an electrochemical signal. One hour later, the recording medium was replaced and fresh D-serine was newly added. The microelectrode response was thus evaluated every hour for 6 hours, during which it was almost constantly exposed to 5 µM D-serine. Under these conditions, response to D-serine remained stable (n=4, data not shown), indicating that (1) the enzyme was strongly fixed to the platinum wire and (2) the microbiosensor could be exposed to physiological D-serine concentrations for several hours without any alteration of its sensitivity.

Storage stability was evaluated for microbiosensors kept at -20°C immediately after manufacturing. Microbiosensors were tested for their response to 5 µM D-serine 8, 21, 28, 35, 84 and 160 days after manufacturing. They were dried and frozen between each test. Response to D-serine remained unchanged (n=3, data not shown), showing only 2% loss in sensitivity after more than 160 days of storage.

Selectivity

The preceding data demonstrate that our microbiosensor can reliably detect D-serine concentrations as low as a few tens of nM for several hours in standard solutions. However, in order to detect D-serine in complex biological media, it is necessary to achieve selective measurements avoiding the detection of other endogenous molecules present *in vivo*. Endogenous molecules can interfere in two ways: (1) direct oxidation on the surface of the platinum wire and

(2) oxidation by the enzyme accompanied by H_2O_2 production. Both kinds of interference were investigated:

1- Endogenous oxidizable molecules

The major endogenous molecules susceptible to oxidation at a platinum surface at +500 mV vs. Ag/AgCl reference electrode are serotonin (5-HT) and its metabolite 5-hydroxyindolacetic acid (5-HIAA), dopamine and its metabolite dihydroxyphenylacetic acid (DOPAC), L-cysteine, ascorbic acid and uric acid. Biosensors using enzymes directly coated on a bare platinum wire or carbon fiber typically produce strong interfering oxidation currents in response to physiological concentrations of these endogenous molecules. For example, microbiosensors consisting in RgDAAO directly deposited on a bare Pt wire showed such interfering responses (Fig. 4A). An efficient strategy to overcome these non-specific signals is to deposit a layer of electropolymerized PPD onto the Pt wire[30, 39, 57, 58]. This process is highly reproducible and generates a thin (10 nm), uniform membrane. The PPD layer forms a steric barrier that allows H_2O_2 diffusion but blocks larger molecules[59]. It has several advantages, including an easily controllable thickness, a good reproducibility and a good uniformity of the film[57]. Moreover, electropolymerized films of m-phenylenediamine show superior selectivity compared to those prepared with its p- or o- isomers[30]. RgDAAO microbiosensors manufactured from a poly-m-phenylenediamine covered Pt wire showed a dramatic reduction (97-99%) in the interfering responses produced by endogenous oxidizable molecules (n=4, Fig. 4B). By contrast, D-serine detection was only reduced by 12%.

We also tested the operational stability of the PPD layer. As shown in Fig. 4C, the small response to 20 µM 5-HT remained stable for 6 hours (in PBS containing 5 µM D-serine at room temperature), indicating that the PPD layer retained its ability to reject 5-HT over several hours.

However the PPD layer had a tendency to lose its selectivity over several days, even when stored at -20°C. We found that it could be reactivated by dipping the microbiosensor for 1-2 s in a 100 mM m-phenylenediamine solution, at 700 mV *vs.* Ag/AgCl reference electrode. This procedure barely affected the sensitivity of the microbiosensor and restored the original selectivity. Overall, these results show that interfering responses caused by endogenous oxidizable molecules are reduced by more than 97% by an electropolymerized film of m-phenylenediamine. Importantly, this selectivity layer decreases the responses to physiological concentrations of these interfering molecules to a negligible level compared to D-serine oxidation currents.

2- Other D-amino acids

Free RgDAAO in solution preferentially oxidizes neutral D-amino acids (such as D-alanine, D-proline, D-cysteine, D-valine and D-serine), shows low activity towards basic ones and is practically inactive on acidic D-amino acids[51]. As expected, our RgDAAO microbiosensors generated large electrochemical responses not only to D-serine, but also to other D-amino acids, with the exception of D-aspartate and D-glutamate (6.5% and 1.7% respectively of the D-serine response at the same concentration, n=4, Fig. 5A). Glycine also generated very small responses (3.5% of D-serine response, n=4, Fig. 5A) whereas L-amino acids were not detected (<1.5% of D-serine response for L-serine and L-cysteine, n=4, Fig. 5A). Therefore, and consistent with earlier reports[54], RgDAAO showed a broader substrate specificity when fixed on the microbiosensor than the free form, in particular, an increased activity towards basic amino acids.

In a recent study, Hamase et al.[53] showed that D-serine was the predominant D-amino acid in the CNS, followed by D-aspartate (about 10% of D-serine contents), which is not detected by the microbiosensor, and by D-alanine (<3% of D-serine contents), which is a good substrate

for RgDAAO (Fig. 5B). Therefore, the new RgDAAO microbiosensor would be selective for D-serine when used in the CNS, since interference from endogenous D-amino acids is negligible compared to D-serine signals.

Validation of the microbiosensor on brain extracts

To confirm the selectivity of the microbiosensor, we measured D-serine concentrations in brain extracts, comparing amperometric recordings and HPLC analysis. Both methods yielded comparable D-serine concentrations (130 ± 7 µM for HPLC vs. 139 ± 8 µM for the microbiosensor, n=6, NS, Fig. 6C): amperometric detection using the microbiosensor differed from HPLC analysis by only 6%. To better quantify the amount of non-specific signals detected by the microbiosensor in the brain extracts, we eliminated D-serine (and other DAAO substrates) by incubating the sample for 2 hours with 20 U of RgDAAO and 200 U of catalase. This enzymatic treatment completely eliminated the D-serine peak in the HPLC chromatograms (Fig. 6A) and reduced the amperometric signal by 95.0 ± 1.3% (n=6, $p<0.01$, Fig. 6B). This result confirms that the sum of interfering signals detected by the microbiosensor in brain extracts is about 5-6% of the D-serine signal, and that our RgDAAO microbiosensor specifically detects D-serine when used in the CNS.

***In vivo* detection of D-serine**

We then implanted our microbiosensors in the frontal cortex of anesthetized rats. The RgDAAO microbiosensor was implanted close to a control biosensor (devoid of DAAO) used to control for non-specific variations in oxidation current. Both microbiosensors were coated with an additional layer of Nafion to optimize biocompatibility (see Experimental Section).

Implantation in the CNS produced a small decrease in sensitivity (-13.2%, 10.05 ± 1.03 pA/µM D-serine before, 8.72 ± 0.97 pA/µM after *in vivo* implantation, n=8). This decrease in sensitivity is common to virtually all microbiosensors and generally occurs during the first 15 min following insertion into the brain[60].

About 1 hr after implantation into the CNS, the background currents stabilized, showing a difference of 47.2 ± 7.5 pA (n=6) between the control and RgDAAO microbiosensor. This difference was not detected in calibrations made in PBS or in an artificial extracellular medium of ionic composition close to that of the rat cerebrospinal fluid (2.3 ± 1 pA, n=7). Because the control electrode and the RgDAAO microbiosensor were equally sensitive to endogenous oxidizable molecules like H_2O_2, ascorbate, etc., and because D-serine is the predominant D-amino acid in the brain, the difference in background current mostly reflected the ambient basal D-serine concentration in the frontal cortex. To estimate the D-serine concentration represented by this difference in background currents, we calibrated our microbiosensors in artificial extracellular solution. In this medium, the sensitivity of the microbiosensor was unchanged compared to PBS. Moreover, addition of a physiological concentration (400 µM) of ascorbate in the recording medium did not change the sensitivity of the microbiosensor (n=3, data not shown). Using these calibration experiments, we estimated the basal extracellular D-serine concentration in the rat frontal cortex under ketamine-xylazine anesthesia at 3.1 ± 0.4 µM. This value is of the same order of magnitude as other estimations based on microdialysis experiments (6-8 µM)[20-22].

We then injected 1 g/kg D-serine intraperitoneally. It induced a steady increase in oxidation current at the RgDAAO microbiosensor, but not at the control sensor, indicating that the electrochemical signal detected by the RgDAAO microbiosensor was specific for D-serine. This increase in electrochemical signal was most probably due to the diffusion of D-serine across

the blood-brain barrier. Three hours after the injection, the increase in oxidation current at the RgDAAO microbiosensor was 65.3 ± 14.2 pA whereas the background current at the control microbiosensor decreased by 5.8 ± 1.8 pA (n=6, Fig. 7). Using the same calibration experiments in artificial cerebrospinal fluid, we estimated that the D-serine extracellular concentration increased by 4.8 µM, to reach a final 7.9 ± 1.2 µM at the end of the 3 hour recording following the peripheral injection. This modest increase in D-serine concentration is in agreement with previous measurements made post-mortem on whole tissue[61]. However, these data contrast with an earlier report using microdialysis, that showed a much faster 6-fold increase in D-serine levels following the intraperitoneal injection of a smaller dose of D-serine (50 mg/kg)[62]. Indeed, implantation of microdialysis probes in the brain usually produces significant tissue damage, especially to neighboring blood vessels[24, 25]. This problem might have led to an overestimation of D-serine diffusion into the brain. In this respect, biosensors of micrometric size seem preferable for studies requiring integrity of the blood-brain barrier[25]. In particular, the slow and modest increase in D-serine levels detected by our microbiosensors after the peripheral D-serine injection, suggests that damage to the blood-brain barrier produced upon implantation of the electrodes in the frontal cortex was indeed minimal.

Conclusion

Overall, these data demonstrate that D-serine levels in the CNS can be monitored using platinum-wire microbiosensors coated with the enzyme RgDAAO. This is the first report, to our knowledge, showing successful detection of D-serine concentrations *in vivo* at the micromolar level using an amperometric biosensor. Compared to previous DAAO biosensors used for D-amino acid detection in food, D-serine detection *in vivo* requires an increased selectivity, a higher

sensitivity, and miniaturization to allow implantation in the CNS of small laboratory animals. These technical challenges have been overcome through (1) the use of RgDAAO, a more active and more selective enzyme than the previously used pkDAAO, (2) the development of micrometric platinum wire electrodes for H_2O_2 monitoring, and (3) the use of a highly selective PPD layer to block the non-specific oxidation of endogenous oxidizable molecules.

This new device will now pave the way for a better understanding of the physiological and pathological situations that lead to changes in D-serine levels in the CNS as well as the cellular mechanisms of D-serine release and elimination. It will also provide a powerful analytical tool for screening new pharmacological agents designed to modify D-serine metabolism in the CNS, in order to improve current treatments for neurological and psychiatric disorders such as schizophrenia[62].

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FIGURE CAPTIONS

Figure 1: Design of the biosensor. A. Photomicrograph of the tip of a RgDAAO microbiosensor. The enzymatic layer appears as a translucid, yellow membrane. **B.** Schematic representation of the D-amino acid oxidase (RgDAAO) microbiosensor. A platinum wire is covered with a layer of poly-m-phenylenediamine (PPD) and an enzymatic membrane of RgDAAO. **C.** Enzymatic reaction allowing D-serine detection at the microbiosensor: D-serine is oxidized into hydroxypyruvate by RgDAAO with equimolar production of hydrogen peroxide (H_2O_2) that diffuses through the PPD layer and is oxidized by the platinum wire. H_2O_2 oxidation gives rise to two electrons detected by the patch-clamp amplifier.

Figure 2: D-serine detection. A. Response of the biosensor to changes in D-serine concentration. The electrode was rapidly transferred from phosphate buffered saline (PBS) to a 500 nM or 1 µM D-serine solution and back to PBS. The change in D-serine concentration was detected as a step in the oxidation current recorded by the electrode **B.** Response time. The electrode was switched from 0 to 10 µM D-serine. The response time was defined as the time between 10% and 90% of the current step. In order to obtain a higher time resolution, the signal was averaged over 10 ms (instead of 1 s) **C.** Calibration curves of a microbiosensor at three oxygen concentrations (N_2-saturation, air equilibrated and O_2-saturation) in the 0-50 µM D-serine concentration range. Except for the N_2-saturated medium, the oxidation current shows a linear dependence on the D-serine concentration. **D**. Calibration curves of the microbiosensor in the 0-

2.5 mM D-serine concentration range. The calibration was made in N_2-saturated, in air equilibrated and in O_2-saturated PBS. The relationship between the oxidation current and D-serine concentration fits with a classical Michaelis-Menten equation in which O_2 is not-limiting in catalysis.

Figure 3: pH and temperature dependence. A. pH-dependence of D-serine detection. pH was adjusted in 0.01 M PBS from 5.4 to 8.0 and 0.01 M borate from 8.3 to 11.5. Data are expressed as a percentage of the response at pH 8.0 (n=4). **B**. Temperature-dependence of the microbiosensor response (n=4). All measurements were performed in 10 µM D-serine, PBS 0.01M, pH 7.4.

Figure 4: Selectivity of the biosensor. Responses of the biosensor to physiological concentrations of different interfering molecules present in the CNS, without (**A**) and with (**B**) the PPD layer (n=4). The PPD layer dramatically decreased the sensitivity of the biosensor to interfering substances. **C**. Stability of the PPD layer. Electrochemical responses to 5-HT remained low for at least 6 hours of *in vitro* experiment at room temperature (n=4, measuring conditions: PBS 0.01 M, pH 7.4).

Figure 5: Detection of other amino acids. A. Detection of the D-isomers of the 20 standard amino acids, as well as glycine, L-serine and L-cysteine (10 µM). Data are expressed as a percentage of the response obtained with 10 µM D-serine (n=4). As expected from the biochemical properties of RgDAAO, the biosensor detected all D-amino acids that we tested, except D-aspartate and D-glutamate. Glycine and L-amino acids produced negligible responses.

B. Amounts of the most abundant D-amino acids in the mouse brain as reported by Hamase *et al*[53]. The predominant D-amino acid is D-serine (red), followed by D-aspartate (blue) and D-alanine (green).

Figure 6: D-serine detection in brain extracts. A. Example chromatogram showing separation between D-serine (elution time = 12 min) and L-serine (= 13 min). The D-serine peak completely disappeared after incubation with RgDAAO and catalase. **B**. Example of electrochemical responses recorded by the microbiosensor upon addition of a brain extract (40× dilution) and 3 µM D-serine. The response almost disappeared when the brain extract was preincubated with RgDAAO and catalase. **C**. Summary of the results of D-serine concentrations in brain extracts estimated by HPLC and the RgDAAO biosensor. Both techniques yielded comparable results, thus indicating an excellent selectivity for D-serine in brain extracts. * significant difference with D-serine levels following DAAO incubation ($p<0.05$). Data are expressed as mean ±SEM (n=6).

Figure 7: D-serine detection *in vivo*. Effect of an intraperitoneal injection of D-serine (1 g/kg body weight) on the electrochemical signal detected in the frontal cortex by a RgDAAO microbiosensor and a control (BSA) microbiosensor implanted in its vicinity (about 0.5 mm). Background currents were systematically higher at the RgDAAO microbiosensor compared to the control electrode, revealing a detectable basal extracellular D-serine concentration. Peripheral D-serine injection produced a specific increase in oxidation current at the RgDAAO microbiosensor, reflecting D-serine diffusion across the blood brain barrier. Data are expressed as mean (solid line) ±SEM (shading) of 6 experiments.

Table 1: Response of microbiosensors produced with pkDAAO or RgDAAO on different D-amino acids. Sensitivity to D-serine, D-aspartate and D-alanine were assessed for the two microbiosensors. RgDAAO microbiosensors show greater sensitivity and improved selectivity for D-serine compared to pkDAAO ones (n=4).

Figure 1

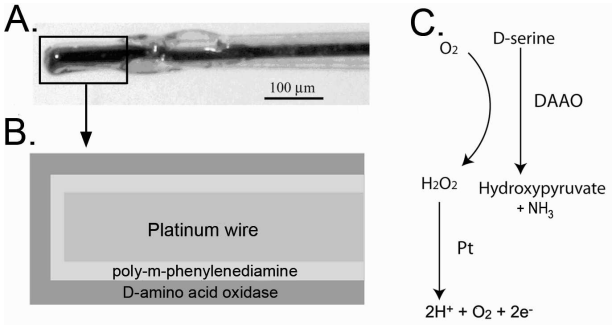

Figure 2

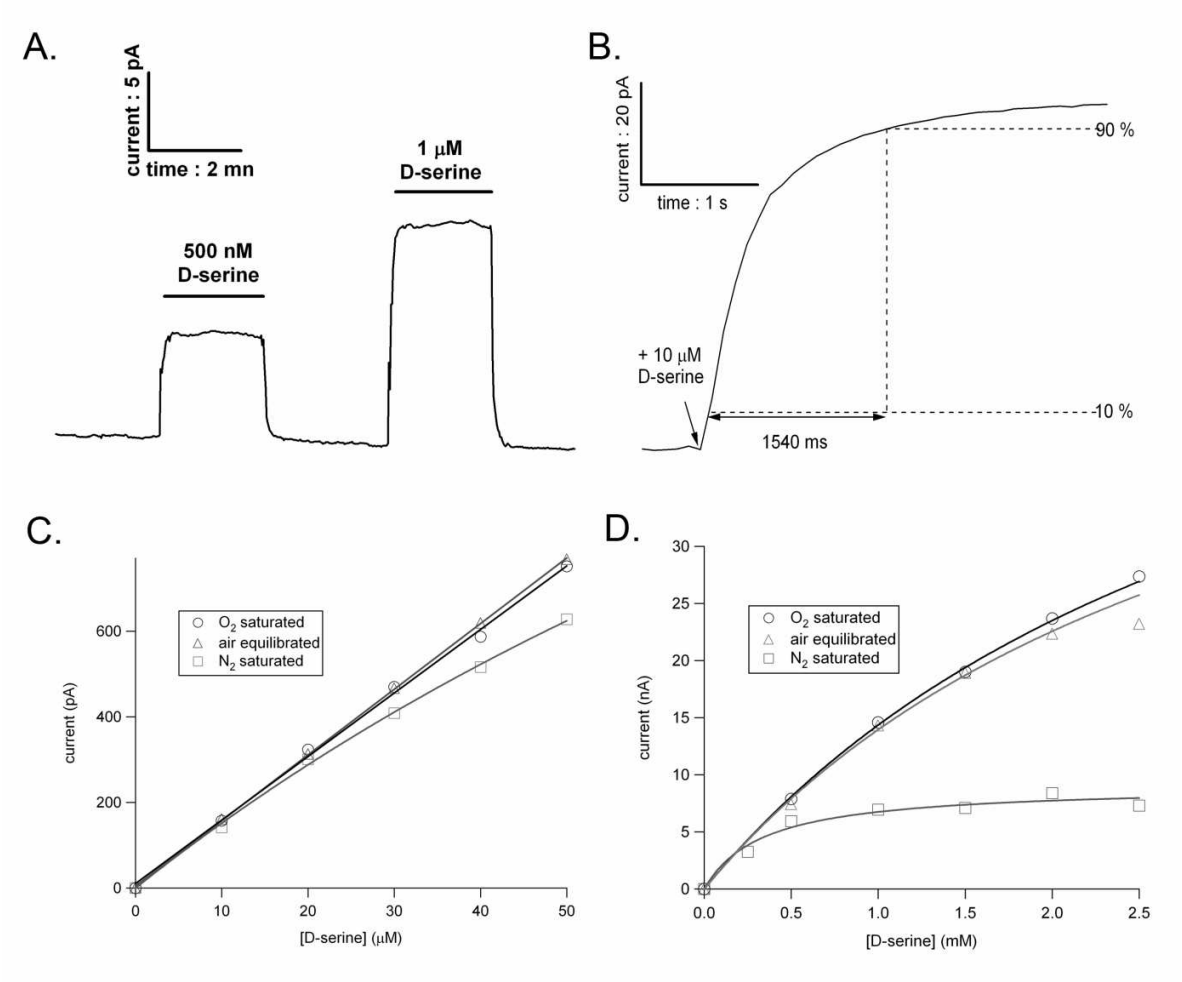

Figure 3

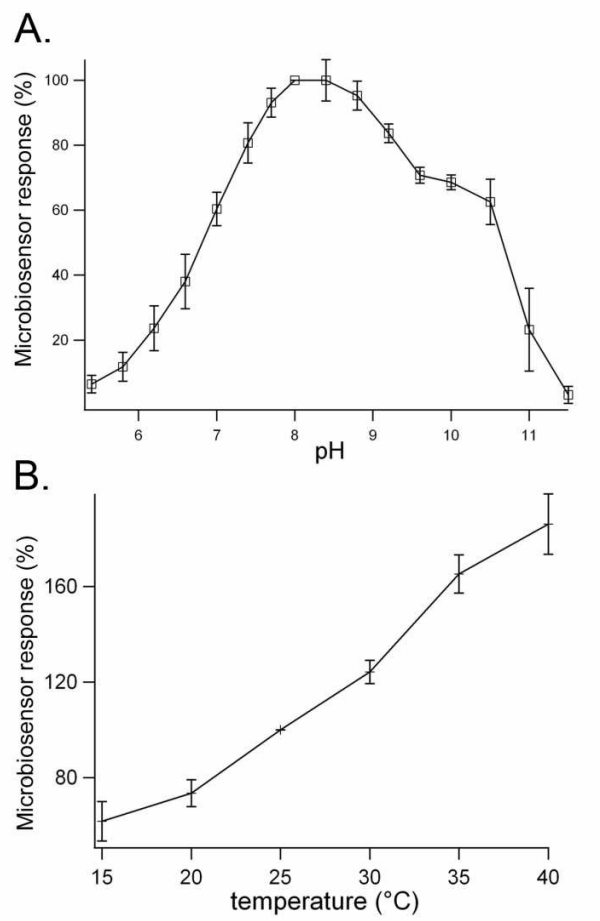

Figure 4

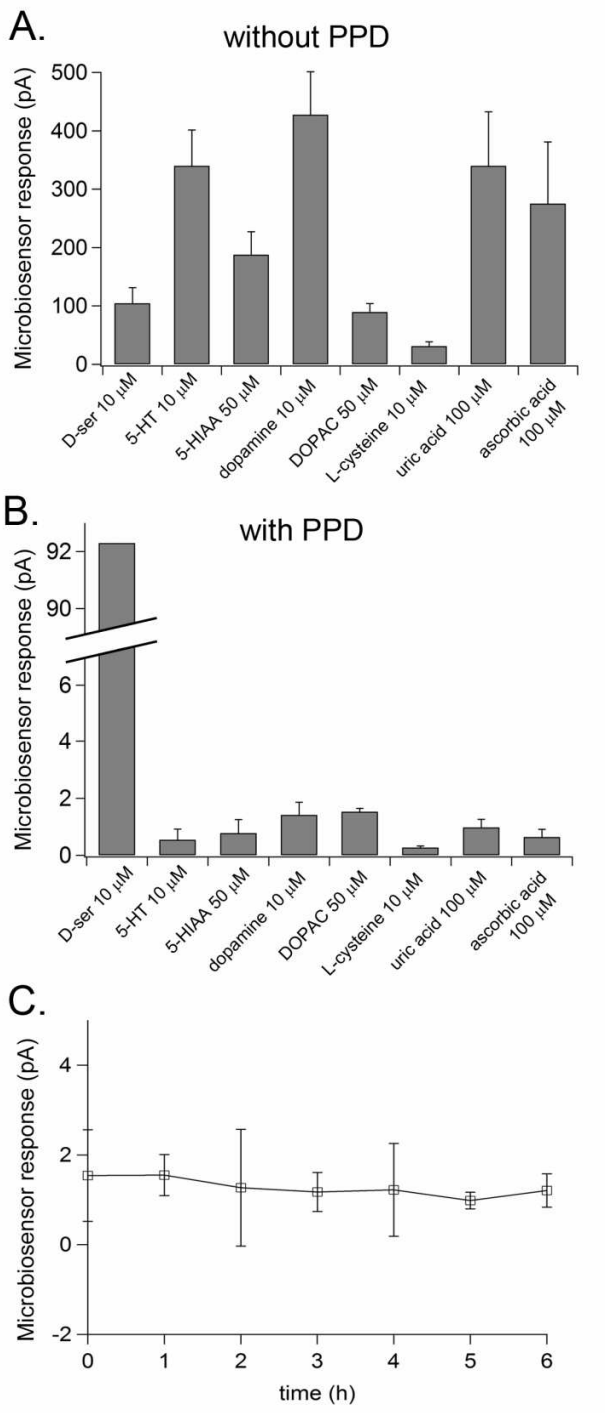

Figure 5

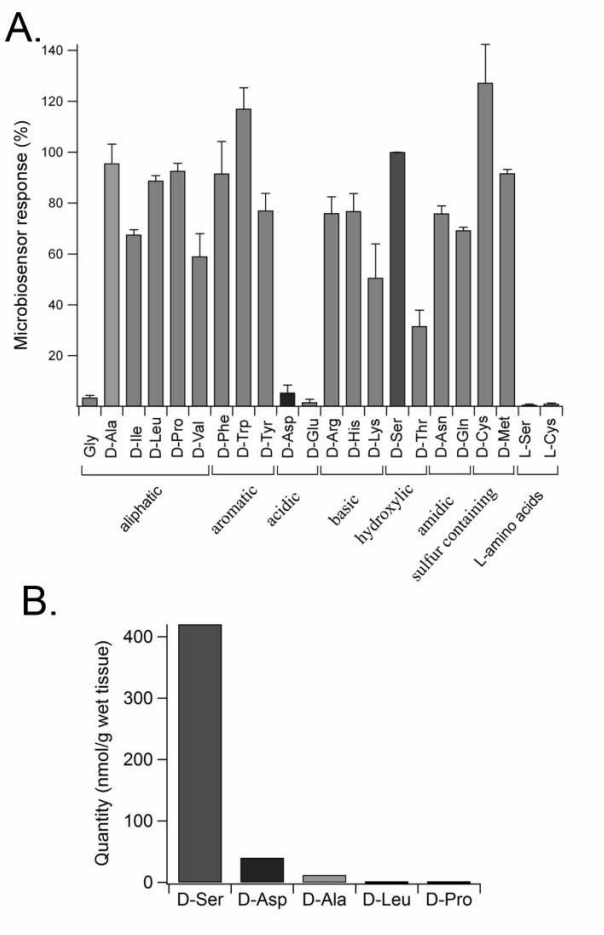

Figure 6

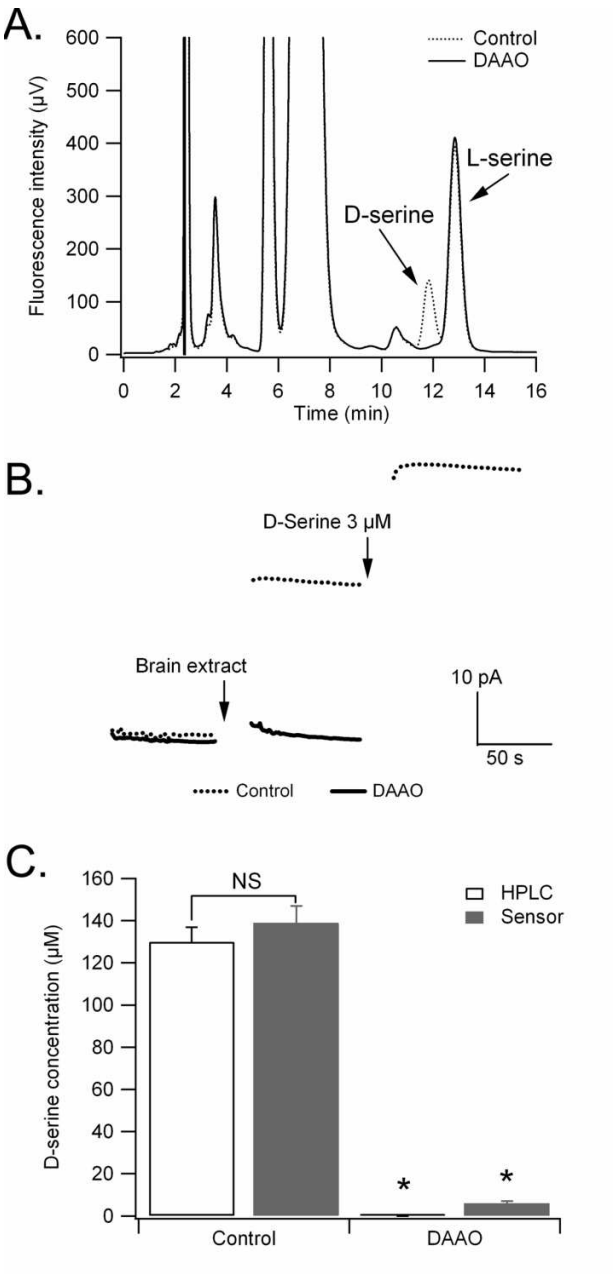

Figure 7

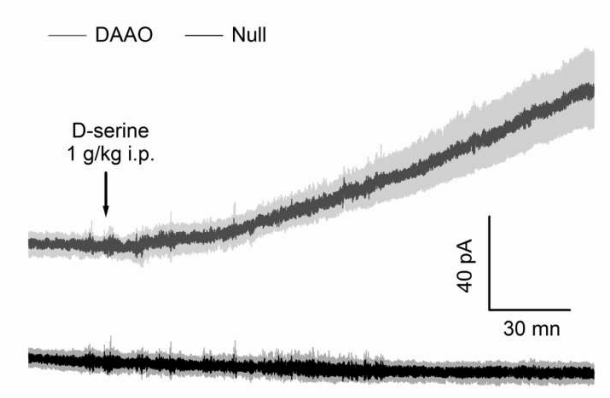

Table 1

Substrate	pKDAAO microbiosensor		RgDAAO microbiosensor	
	Sensitivity (pA/µM)	% of D-serine detection	Sensitivity (pA/µM)	% of D-serine detection
D-Ser	6.5 ± 2.0	100%	9.2 ± 3.4	100%
D-Asp	2.5 ± 0.8	38.6%	0.5 ± 0.2	5.5%
D-Ala	10.8 ± 2.9	165%	9.6 ± 1.4	104%