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INVITED REVIEW

Amperometric microbiosensor as an alternative tool for investigation of D-serine in brain

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Abstract This paper discusses the application of a reagentless, selective microbiosensor as a useful alternative tool for monitoring D-serine in neural samples. The main components of the 125-μm-diameter disk biosensor were D-amino acid oxidase for D-serine sensitivity (linear region slope, 61 ± 7 μA cm^{-2} mM^{-1}; limit of detection, 20 nM), and poly-phenylenediamine for rejection of electroactive interference. The response time of the biosensor was of the order of 1 s, ideal for 'real-time' monitoring, and detection of systemically administered D-serine in brain extracellular fluid is demonstrated. Exploitation of this probe might resolve queries involving regulation of D-serine in excitotoxicity, and modulation of *N*-methyl-D-aspartate receptor function by D-serine and glycine in the central nervous system.

Keywords D-Serine · Microbiosensor · D-Amino acid oxidase · Brain · Fixed potential amperometry

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Introduction

D-Serine has been investigated along many scientific lines, in part due to its high content in the cerebral cortex, hippocampus and striatum of rodent and human forebrains (Martineau et al. 2006), which had led to an increased understanding of D-serine neuromodulation over the past two decades (Hashimoto et al. 1992a, b). The existence of D-serine in the central nervous system (CNS) is significant not only because it is an unusual isomeric form of an amino acid, but more importantly because it fulfills the criteria of a ligand for the glycine binding site at *N*-methyl-D-aspartate receptors (NMDAr) involved in excitatory neurotransmission and cognitive function (Wolosker et al. 2002). Modulation of ligand binding to the glycine site of NMDAr seems to sustain long-term synaptic plasticity, which may serve as a cellular mechanism underlying learning and memory (Martineau et al. 2006). D-Serine thus modifies the function of NMDAr in transmission which further modulates neural development.

Such a role is particularly notable in the cerebellum. Here, the Bergmann glial cells direct the migration of granule cells by a mechanism which is dependent on glycine site occupancy at NMDAr. D-Serine is released from Bergmann glia and binds to the NMDAr of the granule cell leading to granule cell migration from the external granular layer to the internal granular layer (Schell et al. 1997; Kim et al. 2005). This atypical amino acid can serve as a gliotransmitter, modulating neurotransmission at glutamatergic synapses, and acting as a motility-promoting signal important for development and maturation of the CNS. Gliotransmisson is a process in which astrocytes are dynamic elements that influence synaptic transmission and synaptogenesis. The best known gliotransmitters are glutamate and ATP. D-Serine has been demonstrated to act as

a gliotransmitter in glutamatergic synapses, and its levels are physiologically linked to NMDAr distribution more closely than glutamate itself. This is due to the fact that glutamate does not have a biosynthetic apparatus that guarantees the selective localization of transmitter to the synapse and glutamate concentration does not parallel that of NMDAr (Oliet and Mothet 2009).

The distribution of D-serine is not only confined to the glial lineage but also has been initially observed in dendrites and axons of some cortical and brainstem neurons (Kato et al. 2011). Immunochemical staining with new antibodies and prolonged incubation showed the presence of significant amounts of D-serine in primary neuronal cultures and neurons from brain sections of young and adult species of Sprague–Dawley rats. Strong D-serine immunoreactivity is recovered in neuronal cell bodies (Miyoshi et al. 2011). The presence of D-serine in neurons might correlate with high serine racemase levels in these cells (Shoji et al. 2006; Foltyn et al. 2010; Ding et al. 2011). This means that the cellular distribution of D-serine in neurons of the CNS may be developmentally regulated. This view is supported by the high levels of D-serine detected in brain from birth to postnatal day 21 (Iizuka et al. 2011). On the other hand, D-serine levels are very low and mainly localized in neuronal cell bodies and dendrites in mature rats. Rosenberg et al. (Rosenberg et al. 2010) revealed that D-serine is derived from neurons in the cerebral cortex which contributes to the regulation of NMDAr transmission.

Thus, D-serine seems to fulfill some of the criteria defining functionality as a neurotransmitter, being distributed similarly to NMDAr and functioning as an endogenous ligand for its glycine site. In addition, D-serine is synthesized by serine racemase (Schell et al. 1997) and metabolized by D-amino acid oxidase (DAAO) (Nagata 1992; Hashimoto et al. 1993) acting as an inactivating hydrolytic enzyme. The existence of a biosynthetic pathway, a target receptor (Turpin et al. 2012), vesicular release (Mothet et al. 2005; Martineau et al. 2008), uptake system (Bauer et al. 2005; Rutter et al. 2007) and degradative enzymes supports the arguments that D-serine is indeed a neurotransmitter.

D-serine dysregulation might also contribute to NMDAr dysfunction that occurs in neuropsychiatric and neurodegenerative diseases (Hashimoto et al. 2004; Simonin et al. 2006; Thompson et al. 2012). The harmful effects of excessive D-serine will activate NMDAr and consequently result in massive calcium influx into the neuronal cell. NMDAr over-activation is the main factor in excitotoxicity which occurs following stroke and neurodegenerative diseases (Katsuki et al. 2007; Wolosker et al. 2008).

Excitotoxicity depends on NMDAr activation. Neurotoxicity induced by amyloid β-peptide (Aβ) is exacerbated by the release of D-serine. Neurotoxicity induced on primary hippocampal neurons by a primary microglia-conditioned medium treated with 15 μM Aβ (containing high levels of D-serine) was rescued when the medium was treated by DAAO and 5,7-dichlorokynurenic acid (DCKA), an antagonist of the glycine modulatory site of NMDAr. Further evidence that D-serine plays an essential role in Aβ-induced neurotoxicity originates from the observation that serine racemase knockout mice showed a 90 % decrease in forebrain D-serine content as well as a reduced neurotoxicity induced by NMDAr and Aβ peptide injection in the forebrain (Wu et al. 2007).

Serine racemase inhibitors might decrease NMDAr activation and therefore be used as a new strategy to prevent stroke damage and cell death in neurodegenerative diseases (Wolosker et al. 2008). The D-serine dysfunction in long-term potentiation (LTP) might also cause cognitive deficits. This is further supported by the findings that aged rats displayed low levels of hippocampal D-serine and serine racemase expression (Mothet et al. 2006). On the other hand, LTP in young rats is not enhanced by exogenous D-serine. Thus, LTP impairment observed in aged rats appeared to be caused by deficits in local D-serine synthesis.

A recent study reported that endogenous D-serine mediates motoneuron cell death by excessive stimulation of spinal cord NMDAr in amyotrophic lateral sclerosis (ALS). Thus, inhibitors of serine racemase may provide a new neuroprotective strategy against ALS. D-Serine administration has ameliorated the symptoms of schizophrenia when associated with conventional neuroleptics (Kantrowitz and Javitt 2010). Schizophrenic patients display a higher ratio of L-serine to D-serine in the blood and cerebrospinal fluid (Bendikov et al. 2007). Given this evidence for the important role of D-serine in the CNS, the use of a multifunctional device which includes a D-serine sensor will be beneficial in drug delivery or the invasive intensive care monitoring of both strokes and traumatic brain injury patients where microdialysis is being used at present (Kim et al. 2005).

Analysis of D-serine in extracellular fluid

D-Serine in the brain was first detected and measured accurately using high-performance liquid chromatography (HPLC) and gas chromatography (GC) (Hashimoto et al. 1992b, 1995). Brain microdialysates were perfused and analyzed with liquid chromatography mass spectrometry (LCMS) using Marfey's reagent, giving peak separation within 2 min. Hashimoto and co-workers also made a key contribution in separating serine enantiomers. They showed that D-serine is heterogeneously distributed throughout the rat brain with a pattern resembling that of the NMDA subtype of glutamate receptors. A capillary zone

electrophoresis method in determining serine enantiomers in rat cerebral tissue has also been reported with a limit of detection of 0.3 μM and D-serine peak at 40 min (Zhao et al. 2005). Capillary electrophoresis (CE) has many advantages such as higher separation efficiency and small sample volume. Microdialysis–CE-laser-induced fluorescence has been reported for the determination of D-serine in dialysate collected from rat striatum (Ciriacks and Bowser 2006). This method employed a microdialysis probe coupled with separation capillary and UV laser-induced fluorescence detection for sensitive and high-throughput analysis. The D/L-serine tagged with fluorescein-5-isothiocyanate (FITC), dansyl chloride, *o*-phthalaldialdehyde (OPA) and 3-(4-carboxybenzoyl)-2-quinoline-carboxalaldehyde were resolved by chiral CE. However, derivatization of D/L-serine with FITC or dansyl chloride tends to produce various by-products, which further complicate the subsequent separation. The fluorescence intensity of amino acids derivatization with OPA depends on the thiol used such as *N*-isobutyryl-L-cysteine (Miyoshi et al. 2009) and *N*-tert-butyloxycarbonyl-L-cysteine (Radzishevsky and Wolosker 2012). Stereoselectivity was then achieved either by pre-column treatment with DAAO (Oguri et al. 2005) in acidic aqueous medium or incorporating DAAO in postcolumn separation (Kato et al. 2011; Miyoshi et al. 2011). Although derivatization reaction and two-dimensional separation takes a longer analysis time, the methods give a low limit of detection for D-serine that is 0.025 nmol/g tissue or mL fluid for samples of low concentration of D-amino acids (Miyoshi et al. 2009, 2011). However, the retention peaks of D/L isomers are too close, making these analytical separation methods only suitable for monitoring endogenous D-serine synthesis and release from primary cultures due to higher level of L-glutamine and L-serine present in the cells and culture medium (Radzishevsky and Wolosker 2012). In addition, microdialysis coupled with various analytical techniques (HPLC and CE), suffers from dilution of perfused dialysates from the extracellular fluid (ECF). The choice of technique is restricted by its time resolution if correlation with in situ neurochemical concentrations in the brain is to be achieved.

D-Serine staining (Schell et al. 1997) using antibodies to highlight D-serine localization has revealed that D-serine is mostly associated with astrocytes that ensheathe synapses and is particularly enriched in gray matter. D-Serine immunoreactivity has also been observed also in dendrites and axons of cortical neurons (Kato et al. 2011). Although localization analysis, using polyclonal antibodies and histoimmunochemistry, offers great sensitivity to D-serine, it gives little information about the function of endogenous D-serine in brain.

Martineau et al. (2006) suggested that there remains much to be done to delineate the respective contributions of D-serine and glycine at glutamatergic synapses in physiological and pathological conditions. Therefore, development of new tools to visualize D-serine in vivo will help define the role of this agonist in regulating NMDA-receptor-dependent cell death, cell survival and physiological pathways. Electrophysiological analysis detected an increment in NMDA current after being diminished by DAAO, by application of exogenous D-serine (Turpin et al. 2012). Biosensor technologies have been refined progressively and have been used to analyze chemical signaling in the brain (Sasabe et al. 2012). This is due to the fact that biosensors offer the possibility of monitoring rapid and transient changes in extracellular neurochemical concentrations.

The primary reason for developing biosensors for in vivo application is the ability to measure biologically important compounds that are either electroinactive or only poorly electroactive at specific potentials, such as glucose, lactate, glutamate as well as D-serine. Biosensors provide selectivity, rapid temporal response and excellent sensitivity, making these probes an interesting choice in the study of transient neurochemical changes (Sasabe et al. 2012).

D-Serine implantable microbiosensor

Evaluating D-serine only in terms of localization does not infer its function. The ECF baseline concentration of D-serine is very much less than those of glucose and lactate, making in vivo detection of D-serine more challenging. Although the HPLC and CE methods are sensitive and accurate, they have poor time resolution (~2 min) (O'Brien et al. 2005) which are unsuitable in neurotransmission studies.

The determination of D-serine in rat cortex by incorporating D-amino acid oxidase (DAAO) from recombinant *R. gracilis* has been reported (Singh et al. 2012). Such a device is based on stereospecific oxidative deamination of neutral D-serine, catalyzed by DAAO to the corresponding imino acid, which is the rate-limiting step of the overall reaction. The reduced flavin adenine dinucleotide ($FADH_2$) produced is oxidized by oxygen to generate hydrogen peroxide prior to imino acid dissociation from the enzyme-active center producing α-keto-3-hydroxypropanoic acid and ammonium ions through a non-enzymatic reaction (Pilone 2000). Although the presence of DAAO provides enantioselectivity toward D-serine, implantable biosensors must also have excellent interference rejection capacity to be useful in in vivo applications (O'Neill et al. 2008). This selectivity can be achieved by incorporation of a permselective membrane in the biosensor design such as cellulose acetate, Nafion (Naf) and electrosynthesized polymeric matrices, e.g., polypyrrole and polyphenylenediamine.

As previously reported (Killoran and O'Neill 2008), poly-*ortho*-phenylenediamine (PPD) is the best phenylenediamine isomer being used in the design of a permselective layer for micro implantable Pt/Ir based biosensors. This isomer is superior in blocking ascorbic acid (AA) compared to the *meta* isomer (Schuvailo et al. 2006) for AA levels greater than 200 μM. Furthermore, stability studies indicated that the permselectivity of PPD formed from *meta* isomers degraded rapidly (Killoran and O'Neill 2008), supporting the choice of using the *ortho* isomer in brain implantable sensors. Glutaraldehyde (GA) has been applied on the PPD surface as an amine-reactive cross-linker. In a comparative study of different enzyme and polymer configurations (Zain et al. 2010), the Pt_D/PPD/GA/DAAO design not only gave the best linear region slope (LRS, i.e., analyte sensitivity) on account of its high J_{max} (good enzyme loading) and moderate value of K_M (high enzyme substrate affinity) on account of the relationship: LRS = J_{max}/K_M (O'Neill et al. 2008), but it also achieved a relatively good biosensor efficiency; see below for a full discussion of these parameters.

Enzyme kinetics and current response

Enzymes are complex molecular machines that operate through a great diversity of mechanisms. Kinetic measurements of enzymatically catalyzed reactions are among the most powerful techniques for elucidating their catalytic mechanisms. The generalized reaction for an enzyme (E) producing a product (P) involving both substrate (S) and co-substrate (O_2) is summarized as follows:

$$S + E/FAD \rightarrow P + E/FADH_2, \quad (1)$$

$$E/FADH_2 + O_2 \rightarrow E/FAD + H_2O_2, \quad (2)$$

$$H_2O_2 \rightarrow O_2 + 2H^+ + 2e^-. \quad (3)$$

The two-substrate form of the Michaelis–Menten equation for the overall rate of reaction, υ, is given by (O'Neill et al. 2008):

$$\upsilon = \frac{\upsilon_{max}}{1 + \frac{K_M(S)}{[S]} + \frac{K_M(O_2)}{[O_2]}}. \quad (4)$$

If the concentration of the co-substrate is large and constant, Eq. (4) can be simplified to:

$$\upsilon = \frac{\upsilon'_{max}}{1 + \frac{K_M(S)}{[S]}}. \quad (5)$$

Equation 5 represents the rate of product formation, which in this case reflects the rate of H_2O_2 generation. The H_2O_2 generated in the enzyme layer is species detected at the electrode surface, but its bulk concentration is zero; it is the flux of H_2O_2 to the surface that determines the current. Converting the rate into current can be achieved by multiplying both sides by $\alpha z F$ (Area), where α is the fraction of the flux that is oxidized on the Pt surface (some H_2O_2 is lost to the bulk), z is the number of electrons involve in the oxidation and F is the Faraday constant. Consistent with this analysis, the biosensor current responds hyperbolically to the bulk concentration of enzyme substrate, and υ'_{max} is related to I_{max} (maximum current) through the I_s term (Eq. 6). The value of K_M, the concentration of substrate that yields ½ υ_{max}, does not change with this conversion. Biosensor responses (I_S), can be converted to current density (J_S) and plotted against substrate concentration [S].

$$I_S = \frac{I_{max}}{1 + \frac{K_M(S)}{[S]}}, \quad (6)$$

$$J_S = \frac{J_{max}}{1 + \frac{K_M(S)}{[S]}}. \quad (7)$$

J_S is the current density for the biosensor current response per unit area and it measures the overall rate of enzyme reaction (Eq. 7). J_{max} is the J_s value at enzyme substrate saturation. Different values of J_{max}, determined under the same conditions, reflect differences in the activity of enzyme on the surface (k_2 [E]; see Reaction 8).

The Michaelis constant, K_M, is defined in terms of the rate constant for the generalized reactions describing the conversion of substrate (S) to product (P), catalyzed by enzyme (E); see Eq. 9. When Eq. 7 is used to approximate the two-substrate case, however, the K_M is more complex, containing co-substrate terms. K_M is then the apparent Michaelis constant and defines the concentration of substrate that gives half the J_{max} value. Hence, changes in K_M are sensitive to the binding constant, k_1, and have been interpreted in terms of barriers to enzyme substrate binding (O'Neill et al. 2008), as well as changes in oxygen demand (McMahon et al. 2007).

The overall reaction between the enzyme-active site and its substrate forms a complex with the enzyme that subsequently breaks up to products and enzyme:

$$E + S \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} ES \xrightarrow{k_2} E + P, \quad (8)$$

$$K_M = \frac{k_{-1} + k_2}{k_1} \quad (9)$$

where, E, S, ES and P are enzyme, substrate, enzyme substrate complex and products, respectively. More specifically, E refers to the enzyme-active site rather than enzyme molecules. Rate constants are represented by k_1,

Table 1 Comparison of microbiosensors for D-serine detection in vivo

	Pernot et al. (2008), Thompson et al. (2012)	Zain et al. (2010)
Electrode geometry	Pt fiber	Pt disk
Anion barrier	*m*-Phenylenediamine and Nafion	*o*-Phenylenediamine and Nafion
In vitro LOD	16 nM	20 ± 0.07 nM
Response time	2 s	<1 s
Sensitivity	9.2 ± 3.4 pA mM^{-1}	61 ± 7 μA cm^{-2} mM^{-1}
DAAO origin	Yeast of *Rhodotorula gracilis*	Porcine kidney

k_{-1} and k_2. According to this model, when the substrate concentration is at a value where all enzyme-active sites have been completely converted to the ES form, the second step of the reaction becomes rate limiting and the overall reaction rate becomes insensitive to further increases in substrate concentration (O'Neill et al. 2008). The apparent Michaelis constant for S is also useful for defining the linear range of response to S (up to ½ K_M), as well as the slope in the linear region, i.e., LRS ≈ J_{max}/K_M; see Eq. 10.

$$\underset{\mathrm{Lt}[S]\to 0}{J_s} = \frac{J_{max}}{1+\frac{K_M}{[S]}} \approx \frac{J_{max}}{K_M}[S] \tag{10}$$

Several DAAO-based biosensors have been developed for D-amino acid determinations (Arai et al. 1998; Sarkar 2000; Dominguez et al. 2001; Wu et al. 2004; Sacchi et al. 2012) with relatively high limit of detection (25 μM–0.03 mM).

The constant potential amperometry (CPA) technique has been applied to enable continuous D-serine monitoring in the brain (Pernot et al. 2008; Zain et al. 2010; Thompson et al. 2012) as summarized in Table 1. The measured current obtained provides a continuous analog record of D-serine concentration in the vicinity of the microbiosensor. A higher response time (>1 s) was observed for the cylinder-based biosensors and might be due to excessive electrode treatment with Nafion® (Machado et al. 2008). These probes are able to detect D-serine due to high concentrations in the cerebral cortex, hippocampus and striatum of rodent and human forebrain.

D-aspartate and D-alanine are other D-amino acids prevalent in CNS, but D-serine levels in brain ECF are about two orders of magnitude higher than those of D-alanine (Morikawa et al. 2001; Wolosker et al. 2002).

Biosensor application in D-serine detection in vivo

Given the success of the in vitro characterization described (Zain et al. 2010) for the design Pt_D/PPD/Naf/GA/DAAO, this and other configurations were implanted in rat brain striatum, at coordinates (skull level between lambda and bregma): A/P +1 from bregma, M/L +1.3 and D/V −4 (from dura) positioned by a stereotaxic frame. Mean baseline currents taken from 0 to 30 min at 40 Hz data sampling rate for (1) bare Pt_D, (2) Pt_D/PPD and (3) Pt_D/PPD/Naf/GA/DAAO were: 1.30 ± 0.08, −0.10 ± 0.04 and 0.35 ± 0.05 nA ($n = 6$), respectively. A current response was obtained following microinjection of D-serine next to the implanted Pt_D/PPD/Naf/GA/DAAO biosensors in order to validate that the DAAO remained immobilized and functional on the biosensor surface. Both the high concentrations of infused D-serine needed and the current collapse following the infusion were associated with diffusion and ECF tissue uptake of substrate. Such signals were not observed when a "blank" sensor (same design as Pt_D/PPD/Naf/GA/DAAO, but omitting the enzyme: Pt_D/PPD/Naf/GA) was implanted ($n = 6$) (Zain et al. 2010).

The use of a differential measurement between the biosensor and electrode devoid DAAO, which is electrically and physically equivalent (i.e., similar electrochemical environment, size, PPD thickness, etc.), clearly indicates that those signals produced are from the bioreaction between DAAO and D-serine. This self-referencing method allows the removal of all nonspecific currents, especially from AA and other electrooxidative neurochemicals in the ECF.

D-Serine in vivo signals need to be verified in terms of extracellular changes in D-serine concentration in brain. This signal identification is important as currents can arise from various sources. Changes in tissue impedance by electrolysis, double-layer charging and heterogeneity of ECF complex environment can contribute to the electrochemical responses (O'Neill et al. 1983). Using constant potential amperometry to validate the response (current), the biosensors were alternately changed and held for 30 min at constant potential (0 mV) at which H_2O_2 does not oxidize. The zero potential is insufficient to electrolyze H_2O_2 at this regime; so the current resulting from DAAO-D-serine bioreaction was abolished at 0 mV.

A 0.27 μmol/g free D-serine concentration of wet weight tissue of telencephalon, diencephalon and midbrain was first reported (Hashimoto et al. 1992b) and high content of D-serine (0.40 μmol/g) was further discovered in the forebrain (Morikawa et al. 2001). An exact concentration value of extracellular D-serine is still in question because of the uncertainty of D-serine synaptic production and uptake in extracellular fluid correlation to synaptic concentrations

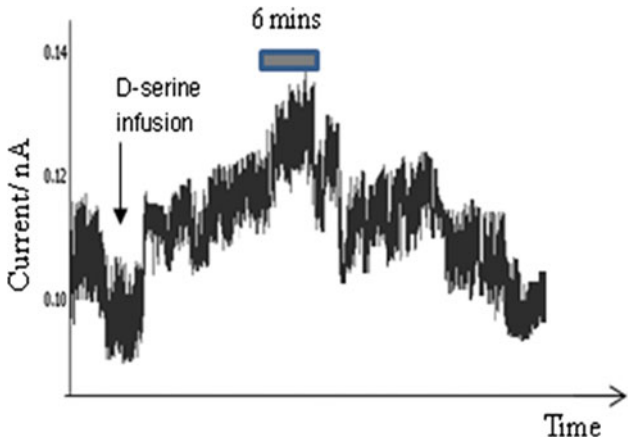

Fig. 1 Time course of changes in concentration of D-serine (observed as changes in baseline-subtracted biosensor current response) in forebrain using Pt_D/PPD/Naf/GA/DAAO implanted at A/P +1 from bregma, M/L +1.3 and D/V −4 (from dura) and poised at a fixed potential of +700 mV versus Ag/AgCl. The *bar* highlights the maximum current reading over a 6-min period (0.133 ± 0.005 nA) after intravenous administration of 10 mmol/kg of D-serine in Sprague–Dawley rats, $n = 3$

and not to total tissue concentration (Nagata et al. 1994; Ciriacks and Bowser 2006).

Systemic administration of D-serine

To obtain further insights into the distribution and metabolism of exogenous D-serine, the effect of intravenous (i.v.) administration of D-serine (10 mmol/kg) on the biosensor signal of D-serine in the forebrain of adult rats was studied. As shown in Fig. 1, the administration produced a significant augmentation of D-serine levels at the implantation site (A/P +1 from bregma, M/L +1.3 and D/V −4 from dura) 12 min after D-serine i.v. administration. The gradual current rise from 97 ± 3 pA at $t = 35$ min reached a maximum current value of 133 ± 6 pA, from $t = 47$ to $t = 53$ min. Approximately 30 min after D-serine i.v. administration, the elevated current declined to baseline values. This may be due to the fact that D-serine had been metabolized by endogenous DAAO, or taken up into the intracellular compartment. Histology of brain slices were performed with thionine to aid visualization of the electrode-induced lesion in the brain (not shown), but probes of these dimensions do not cause a major gliotic reaction of the tissue (Duff and O'Neill 1994). Blood was eventually absorbed on the biosensor which contributed to the fouling effect of the biosensor in denaturing DAAO after continuous 8 h of in vivo usage.

The major strength of the probe is its ability to monitor chemical dynamics in real time. In addition to being reagentless, the biosensor has also shown in vitro signal increase between 10 and 90 % in less than 1 s, i.e., a fast response time. This criterion is important in monitoring chemical activity in brain as compared to other techniques for brain D-serine analysis with longer analysis time (Grant et al. 2006; Schuvailo et al. 2006; Tanaka et al. 2007).

Conclusion

Studies have demonstrated high temporal resolution microbiosensor detection for D-serine in the brain in vivo. The time resolution of the technique provides an unprecedented ability to monitor D-serine in real time within a behavioral context related to D-serine release and uptake. This assay involved no sample pretreatment and direct measurement was done in real time of the chemical events in the brain.

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