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The selective serotonin reuptake inhibitor fluoxetine reduces striatal in vivo levels of voltammetric nitric oxide (NO): A feature of its antidepressant activity?

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ABSTRACT

Voltammetric (electrochemical) methodologies such as differential pulse voltammetry and amperometry used together with electrically and chemically treated carbon fibre micro-electrodes (mCFE) allow selective monitoring of nitric oxide (NO). Preliminary in vitro studies have shown that the selective serotonin reuptake inhibitor (SSRI) antidepressant paroxetine inhibits constitutive nitric oxide synthase (cNOS) activity in animals and humans and that another SSRI such as fluoxetine reduced NO release in the media of synovial cells. The aim of this work was to verify by means of amperometry and specifically treated mCFE the capability of fluoxetine to alter the in vivo release of central NO, in the attempt to further clarify such putative antidepressant mechanism of action of this SSRI compound. The in vivo results support the chemical NO related nature of the endogenous amperometric signal evoked by NMDA injection in the striatum of anaesthetised rats, as pre-treatment with NOS inhibitor L-NAME prevented its appearance. Subsequently fluoxetine treatment resulted in decreased striatal NO, further supporting in vitro studies proposing a link between the serotonergic system and the NO system.

Nitric oxide (NO) is a highly reactive free radical and an important biological intra-cellular messenger either in periphery or in the central nervous system.

NO is produced by nitric oxide synthase (NOS) during the conversion of L-arginine (NOS substrate) to citrulline. In the brain, NO is a reactive volume transmitter produced in response to neuronal activation [6], mainly by increased activity of glutamate *N*-methyl-D-aspartate (NMDA) receptor [17].

NO has short half-life (0.2–120 s [9,15,25,30]) that makes its direct measurement in biological models very difficult also because of its low concentration (<0.1 μmol/L) and fleeting existence: the shortest half-life of NO measured in perfusion cascades is 3–6 s [4]. In addition, NO reacts extremely fast with superoxide and other active free radicals and slower with molecular oxygen [2].

Thus, most of the techniques for assaying NO use indirect methods relying on measurements of secondary species such as nitrites, nitrates or L-citrulline, an endogenous co-product [1]. These indirect not real time methods suffer from poor sensitivity and selectivity [24].

More recently electrochemical methods such as microdialysis or voltammetry have been introduced. They are based upon the feasibility of monitoring NO release following stimulation of NMDA receptors. For instance, in microdialysis studies, the NMDA-induced NO release was assessed indirectly by measuring the accumulation of its chemically stable secondary products: nitrites and nitrates in perfusate samples [21]. Clearly, this prevents the detection of changes of NO release occurring within a time scale of milliseconds–seconds [3,19].

On the other hand, voltammetric methods allow sufficient time resolution to detect real time NO release. Indeed, by using amperometry, we were able to perform measurements of NO in time intervals of fraction of seconds at a selective oxidation potential when used together with specifically treated mCFE then avoiding measuring the accumulation of stable metabolites (nitrites, nitrates). With such methodology we demonstrated the induced NO formation in vitro (ex vivo) when adding in the lumen of rodent aortic ring NO releasers such as NMDA, Substance P or vaso-active compounds such as lacidipine or other dihydropyridines [10,13]. Furthermore we have pharmacologically verified the chemical nature of the oxidation signal related to NO and monitored in vivo in the rat striatum using NO releasers or NOS inhibitors as well as NMDA receptor antagonists [9,12].

NO involvement in cellular memory processes and synaptic plasticity makes it an ideal regulator of short and long term adaptive changes associated with mood regulation [19] and evidence for its involvement in schizophrenia [31] and anxiety disorders [27] have been recently introduced. In particular, in vitro studies have shown that SSRI antidepressant paroxetine inhibits constitutive NOS activity in animals and humans. In addition, it was reported

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that fluoxetine reduced NO release in the media of synovial cells stimulated by interleukin-1 alpha plus tumor necrosis factor alpha [32]. Successively, Luo and Tan [22] have shown that chronic mild stress (CMS) physically deforms neurons in the rat hippocampal formation and that fluoxetine can renormalize those neurons via the inhibition of NOS over-expression, otherwise origin of an over-production of NO, which lead to the morphological abnormality in such rat model (CMS) of human depression. This finding has been recently supported by observation that nNOS over-expression is essential for chronic stress-induced depression and that exposition to CMS resulted in behavioral changes typical of depression i.e. behavioral despair [34].

The aim of the present in vivo work was to study the capability of fluoxetine to alter NMDA-stimulated release of NO monitored in the striatum of anaesthetised rat, by means of in vivo voltammetry and specifically treated mCFE [9,12,25]. The attempt is to analyse the involvement of central NO within the antidepressant mechanism(s) of action of such SSRI compound.

Adult male rats (250 g) supplied by Charles-River (Italy) were kept in temperature and humidity-controlled rooms (22 °C, 50%) with lights on from 0700 to 1900 h with water and food available *ad libitum*. Amperometric analyses were carried on in the striatum of anaesthetised rats (urethane, 1.2 g/kg i.p.) held in a Kopf stereotaxic frame throughout the experiments. All procedures were carried out according to the Italian law (Legislative Decree no.116, 27 January 1992), and the European Directive 86/609/EEC, fully compliant with GlaxoSmithKline policy on the care and use of laboratory animal and codes of practice. All efforts were made to minimize the number of animals used and their suffering.

NO, sodium nitrite (Carlo Erba, Milan, Italy), NMDA, L-N^G-nitro-arginine methyl esther (L-NAME) (all from Tocris, Cambridge, UK) were dissolved in aCSF and injected locally into the rat striatum (volume: 1 μL during 30 s).

Voltammetry was applied by means of a μAutolab polarograph (EcoChemie, The Netherlands) linked to an IBM PC computer equipped with a General-Purpose Electrochemical System Software (GPES) package [8]. Measurements were performed with a three-electrode potentiostat system made of a electrochemically coated in HCl 0.1 M silver wire (Clark Instruments, diameter 100 μm) to obtain a silver/silver chloride (Ag/AgCl) reference electrode, a silver wire auxiliary (counter, diameter 100 μm) electrode and a 30 μm diameter mCFE (working electrode) constructed and treated as described earlier [7,12,25]. Briefly, at first the active tip of the mCFE was electrically treated i.e. it was submitted to three consecutive 20-s periods of electrical pulse (70 Hz; 0–3 V, 0–2.5 V and 0–1.5 V, respectively. Then the tip of the mCFE was coated with nafion [11] and electro-polymerized with ortho-phenylenediamine (Nafion-oPD mCFE) in order to selectively monitor NO [16]. Indeed, such treatment affects the active tip (500 μm length) of the 30 μm diameter carbon fibre [26] that protrudes from the end of the glass pipette and was confirmed as having increasing sensitivity, selectivity and reliability of the micro-sensor to selective detection of NO when using DPV as well as amperometry [9,12,25].

Subsequently, amperometric parameters were selected to achieve real time selective measurement of NO oxidation current as described [9,12]. In particular, here the use of short range differential amperometry applied within +500 and +550 mV as mentioned above and described earlier [23] helps avoiding detection of ascorbic acid, dopamine, serotonin, their metabolites uric acid, i.e. the compounds monitored with DPV at oxidation potentials between −100 mV and +450 mV [7]. In fact, it measures a differentiated current due to NO oxidation as the contribution of other oxidizable compounds was eliminated by the differentiation provided that the difference between their oxidation potentials and the NO were sufficient (that is more than 100 mV) [23].

Furthermore, in vivo oxidation of neuropeptides has been described at +700 mV [7], thus also such compounds are not detected in the present electrochemical configuration.

This amperometric method is accurate in evaluating the amount of species that can be oxidised and it is quite in "real time" as the corresponding current level can be measured in time intervals of 0.1 s or less. Then, following DPV in vitro calibration to determine the specific oxidation potential of the compounds tested as described [9,12,25] selective amperometric analyses of NO or nitrites were performed in the striatum of anaesthetised rats.

For in vivo studies the reference and auxiliary electrodes prepared from silver wire (100 μm diameter) as described above, were inserted between the bone and the dura mater through holes (200 μm diameter) that were drilled in the parietal bone of the rat head. The mCFE was positioned in the striatum under stereomicroscopy, with coordinates: AP, 1.2; ML, 2.5; DV, 4.5 mm from bregma. All electrodes were connected with electrical wires to the polarograph.

A Hamilton needle (diameter 100 μm) for local injection was also positioned in the striatum approximately 250 μm apart of mCFE. Amperometric scans were then performed, each scan lasting at least 200 s, in six groups of anesthetised rats ($n = 5$ each):

Group 1: received vehicle (aCSF, 1 μL/30 s) 10 min before the local intrastriatal injection of NMDA (1 μL, 100 μM);
Group 2: received L-NAME (1 μL, 100 μM) 10 min before the local intrastriatal injection of NMDA (1 μL, 100 μM);
Group 3: received a systemic treatment with vehicle (aCSF, 600 μL i.p.) 90 min before the local injection of the solvent of NMDA: aCSF (1 μL/30 s). 15 min later the NOS inhibitor L-NAME was also locally injected (1 μL, 100 μM).
Group 4: received vehicle systemically (aCSF, 600 μL i.p.) 90 min before local intrastriatal NMDA challenge (1 μL, 100 μM).
Group 5: received fluoxetine systemically (20 mg/kg i.p.) 90 min before local intrastriatal NMDA challenge (1 μL, 100 μM).
Group 6: received two local intrastriatal NMDA challenges (1 μL, 100 μM) 90 min apart.

At the end of each experiment, local intrastriatal injection of 1 μL/100 μM sodium nitrite followed approximately 5 min later by local injection of exogenous NO (approximately 0.3 μM) both dissolved in aCSF were performed as control for the proper functioning of the mCFE system, as described previously [9,12].

The anatomical localization of the probes was performed at end of each experiment as described [8].

The statistics were calculated from the raw data using ANOVA with STATISTICA software version 6.0. Raw values are peak heights expressed in nanoAmperes (nA). Bonferroni multiple comparison test was used to calculate the statistical significance of differences between groups. Statistical significance was set at $p < 0.05$.

Injection of 1 μL/100 μM NMDA in the striatum of the anaesthetised animal generated a transient and sharp oxidation signal (2.81 ± 0.32 nA over baseline, mean ± SEM, $n = 20$) (see as example Fig. 1 trace a). The NMDA-induced NO signal appeared about 10–15 s after the injection and lasted about 20–25 s. In contrast, intrastriatal injection of vehicle (i.e. aCFS) 10 min before local NMDA application, did not affect the current baseline (see for instance trace c in Fig. 1). Additionally, two successive NMDA injections made 90 min apart were followed by similar oxidation signals (see as example Fig. 3). The time interval of 90 min between NMDA injections was chosen to avoid possible desensitisation processes of the response by repeated NMDA applications [12].

In other rats (group 2) intrastriatal application of the non-selective NOS inhibitor L-NAME (1 μL, 100 μM 10 min beforehand markedly and significantly ($p < 0.05$) reduced the NMDA-stimulated NO release to 0.60 ± 0.48 nA (mean ± SEM) as

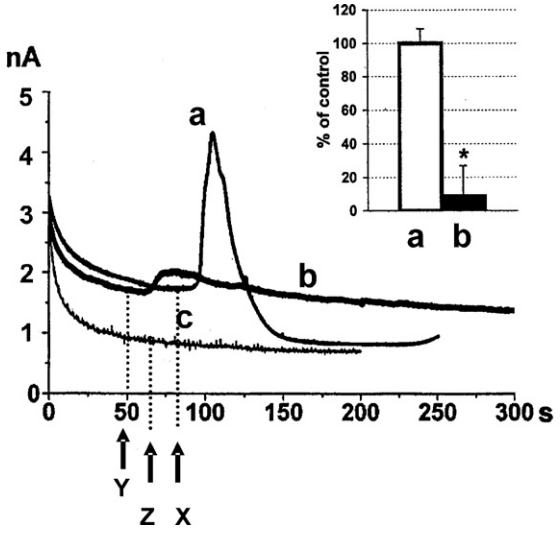

Fig. 1. Trace a: obtained in one rat (i.e. of group 1) receiving focal application to the striatum of vehicle (aCSF, 1 μL/30 s) 600 s before the local injection of μL of 100 μM NMDA (arrow X); trace b: obtained in one rat (of group 2) receiving focal application to the striatum of L-NAME (1 μL, 100 μM) 600 s before the local injection of NMDA (1 μL, 100 μM, arrow Y); and trace c: obtained in one rat (of group 3) receiving focal application to the striatum of vehicle (1 μL aCSF, arrow Z) 5400 s after systemic treatment with vehicle (600 μL aCSF i.p.). A second intrastriatal application was performed with L-NAME (1 μL, 100 μM) 900 s after the first focal injection with aCSF (not shown). Trace c is very similar in shape to the representative evolution in time of the signal when no treatments are performed. It appears that after 50 s recordings and until 300 s (and more) the digression of baseline current is inferior to 0.5 nA, therefore we consider that as stable baseline. In all traces only the ascending side of the signal was considered and measured. Representative amperometric striatal traces obtained in single animals. Inset: histograms show the mean ± SEM of the data (peak height) collected in the various groups of rats. Data were averaged and were expressed in percent of the averaged NO levels measured in nA. Asterisks: $p < 0.05$ as determined by one-way ANOVA and Bonferroni' post hoc multiple comparison test.

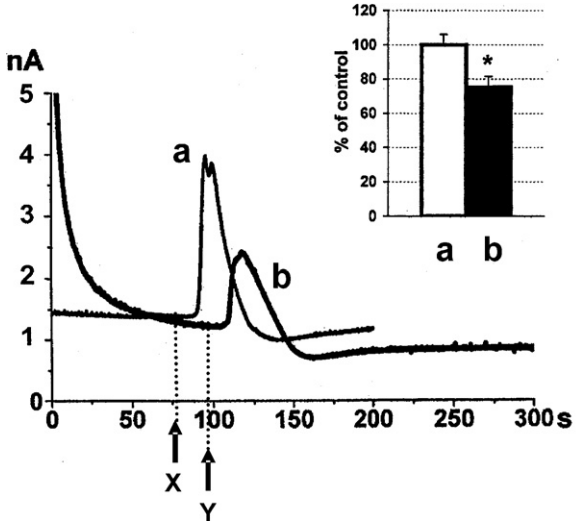

Fig. 2. Trace a: obtained in one rat (of group 4) receiving intrastriatal application of NMDA (1 μL, 100 μM, arrow X) 5400 s after systemic treatment with vehicle (600 μL aCSF i.p.); and trace b: obtained in one rat (of group 5) receiving fluoxetine (20 mg/kg i.p.) 5400 s before intrastriatal NMDA challenge (1 μL, 100 μM, arrow Y). Representative amperometric striatal traces obtained in single animals. Inset: histograms show the mean ± SEM of the data (peak height) collected in the various groups of rats. Data were averaged and were expressed in percent of the averaged NO levels measured in nA. Asterisks: $p < 0.05$ as determined by one-way ANOVA and Bonferroni' post hoc multiple comparison test.

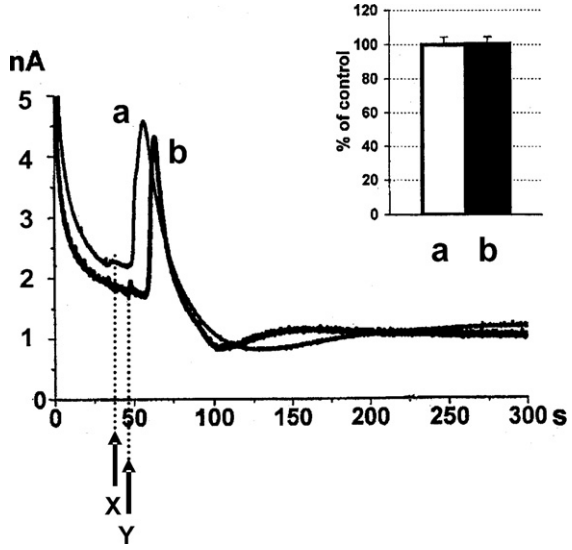

Fig. 3. Traces obtained in one rat (of group 6) receiving two successive NMDA intrastriatal injections made 5400 s apart (arrow X and Y, respectively). Representative amperometric striatal traces obtained in single animals. Inset: histograms show the mean ± SEM of the data (peak height) collected in the various groups of rats. Data were averaged and were expressed in percent of the averaged NO levels measured in nA. Asterisks: $p < 0.05$ as determined by one-way ANOVA and Bonferroni' post hoc multiple comparison test.

shown in Fig. 1 (see as example trace b). On the other hand, in the rats of group 3 intrastriatal addition of such NOS inhibitor had no significant effect on baseline oxidation current (not shown).

Data gathered in another group of animals (group 5) showed that fluoxetine given 90 min before local treatment with NMDA to stimulate NO release was responsible for a significant ($p < 0.05$) reduction of the amperometric NO related signal. The reduction reached approximately 68 ± 6% (mean ± SEM) of control values (see as examples traces a versus trace b, respectively, Fig. 2).

At the end of each experiment, and similarly to in vitro experiments, at potential +550 mV the application in the striatum of 1 μL of 0.3 μM exogenous NO, but not that of 100 μM sodium nitrite, caused the generation of a sharp, transient DCA oxidation signal. Typically, the signal appeared about 5–8 s after the injection, had an intensity of about 20 nA and a peak width at half height of approximately 10 s.

It appears that following intrastriatal injection of NO (1 μL 0.3 μM) the in vivo related NO signal reached about 12.1 ± 1.4 nA ($n = 30$) while in vitro calibration, addition of the same amount of NO in 3 mL solvent resulted in a 25.3 ± 2.6 nA oxidation signal ($n = 10$ mCFE). The NO related signal determined by fluoxetine treatment appeared to be approximately 2.5 ± 0.12 nA ($n = 10$) therefore suggesting that the fluoxetine-stimulated NO release corresponds approximately 3 nM concentration. However, it is relatively awkward to compare in vivo and in vitro results because of the obvious physical and chemical differences between in vitro and in vivo constituents (aCSF versus brain tissue) that may intervene differently upon the NO molecule. Nonetheless, being a gas, NO diffuses quickly and in spite of its short half-life [15,30] can spread over considerable distances and affect a large number of structures. For instance, in our setting (time between the injection of exogenous NO and the appearance of the signal approximately 5 s; distance between the injection needle and the mCFE approximately 250 μm) we evaluated in 50 μm/s the velocity of diffusion in tissue. This result is in good agreement with theoretical predictions [30] proposing that exogenous NO can rapidly reach the striatal mCFE.

Various studies have proposed that endogenous NO contributes to the pathogenesis of depression. For instance, conditions of reduced endogenous NO production: i.e. via treatment with NOS inhibitors such as L-NNA, L-NAME, or 7-nitroindazole have shown evidence of antidepressant activities in the forced swimming test (FST) which are prevented by pre-treatment with L-Arginine [20,33]. Additionally, recent observations have proposed a link between depression and a lack of hippocampal neurogenesis and that endogenous NO contributes to CMS-induced depression by suppressing hippocampal neurogenesis [34]. Furthermore, clinical studies have revealed significantly higher plasma nitrate concentrations, an index of NO production, in depressed patients [27].

More than a decade ago, Harvey [19] reported that:

(i) stress-mediated dopamine release, with subsequent depletion of serotonin, results in excessive glutamatergic activity and NO synthesis;
(ii) NMDA receptor antagonists display antidepressant efficacy in animal models.

Successively, the direct involvement of serotonergic function in the antidepressant-like effects of NOS inhibitors has been further described [18]. In particular, it was observed that sub-effective dose of 1-(2-trifluoromethylphenyl)-imidazole (TRIM), a novel neuronal NOS inhibitor, selectively augmented the behavioural effect (i.e. decreased immobility in the FST) of antidepressants acting on serotonergic system, especially that of the SSRIs fluoxetine and citalopram [28].

In the present study, we have at first re-confirmed the chemical nature of the endogenous in vivo amperometric signal evoked in the striatum of anaesthetised rats by NMDA injection, via assessing the effect of the NOS inhibitor L-NAME on the NMDA-induced response. The addition of such a compound prevented the appearance of NO related oxidation signal while pre-treatment with vehicle did not. This is shown in Fig. 1 where trace a indicates the effect of NMDA injection, while trace b shows the influence of L-NAME pre-treatment on the successive local NMDA application, respectively.

Further confirmation of previous works proposing that the treated mCFE is selectively monitoring NO [9,10,13] is the lack of oxidative signal in presence of the exogenous nitrites either in vitro and in vivo (not shown). In particular, the electrical treatment modifies profoundly the physical and chemical state of the carbon fibre [26] i.e. it determines a large presence of COO^- groups on the surface of the carbon fibre [14] which therefore can easily adsorb NO intermediates such as NO^+. In addition, the increased roughness of the carbon fibre amplifies the interaction with the small sized NO molecule [29]. Thus, higher signal than the "diffusion" peak can be monitored with amperometry in case of good adsorption at the surface of the mCFE of the starting material (NO) and/or the initial oxidation reaction product (i.e. NO^+) [5].

We have also demonstrated that NO can have a different oxidation potential than that of nitrites, and this either in vitro, ex vivo functional and in vivo pharmacological experiments. Briefly:

- In vitro at a potential of +550 mV and at room temperature, the addition during the DPV scan of different amount of saturated NO solution (2 mM in PBS) to a well containing 3 mL of PBS resulted in the occurrence of an amperometric signal. The amplitude of the signal was concentration dependent. In contrast to NO, the addition of 100 μM sodium nitrite, as well as that of solvent produced no significant modification of basal current levels at this potential. Only at higher potential (i.e. +650/+750 mV) the addition of sodium nitrite caused an elevation of the oxidation current trace [9].
- Ex vivo functional studies have shown that addition of NO to an adrenaline contracted aortic ring but not that of nitrites, resulted in the expected ring relaxation and the concomitant appearance of a DP voltammetric signal at +550 mV when positioning the active tip of the mCFE parallel and in contact with the endothelium of the aortic ring [9].
- Pharmacological in vivo experiments demonstrated that the amperometric signal monitored at +550 mV in the rat striatum is responsive to glutamatergic stimulation (NMDA, substance P) or inhibition (L-NAME, L-NMMA) of NO synthase [9,10,25]. These selective pharmacological data, together with the matching results following the treatment with the competitive or the non competitive NMDA receptor antagonist AP5 or dizolcipine [12] further confirm that increased activity of glutamate NMDA receptors represents the dominant mechanism by which NO is generated in the brain [17].

Consequently, the present data showing in vivo decrease in endogenous NO following fluoxetine as measured in situ and in real time by amperometry together with selective mCFE further support the relationship between the serotonergic system and the NO system.

Finally, our original in vivo findings confirm that voltammetry with specifically treated micro-sensors can measure NO in vivo quite in real time and that with this methodology it is possible to monitor interaction(s) between SSRIs and NO. Further in vivo work, i.e. in animal models of depression, would be useful to elucidate the role of NO within the clinical antidepressant effect of SSRIs.

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