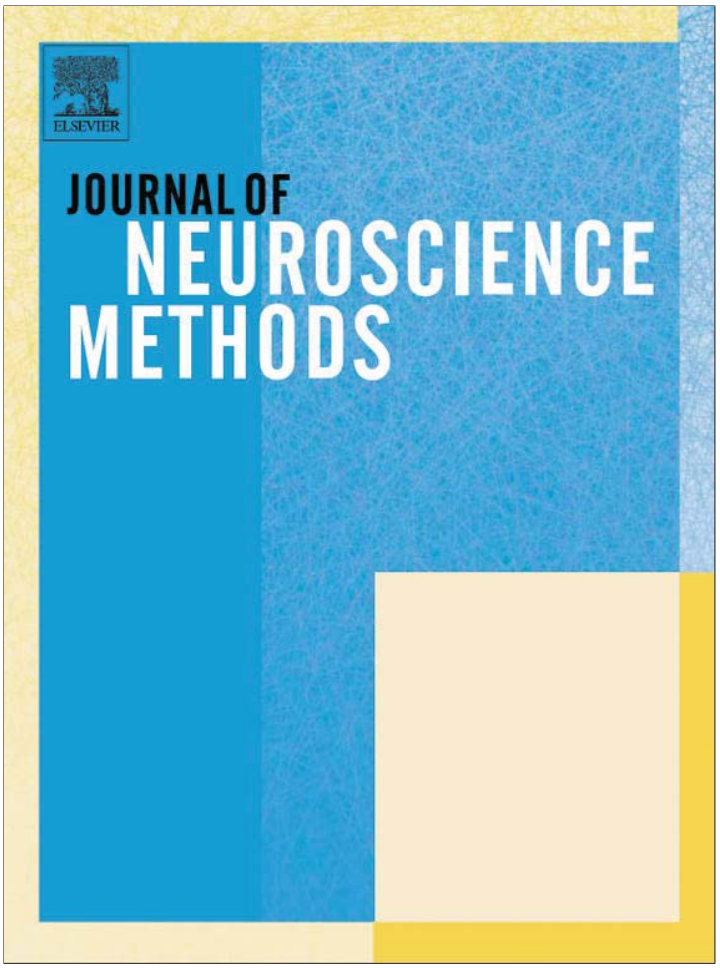

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Journal of Neuroscience Methods 209 (2012) 13–21

Contents lists available at SciVerse ScienceDirect

Journal of Neuroscience Methods

journal homepage: www.elsevier.com/locate/jneumeth

Basic Neuroscience

Brain nitric oxide: Regional characterisation of a real-time microelectrochemical sensor

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ARTICLE INFO

Article history:
Received 13 April 2012
Received in revised form 17 May 2012
Accepted 21 May 2012

Keywords:
Nitric oxide
Real-time
Prefrontal cortex
Nucleus accumbens
In vivo

ABSTRACT

A reliable method of directly measuring endogenously generated nitric oxide (NO) in real-time and in various brain regions is presented. An extensive characterisation of a previously described amperometric sensor has been carried out in the prefrontal cortex and nucleus accumbens of freely moving rats. Systemic administration of saline caused a transient increase in signal from baseline levels in both the prefrontal cortex (13 ± 3 pA, $n = 17$) and nucleus accumbens (12 ± 3 pA, $n = 8$). NO levels in the prefrontal cortex were significantly increased by 43 ± 9 pA ($n = 9$) following administration of L-arginine. A similar trend was observed in the nucleus accumbens, where an increase of 44 ± 9 pA ($n = 8$) was observed when compared against baseline levels. Systemic injections of the non-selective NOS inhibitor L-NAME produced a significant decrease in current recorded in the prefrontal cortex (24 ± 6 pA, $n = 5$) and nucleus accumbens (17 ± 3 pA, $n = 6$). Finally it was necessary to validate the sensors functionality in vivo by investigating the effect of the interferent ascorbate on the oxidation current. The current showed no variation in both regions over the selected time interval of 60 min, indicating no deterioration of the polymer membrane. A detailed comparison identified significantly greater affects of administrations on NO sensors implanted in the striatum than those inserted in the prefrontal cortex and the nucleus accumbens.

1. Introduction

Since its emergence as a signalling molecule in the brain 20 years ago, nitric oxide (NO) has been implicated in many different functions that are determined by the source of the nitric oxide synthase (NOS) enzyme used in its synthesis. Three isozymes have so far been identified; endothelial NOS (eNOS), neuronal NOS (nNOS) and inducible NOS (iNOS) (Alderton et al., 2001; Bruckdorfer, 2005; Kelm, 1999; Kiechle and Malinski, 1993). Endothelial cells are probably the major, if not the sole, location of eNOS in the brain with emerging evidence of its role in regulating brain function, independent of its role in the vasculature (Garthwaite, 2008). Neuronal NOS is the most abundantly present isoform in the brain and is tightly associated with NMDA receptor function on post-synaptic membranes. The inducible form, which is Ca^{2+} independent and produced in large amounts in response to an external stimulus such as infection or inflammation, carries out its role following release from macrophages (Bruckdorfer, 2005).

NO's diverse functions include a neurological function in synaptic plasticity, neurotransmission, learning and memory (Garthwaite, 2008; Wass et al., 2006a,b), in addition to having a primary role in non-specific immunity (Bruckdorfer, 2005), penile erection (Escrig et al., 1999; Mas et al., 2002) and platelet aggregation inhibition (Radomski et al., 1990). While NO is responsible for normal synaptic transmission, excess levels of NO have been portrayed as being neurotoxic (Barth et al., 1997; Espey et al., 2002). Glutamate neurotoxicity is hypothesised to occur primarily due to release of NO from glial cells, via activation of the ionotropic glutamate NMDA receptor (Dawson et al., 1996). Barth et al. (1997) found that NO produced in response to ischaemia and mediated by glutamate release can cause neuronal cell death, effects which were largely prevented by use of NOS inhibitors.

It is hypothesised that the prefrontal cortex serves a specific function in cognitive control in the brain; impairments of this brain region have been implicated in schizophrenia giving rise to negative symptoms and cognitive dysfunctions associated with the disease (Fejgin et al., 2008; Miller and Cohen, 2001; Tzschentke, 2001). The nucleus accumbens which is part of the ventral striatum is assumed to play a role in reward, emotion and addiction (Saul'skaya and Fofonova, 2009; Saul'skaya et al., 2008; Saulskaya and Fofonova, 2006; Savel'ev and Saul'skaya, 2007; Yananli et al., 2007) and it too is postulated to have a function in the pathophysiology of schizophrenia. Recent findings have postulated that the NO pathway may constitute an interesting target for novel pharmacological therapies in schizophrenia and possibly play a role in the pathophysiology of the disorder. However, this contention

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http://dx.doi.org/10.1016/j.jneumeth.2012.05.023

rests on indirect evidence as suitable tools for the real-time detection of NO in vivo have, until recently, been lacking (Palsson et al., 2009). This has also been highlighted recently by Garthwaite, who expressed a great need for a reliable method of directly measuring endogenously generated NO in tissues with the necessary sensitivity or spatial and temporal precision (Garthwaite, 2008). We have previously reported the in vitro (Brown et al., 2009; Brown and Lowry, 2003) and in vivo (Finnerty et al., 2012) characterisation of a Nafion®-modified Pt sensor designed for real-time monitoring of brain extracellular NO. In vitro findings confirmed that the Nafion®(5/2) sensor had a response time suitable for in vivo monitoring, linearity over the relevant concentration range for NO, freedom from protein and lipid fouling, and minimal interference from a variety of endogenous species, including ascorbic acid, dopamine and serotonin over physiologically relevant concentration ranges. A detailed in vivo characterisation was carried out in the striatum of wistar rats and significant NO changes were recorded against baseline following administrations of stock NO, L-arginine and L-NAME. Ascorbate selectivity studies confirmed minimal deterioration of the Nafion®-modified surface and the stability of the sensor was analysed over 8 days confirming no significant change in baseline. We investigated the application of the NO sensor in a study carried out utilising the psychotomimetic drug phencyclidine hydrochloride (PCP), which was found to induce a dose-dependent increase in prefrontal cortex NO levels, thus corroborating previous indirect evidence of this effect of PCP. In addition, the PCP-induced elevation of NO could be counteracted by pretreatment with the NOS inhibitor, L-NAME, in a dose that has previously been shown to block the behavioural effects of PCP in translational animal models of schizophrenia (Klamer et al., 2004a, 2001; Palsson et al., 2009; Wass et al., 2006a,b). This study has provided the first direct biochemical evidence for an involvement of NO in the effects of the NMDA receptor antagonist PCP.

In the present study an extensive characterisation of the Nafion®(5/2)-modified Pt sensor was carried out in the prefrontal cortex and nucleus accumbens of awake freely moving rats. These two brain regions were chosen due to their high density of NMDA receptors (Monaghan and Cotman, 1985) and their hypothesised roles in the pathophysiology of schizophrenia (Fejgin et al., 2008). Secondly, a comparative analysis was undertaken between the Nafion®(5/2)-modified Pt sensors implanted in the prefrontal cortex, nucleus accumbens and striatum.

2. Materials and methods

2.1. Chemicals and solutions

All chemicals used throughout the experiments were purchased from Sigma Chemical Co. (Dublin, Ireland). A 0.9% solution of saline was prepared by dissolving 0.9 g NaCl in 100 mL doubly distilled water. In all cases, unless otherwise noted all systemic administration of L-arginine (300 mg kg^{-1}), L-N^G-nitroarginine methyl ester hydrochloride (L-NAME, 30 mg kg^{-1}), and sodium ascorbate (2 g kg^{-1}) were made up in a solution of 0.9% saline.

2.2. NO sensor preparation

Nafion®(5/2)-coated Pt disk electrodes were made from Teflon®-insulated platinum/iridium (Pt/Ir 90%/10%) wire (125 μm bare diameter 5T, Advent Research Materials, Suffolk, UK). The electrodes were approximately 4 cm in length and were prepared by carefully cutting 2 mm of Teflon® insulation from one end of the wire and soldering to this end a gold clip which provided rigidity and electrical contact. The other end of the wire acted as the active (disk) surface. The electrode was modified as previously described (Brown et al., 2009; Brown and Lowry, 2003; Finnerty et al., 2012).

2.3. Systemic administrations

All systemic administrations were carried out in 1 mL saline by intraperitoneal (i.p.) injection.

2.4. In vivo implantation and surgery protocol

Male Wistar rats (Biomedical Facility, University College Dublin, Ireland) weighing between 200 and 300 g were housed in a temperature (17–23 °C), humidity and light-controlled (12 h light, 12 h dark cycle) environment with access to food ad libitum prior to surgery. NO sensors were implanted following a previously described procedure (Lowry et al., 1997). Coordinates for the prefrontal cortex and nucleus accumbens with the skull levelled between bregma and lambda, were: A/P + 3.2, M/L ± 0.8 from bregma and D/V − 4.2 from dura and A/P + 1.85, M/L ± 1.3 from bregma and D/V − 6.8 from dura respectively (Paxinos and Watson, 1998). A reference and auxiliary electrode (8T Ag wires, 200 μm bare diameter) were placed in the cortex. The reference potential provided by the bare Ag wire in brain tissue is very similar to that of the saturated calomel electrode (SCE) used in the in vitro characterisation (O'Neill et al., 1998). The electrodes and probe were fixed to the skull with dental screws and dental acrylate (Associated Dental Products, Swindon, UK). The rats were anesthetised with the volatile anaesthesia Isoflurane, placed in a Kopf stereotaxic instrument and kept on a heating pad to prevent hypothermia. A 1 mL/kg injection of the opioid analgesic buprenorphine is administered subcutaneously (s.c.) 30 min after the end of the surgery and the animal allowed to rest. The animal is monitored for the next few hours, before being transferred to a holding bowl where it remains for the duration of the experiment. The animal is allowed to recover for at least 24 h prior to connection to the potentiostat. The desired potential (+900 mV vs. Ag wire) is then applied to the NO sensor and the current is allowed to stabilise for approximately 24 h. Following this period of stabilisation, in vivo measurements were commenced. All experimental procedures were performed under license in accordance with the European Communities Regulations 2002 (Irish Statutory Instrument 566/2002 and U.K. Animals (Scientific Procedures) Act 1986).

2.5. Instrumentation and software

Constant potential amperometry was performed using previously described methods (Brown et al., 2009; Finnerty et al., 2012). All data presented had baselines normalised to zero to show the change in current (ΔI) and reported concentration changes are based on a previously reported protocol (Finnerty et al., 2012). The significance of differences observed was estimated using the Student's *t*-test for paired or unpaired observations where appropriate. Two-tailed levels of significance were used with $p < 0.05$ considered to be significant. All data are presented as mean ± standard error (SEM), with n = number of sensors implanted in 8 animals (saline – prefrontal cortex), 4 animals (saline – nucleus accumbens), 5 animals (L-arginine – prefrontal cortex), 4 animals (L-arginine – nucleus accumbens), 5 animals (L-NAME – prefrontal cortex), 4 animals (L-NAME – nucleus accumbens) 6 animals (ascorbate – prefrontal cortex) and 5 animals (ascorbate – nucleus accumbens). The sample data presented in Figs. 1–4 has been normalised and transformed to the average response obtained for the respective treatments. This removes both inter electrode and inter animal variability by ensuring that the

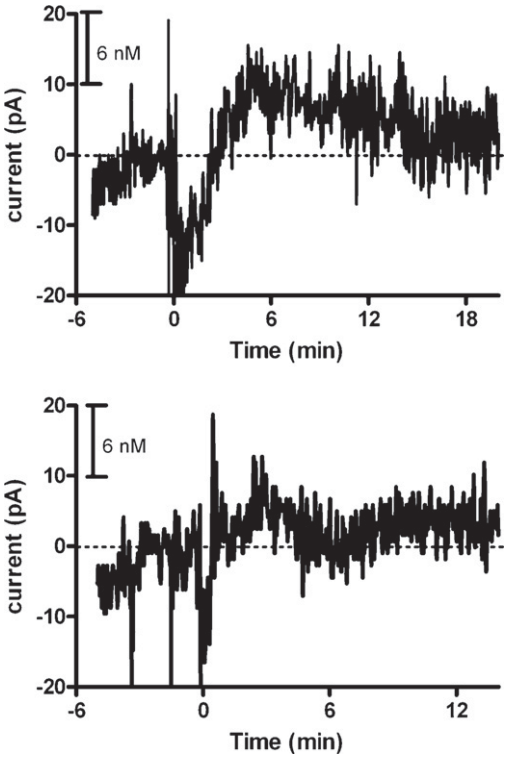

Fig. 1. Typical examples of the effect of saline administration (1 mL i.p. injection) monitored in rat prefrontal cortex (top, average pre-injection baseline = 317 ± 14 pA, n = 17) and rat nucleus accumbens (bottom, average pre-injection baseline = 324 ± 37 pA, n = 8) with a Nafion®(5/2)-modified Pt sensor. Time zero indicates point of injection.

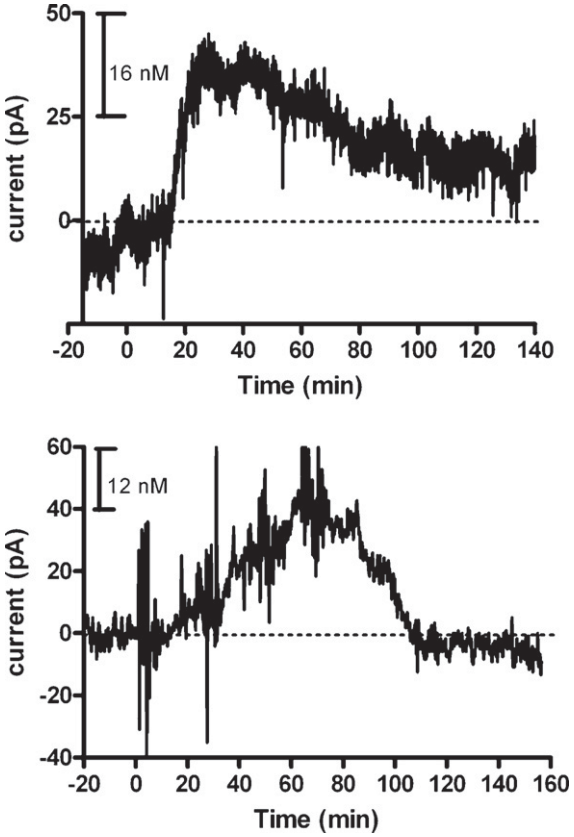

Fig. 2. Typical examples of the effect of L-arginine administration (300 mg kg^{-1}, 1 mL i.p. injection) monitored in rat prefrontal cortex (top, average pre-injection baseline = 394 ± 94 pA, n = 9) and rat nucleus accumbens (bottom, average pre-injection baseline = 245 ± 25 pA, n = 8) with a Nafion®(5/2)-modified Pt sensor. Time zero indicates point of injection.

presented current and concentration changes are representative of the data from all the animals used in each study.

2.6. Experimental conditions

All experiments were carried out with the animal in its home bowl. Implanted electrodes were connected to the potentiostat through a six-pin Teflon® socket and a flexible screened six core cable which was mounted through a swivel above the rats head (Semat Technical) at least 7–8 h prior to the start of the first experiment each day. This arrangement allowed free movement of the animal.

2.7. Voltammetry techniques in vivo

All in vivo experiments utilised constant potential amperometry which involves the application of a constant voltage. The resulting current is directly proportional to the concentration of the analyte at any given time. NO was detected by holding the implanted sensor at the oxidation potential of +900 mV (vs. Ag wire) which has been previously characterised as the optimum potential for NO detection (Brown, 2003; Brown et al., 2009).

3. Results and discussion

3.1. Systemic administrations

Since all administrations were by i.p. injection it was important to examine the effect of normal saline administrations (0.9%) on the oxidation current. We have previously reported a significant but short lived change (22 ± 3 pA, p < 0.001, n = 9) from baseline levels in NO sensor's implanted in the striatum of Wistar rats following saline injection (Finnerty et al., 2012). Similar initial and brief injection effects have also been observed for tissue O_2 (Bolger et al., 2011) and regional cerebral blood flow (rCBF) (Lowry and Fillenz, 2001) during the injection of saline, with a comparable return to baseline levels. A significant increase in the recorded current (13 ± 3 pA, p < 0.001, n = 17) was observed in the prefrontal cortex, reaching a maximum level after 5 ± 1 min (n = 17) and returning to a baseline level after 12 ± 2 min. This short lived increase in oxidation current corresponded to a concentration change of ca. 8 ± 2 nM. A similar affect was observed in the nucleus accumbens following saline administrations. A transient increase in oxidation current (12 ± 3 pA, n = 8) occurred after 4 ± 2 min. This resulted in a significant increase from baseline (p < 0.05) that represented a concentration change of 7 ± 4 nM (n = 8). The current returned to a baseline level after 8 ± 3 min. Typical examples of the effect of saline administration in both regions are shown in Fig. 1. The stress of the i.p. injection stimulates neuronal activation (Vahabzadeh and Fillenz, 1994), increasing rCBF and thus O_2, with the supply of the latter exceeding utilisation. The observed increase in blood flow can be attributed to vasodilation brought about by NO through its physiological function as the EDRF (Ignarro et al., 1987; Palmer et al., 1987). The different roles that NO has to play in the body are dependent on which type of NOS enzyme is used in its synthesis. The constitutive forms (endothelial (eNOS) and neuronal (nNOS)) which are activated by Ca^{2+}, followed by binding to the protein calmodulin, exert their effects through blood flow and neurotransmission respectively (Marletta, 1993). These findings corroborate previous reports that were carried out in the striatum of freely moving rats utilising the amperometric NO sensor (Finnerty et al., 2012).

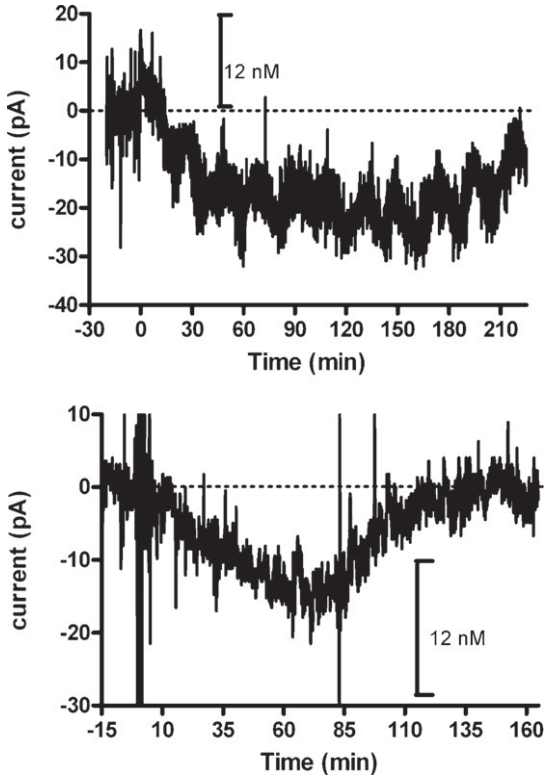

Fig. 3. Typical examples of the effect of L-NAME administration (30 mg kg^{-1}, 1 mL i.p. injection) monitored in rat prefrontal cortex (top, average pre-injection baseline = 311 ± 38 pA, n = 5) and rat nucleus accumbens (bottom, average pre-injection baseline = 407 ± 107 pA, n = 6) with a Nafion®(5/2)-modified Pt sensor. Time zero indicates point of injection.

The reaction of L-arginine with molecular O_2 in the presence of NO synthase results in the formation of NO and L-citrulline in equimolar quantities. Recent studies have demonstrated that arginine availability is an important condition for the physiological functioning of the nitroergic system (Do et al., 2002; Savel'ev and Saul'skaya, 2007). Indirect reports confirm the antioxidant effects of L-arginine in the early and late stages of ischaemia (Maksimovich et al., 2006) and its reduction in brain edema formation and improvement of cortical blood flow in the early phase after a brain trauma (Lundblad and Bentzer, 2007). Previously our group has confirmed in real-time that both systemic and local L-arginine administration significantly increased the NO sensor's signal in the striatum of Wistar rats compared to pre-administration baseline levels (Brown et al., 2009; Finnerty et al., 2012). Typical examples of the effect of L-arginine injections in the prefrontal cortex (top) and nucleus accumbens (bottom) are detailed in Fig. 2. A significant increase was observed in both the prefrontal cortex (43 ± 9 pA, p < 0.01, n = 9) and nucleus accumbens (44 ± 9, p < 0.01, n = 8) in comparison to pre-injection baselines. These corresponded to concentration changes of 27 ± 6 nM (n = 9) and 28 ± 5 nM (n = 8) respectively. The maximum increase was recorded at 42 ± 9 min (n = 9) in the prefrontal cortex before returning to baseline levels after 136 ± 15 min (n = 9). In the nucleus accumbens the maximum response was achieved after 65 ± 10 min (n = 8) and returned to pre-injection levels after 153 ± 12 min (n = 8). These observations confirm that the NO sensor is responding to increased production of endogenous NO in both regions validating previous findings from other groups who reported an increase in NO current accompanying L-arginine perfusion, using an amperometric NO sensor implanted in the hippocampus of rats (Heinzen and Pollack, 2003, 2002). Previously, similar reports have detailed carbon fibre microelectrodes utilised for NO measurements in the corpus cavernosum of urethane-anaesthetised rats recorded enhancements in the NO signal following arginine administration (Escrig et al., 1999; Mas et al., 2002). Also, Carvalho et al. (2004) reported real-time in vitro measurements of NO production in erythrocytes using a commercial NO sensor during stimulation by L-arginine.

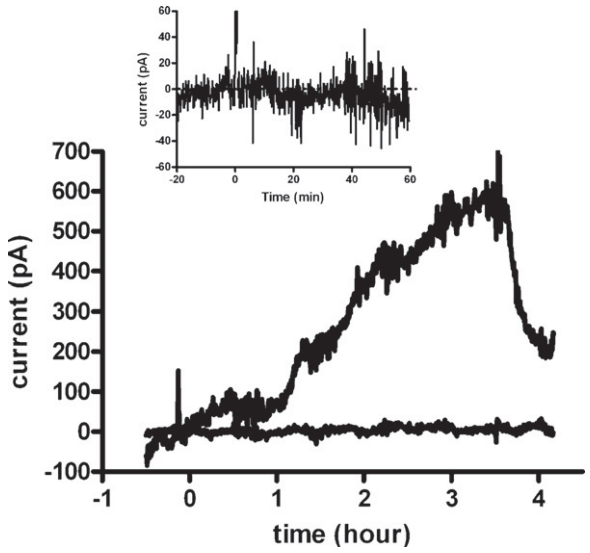

Fig. 4. Typical example of the effect of an i.p injection of sodium ascorbate (2 g kg^{-1}, average pre-injection baseline = 359 ± 56 pA, n = 13) on currents monitored in prefrontal cortex with a Nafion®(5/2)-modified Pt sensor and carbon paste electrode (CPE). Time zero indicates point of injection. Inset: Typical example of the effect of an i.p. injection of sodium ascorbate on a 60 min response of a Nafion®(5/2)-modified Pt sensor implanted in the prefrontal cortex.

L-NAME is a non-selective nitric oxide synthase (NOS) inhibitor which acts by competing with L-arginine for its binding site on the NOS enzyme (Alderton et al., 2001). Microdialysis investigations have provided indirect evidence relating to decreases in co-product L-citrulline levels in the dorsal striatum following local infusion of various NOS inhibitors, L-NAME (Ohta et al., 1994) and in the nucleus accumbens following *N*-nitro-L-arginine (Saulskaya and Fofonova, 2006). L-Citrulline displays much greater chemical stability than NO which has resulted in the development of a series of indirect methods based on studies of citrulline synthesis in the nucleus accumbens for assessing NO production (Saul'skaya and Fofonova, 2009; Saul'skaya et al., 2008; Savel'ev and Saul'skaya, 2007). Recently we reported a significant decrease in NO (91 ± 19 pA, p < 0.05, n = 4) compared to baseline levels following L-NAME administration in the striatum of Wistar rats (Finnerty et al., 2012). A typical example of the effects of a 30 mg kg^{-1} i.p. injection in both the prefrontal cortex and nucleus accumbens are illustrated in Fig. 3. The signal (ΔI) decreased significantly by 24 ± 6 pA (n = 5, p < 0.05) and 17 ± 3 pA (n = 6, p < 0.01) respectively compared to baseline levels. A maximum response was observed at 51 ± 12 min (n = 5) in the prefrontal cortex that returned to baseline levels after 184 ± 72 pA (n = 5). A similar observation was reported in the nucleus accumbens with a maximum decrease occurring at 60 ± 6 min (n = 6) and returning to pre-injection levels after 171 ± 27 min (n = 6). The current changes corresponded to concentration changes of 15 ± 4 nM (n = 5) and 11 ± 2 nM (n = 6) in the prefrontal cortex and nucleus accumbens respectively. These findings corroborate investigations undertaken by Escrig et al. (1999) in the corpus cavernosum whereby local and systemic administrations of L-NAME caused a decrease in NO levels. Indirect reports detailing L-NAME have shown that the NOS inhibitor attenuates or completely blocks the effects of phencyclidine hydrochloride (PCP), an NMDA receptor antagonist that is assumed to carry out its actions through an NO-mediated mechanism (Wass et al., 2006a,b;

Wiley, 1998). A number of behavioural studies incorporating a series of different paradigms have confirmed this, for example, prepulse inhibition, which is the reduction in startle amplitude to a startling stimulus when this stimulus is immediately preceded by a weaker pre-stimulus (Klamer et al., 2001, 2004b). Another is latent inhibition, which is a referral to a phenomenon whereby pre-exposure to a stimulus weakens the subsequent association of that stimulus with a reinforcer in classical conditioning (Klamer et al., 2005; Palsson et al., 2005). We have recently reported direct evidence that 10 mg kg^{-1} L-NAME injections inhibit NO production following systemic administration of PCP (Palsson et al., 2009) confirming the postulations of the previous behavioural investigations. Other paradigms reported include the elevated plus-maze which is based on exploratory behaviour of rats. NO is believed to play a role in learning and memory and L-NAME is shown to induce a learning deficit in this avoidance learning test (Da Cunha et al., 2005). Since nitric oxide has been characterised as the EDRF, it is very closely associated with CBF and cerebrovasodilation. A number of existing reports have reported that L-NAME induced a reduction in levels of CBF that were increased following a period of hyperbaric oxygen exposure by a nitric oxide mediated mechanism (Demchenko et al., 2000, 2001; Hagioka et al., 2005). Yan et al. (2003) also reported a decrease in CBF in response to intravenous infusion of the NO synthase inhibitor at doses of 1, 3, 10 and 30 mg kg^{-1}.

3.2. Interference studies

Previously the Nafion®(5/2)-modified Pt NO sensor demonstrated excellent selectivity towards NO in vitro against a wide range of electroactive interferents (e.g. ascorbic acid, dopamine, DOPAC, NO_2^-, serotonin) found endogenously in brain extracellular fluid (Brown et al., 2009). In vivo investigations in the striatum of freely moving rats displayed excellent rejection characteristics against ascorbate (Finnerty et al., 2012), the most abundantly present interferent reported in the ECF with a hypothesised concentration of ca. 500 μM (Miele and Fillenz, 1996). It is imperative to confirm that the Nafion® membrane has not degraded when placed in the in vivo environment and that the sensor exhibits similar selectivity characteristics to those recorded in vitro (Brown et al., 2009; Brown and Lowry, 2003). With its high concentration and ease of oxidation, ascorbate is probably the simplest molecule to detect and monitor in brain ECF using in vivo voltammetry techniques (Lowry and O'Neill, 2006; O'Neill et al., 1998). For these reasons it was important to investigate the effect of systemic administrations of ascorbate on the Nafion®-modified Pt sensor current in both the prefrontal cortex and nucleus accumbens. The current was monitored over a 60 min period as previous investigations have reported this time frame allows for a maximum response to occur (Finnerty et al., 2012; Lowry et al., 1996). It is hypothesised that any effect following ascorbate injection will have occurred within this period. Fig. 4 (inset) illustrates this effect from a Nafion®(5/2)-modified Pt NO sensor implanted in the nucleus accumbens. There was a slight decrease in the oxidation current over the course of ascorbate injections in both brain regions that can be attributed to baseline drift recorded over the 60 mins. There was no significant difference in signal against baseline levels in the prefrontal cortex (-8 ± 16 pA, $p > 0.05$, $n = 13$) and the nucleus accumbens (-16 ± 17 pA, $p > 0.05$, $n = 10$) recorded over the 60 min period. Fig. 4 displays a comparison between a typical example of the effect of ascorbate injection (2 g kg^{-1}) on a carbon paste electrode and the Nafion®(5/2)-modified Pt NO sensor implanted in the prefrontal cortex. It is clearly evident that there is a difference in the signal elicited from both sensors following administration of the interferent. This comparison provides validation that ascorbate has reached both sensors and no increase at the Nafion®(5/2)-modified Pt NO sensor was observed, confirming that the NO sensor's surface has remained intact. A similar observation was noted in the nucleus accumbens. Collectively these results corroborate previous investigations undertaken in the striatum of freely moving rats (Finnerty et al., 2012).

3.3. Regional comparisons

Table 1 summarises the in vivo characterisation data for Nafion®(5/2)-modified sensors implanted in the striatum, prefrontal cortex and nucleus accumbens of freely moving rats. It is apparent from these investigations that alternative sources of NO production may be responsible for differences observed between brain regions. The constitutive isoforms of NOS (eNOS and nNOS) are the primary source of NO production in the brain lending support to its hypothesised function as a signalling molecule in the central nervous system. iNOS is primarily linked with pathological situations and is rarely present at tonic levels but is expressed in various cell types such as macrophages and microglia (Garthwaite, 2008). It is imperative that we consider regional variations in determining parameters such as NOS activity and NOS expression, highlighting the fact that there may be multiple sources of NO production and inhibition within a tissue matrix. In the dorsal striatum, projection neurons comprise 90% of all the cells; however, interneurons comprise only 10% of striatal cells and are implicated in regulating striatal projection function. It is the interneurons that are the primary source of nNOS in this brain region (Kawaguchi and Emson, 1996; Marin et al., 2000).

The ventral striatum (nucleus accumbens) contains NO generating interneurons that receive excitatory glutamatergic and dopaminergic inputs from the hippocampus and ventral tegmental area respectively (Saulskaya and Fofonova, 2006). A high density of NMDA receptors has been confirmed in the prefrontal cortex (Monaghan and Cotman, 1985) and it is postulated that NO exerts strong influence on glutamatergic neurotransmission by directly interacting with the receptor (Bernstein et al., 2005). Various other reports have indicated that NOS activity was decreased in the hippocampus of aged rats but not in the cortex or cerebellum (Mollace et al., 1995; Vallebuona and Raiteri, 1995), however, alternative investigators reported increased activity in both the hippocampus and cerebellum of aged rats (Chalimoniuk and Strosznajder, 1998). These findings have all been quantified by indirect methods which is a major disadvantage of the vast majority of existing analytical techniques. The Nafion®(5/2)-modified Pt NO sensor described here represents a major advancement in measuring physiologically meaningful NO levels in real-time and over extended periods.

Saline administrations resulted in transient increases from baseline that had returned to pre-injection baselines within a 15 min time frame across all brain regions. It is a well accepted phenomenon that injection stress causes an increase in neuronal activation which is closely linked to CBF (Vahabzadeh and Fillenz, 1994). The larger blood vessels are supplied with nitrergic nerves that once activated, result in NO release, vasodilation and increased blood flow (Garthwaite, 2008; Toda and Okamura, 2003). Fig. 5 (top) compares the effect of saline injections on the oxidation current recorded in the three regions with an elevated response observed in the striatum. A significant difference ($p < 0.05$) was observed when comparing the current changes in the striatum (22 ± 3, $n = 9$) and nucleus accumbens (12 ± 3 pA, $n = 8$) following injections. No significant difference ($p > 0.05$) was observed between the striatum and prefrontal cortex (13 ± 3 pA, $n = 17$) or between the prefrontal cortex and nucleus accumbens in terms of ΔI changes from baseline levels. A number of factors must be taken into account when interpreting these findings. The NO may be generated from eNOS located in the microvascular network and capillary circulation that can be attributed to a rise in CBF following stress brought about by systemic administration. The implantation site of the

Table 1
Summary of in vivo characterisation data for Nafion®(5/2)-modified sensors implanted in the striatum, prefrontal cortex and nucleus accumbens of freely moving rats.

Brain region	Saline			L-Arginine			L-NAME		
	Current (pA)	Max response (min)	Return (min)	Current (pA)	Max response (min)	Return (min)	Current (pA)	Max response (min)	Return (min)
Striatum	22 ± 3 (n = 9)	4 ± 1 (n = 9)	13 ± 4 (n = 9)	71 ± 14 (n = 6)	22 ± 6 (n = 6)	47 ± 15 (n = 6)	−91 ± 19 (n = 4)	52 ± 16 (n = 4)	180
Prefrontal cortex	13 ± 3 (n = 17)	5 ± 1 (n = 17)	12 ± 2 (n = 17)	43 ± 9 (n = 9)	42 ± 9 (n = 9)	136 ± 15 (n = 9)	−24 ± 6 (n = 5)	51 ± 12 (n = 5)	184 ± 72 (n = 5)
Nucleus accumbens	12 ± 3 (n = 8)	4 ± 2 (n = 8)	8 ± 3 (n = 8)	44 ± 9 (n = 8)	65 ± 10 (n = 8)	153 ± 12 (n = 8)	−17 ± 3 (n = 6)	60 ± 6 (n = 6)	171 ± 27 (n = 6)

Nafion®(5/2)-modified Pt NO sensor's may be situated in closer proximity to vasculature circuitry when inserted into the striatum giving rise to the larger NO signals recorded in this region. This may not be the case for the sensors implanted in the nucleus accumbens and prefrontal cortex, providing a possible explanation for the different responses following saline administrations. A similar effect has been observed using carbon paste electrodes for real-time measurements of oxygen whereby, the concentration of oxygen observed can vary depending on the orientation of the electrode relative to the blood vessels and metabolically active sites, and on the depth of penetration into the tissue (Baumgärtl et al., 1989). Since the dimension (typically 100–200 μm) of carbon paste electrodes are greater than the scale of a capillary zone (ca. 70 μm) (Silver, 1965), an average tissue O_2 level is detected (Bolger et al., 2011). This may translate across to observations reported here with the NO sensor since the three-dimensional geometry of the capillary circulation would be just as well suited for delivering NO globally to the electrode as it is for delivering O_2 (Garthwaite, 2008). Other possible sources could be from postsynaptic NO production derived from nNOS, followed by diffusion into the extracellular space. This is a unique property that NO possesses over conventional neurotransmitters in that being a gaseous molecule it can diffuse freely between membranes and has functions both intracellularly and extracellularly. Kennedy (2000) reported that typical excitatory synapses in the brain could have ca. 50 NMDA receptors dispersed over a 400-nm-diameter postsynaptic density. Since NMDA receptors display a high association with nNOS, there is a strong possibility that they are a contributing factor to our observations. Further evidence provided by Monaghan and Cotman confirm high NMDA densities in the striatum, prefrontal cortex and nucleus accumbens of rats (Monaghan and Cotman, 1985).

Systemic administrations of the precursor L-arginine and the NOS inhibitor L-NAME displayed contrasting affects in the different brain regions investigated. Studies in recent years have demonstrated that arginine availability is an important condition for the functioning of the nitrergic system (Savel'ev and Saul'skaya, 2007). Tsikas et al. (2000) reported arginine saturation in cells causes the precursor to be present at concentrations far exceeding the Km value. A topic that instigates much discussion is why supplementation of the substrate enhances NO production in vivo. It is a well documented phenomenon referred to as the "arginine paradox". In the present study L-arginine produced long lasting effects in both the prefrontal cortex and nucleus accumbens that returned to pre-injection levels within a 180 min period. There was no significant difference ($p > 0.05$) between the three brain regions following systemic administrations as illustrated in Fig. 5 (middle), however, the striatal current returned to pre-injection levels much faster than the prefrontal cortex and nucleus accumbens. This is clearly evident from Table 1 and may be attributed to differences between brain regions. Indirect measurements of NO (nitrite and nitrate detection) following L-arginine administration have been well documented using the microdialysis technique. Local (1 mM) and systemic (500 mg kg^{-1}) administrations sufficiently induced an enhancement of NO production in the rat cerebellum (Yamada and Nabeshima, 1997). However, Hara et al. reported contradictory findings from the hippocampus and striatum using a similar indirect measurement. 500 mg kg^{-1} injections of L-arginine significantly increased extracellular hippocampal nitrite and nitrate levels in comparison to saline administrations. In complete contrast to our findings, they found that 500 mg kg^{-1} had no effect on the extracellular levels of the NO metabolites in the striatum (Hara et al., 2004). Their investigations postulate that the striatum might be less responsive to exogenous L-arginine in enhancing NO production via NOS than the hippocampus. The direct measurements using our Nafion®(5/2)-modified Pt NO sensor suggest a very contradictory function in the striatum with sufficient detection

of increases in NO levels (71 ± 14 pA, $n = 6$). Although there were reduced responses observed in the prefrontal cortex (43 ± 9 pA, $n = 9$) and nucleus accumbens (44 ± 9 pA, $n = 8$), they did not differ significantly from the striatum suggesting that the arginine paradox might not be equally applicable in all regions of the brain.

Fig. 5 (bottom) and Table 1 detail the varying effect of L-NAME administration on the Nafion®(5/2)-modified Pt NO sensor in all three brain regions. There are significant differences ($p < 0.01$) reported between the striatum (−91 ± 19 pA, $n = 4$) and the prefrontal cortex (−24 ± 6 pA, $n = 5$) and nucleus accumbens (−17 ± 3 pA, $n = 6$) respectively. No significant difference ($p > 0.05$) was observed between the prefrontal cortex and nucleus accumbens. One possible explanation for the regional variations may be due to the heterogenous distribution of NOS in the rat brain that affects determining parameters such as NOS expression and NOS inhibition within the tissue matrix. Various groups have provided indirect evidence of the effect of the NOS inhibitor on NO levels in the prefrontal cortex using microdialysis investigations (Laitinen et al., 1994, 1997; Pepicelli et al., 2004). L-NAME and another NOS inhibitor L-NARG failed to diminish cGMP levels in the frontal cortex of rats, however, these findings are difficult to interpret as they provide indirect evidence of NO activity in the region. They may provide some corroboration with our investigations since significantly reduced NO inhibition was observed in the prefrontal cortex in contrast to the striatum. Contrasting evidence provided by Fedele and Raiteri (1999) in the rat cerebellum and hippocampus reported that reterodialysis administration of NOS inhibitors markedly decreased extracellular cGMP in both regions. Collectively this evidence postulates that NOS inhibition in the rat prefrontal cortex is controlled in a different way from what occurs in other brain regions, such as the cerebellum and hippocampus. Citrulline monitoring is extensively utilised as an alternative method of determining NO fluctuations indirectly in various brain regions. Ohta et al. (1994) have reported decreases in citrulline in the striatum following local infusion of L-NAME, a finding which lends further support to our observations. Saulskaya and Fofonova (2006) extend these observations by demonstrating that local administrations of a NOS inhibitor (L-NA) in the nucleus accumbens reduces citrulline levels suggesting that there may be tonic levels of NOS present in the nucleus accumbens, that initiate the formation of the co-product of NO production during homeostasis.

Considering that the NO sensors were implanted bilaterally in left and right hemispheres of the nucleus accumbens, it was of interest to compare the effect of injections on contralateral placement in this brain region. Saline injections resulted in transient increases in NO signal in both the left (19 ± 1 pA, $n = 2$) and right (10 ± 4 pA, $n = 6$) hemispheres. There was no significant difference ($p > 0.05$) recorded between the two. A similar observation was reported for L-arginine injections. There was no significant difference ($p > 0.05$) between responses from the Nafion®(5/2)-modified Pt sensors implanted in the left (53 ± 9 pA, $n = 2$) and right (44 ± 11 pA, $n = 6$) sides of the nucleus accumbens. L-NAME administrations produced similar findings from sensors situated contralateral to each other in this region. NO signals in the left hemispheres (−16 ± 3 pA, $n = 2$) displayed no significant difference ($p > 0.05$) from those situated in the right hemisphere (−18 ± 4 pA, $n = 4$) of the accumbens. These findings confirm that there are no significant differences observed between NO signals recorded in opposite hemispheres confirming the viability of the Nafion®(5/2)-modified Pt sensors in contralateral investigations in the rat brain.

All of the aforementioned illustrate the complexity of NO determinations utilising both direct and indirect analytical methods. However, it is our understanding that this report provides the first extensive and in depth comparison of real-time investigations detailing the variations in endogenous NO determined in a variety of brain regions. The Nafion®(5/2)-modified Pt NO sensor detailed within possesses the necessary sensitivity, spatial and temporal precision for specific applications in animal model studies.

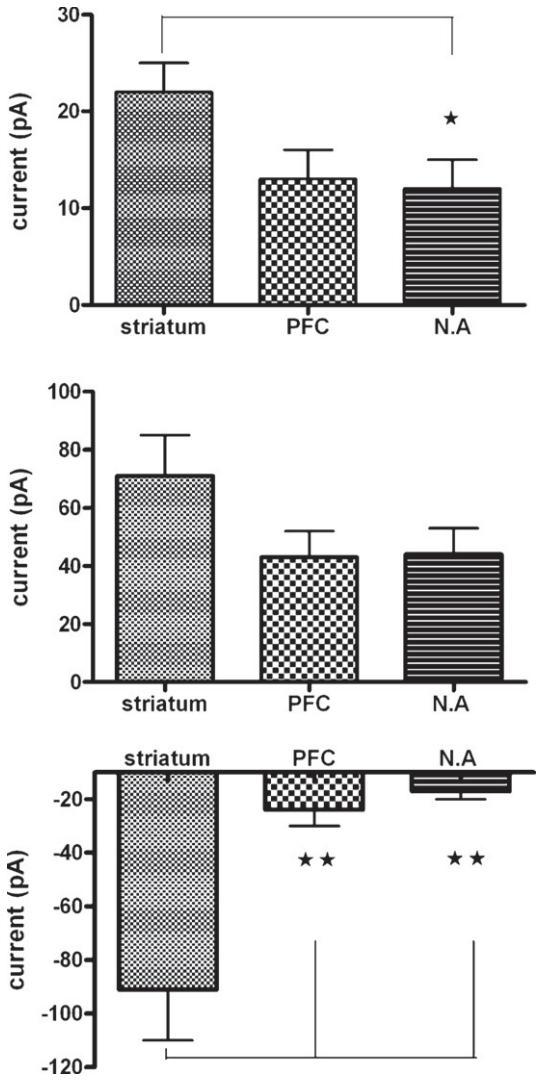

Fig. 5. Comparison of the effect of saline (top), L-arginine (middle) and L-NAME (bottom) on Nafion®(5/2)-modified Pt sensor signal recorded in striatum, prefrontal cortex (PFC) and nucleus accumbens (NA). Data is expressed as mean $\Delta I \pm$ SEM as compared to baseline. (top) ⋆ denotes a significant difference between striatum and nucleus accumbens ($p < 0.05$) and (bottom) ⋆⋆ denotes a significant difference between striatum and prefrontal cortex ($p < 0.01$) and between striatum and nucleus accumbens ($p < 0.01$).

4. Conclusion

We have reported the in vivo characterisation of a Nafion®(5/2)-modified Pt NO sensor in the prefrontal cortex and nucleus accumbens of freely moving rats. Previously we have demonstrated the efficacy of the NO sensor in the striatum using local and systemic administrations. Saline injections caused transient increases in oxidation current in both the prefrontal cortex and nucleus accumbens against baseline levels which was attributed to the stress of the i.p. injection stimulating neuronal activation. L-Arginine injections produced significant increases in the NO signal in both regions. Systemic administrations of the non selective NOS inhibitor L-NAME resulted in significant decreases in the recorded current measured using the Nafion®(5/2)-modified Pt NO sensor. Ascorbate selectivity studies confirmed minimal deterioration of the Nafion®

modified surface. A critical comparison of the in vivo characterisation undertaken in the striatum, prefrontal cortex and nucleus accumbens identified significantly greater affects of administrations on NO sensors implanted in the striatum than the other two regions. However, no definitive explanation can be provided for these interregional differences. In summary we have extensively characterised a highly selective and sensitive NO sensor in three different regions that is capable of measuring physiologically meaningful NO signals in real-time in the brain extracellular fluid.

Acknowledgements

We gratefully acknowledge financial support from the Analytical Chemistry Trust Fund, Royal Society of Chemistry (RSC) Analytical PhD Studentship 03/F/018, Enterprise Ireland (BR/1999/159), Science Foundation Ireland (03/IN3/B376) and the Centre of Applied Science for Health which is funded by the Higher Education Authority under the Programme for Research in Third Level Institutions (PRTLI) Cycle 4.

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