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An Investigation of Hypofrontality in an Animal Model of Schizophrenia Using Real-Time Microelectrochemical Sensors for Glucose, Oxygen, and Nitric Oxide

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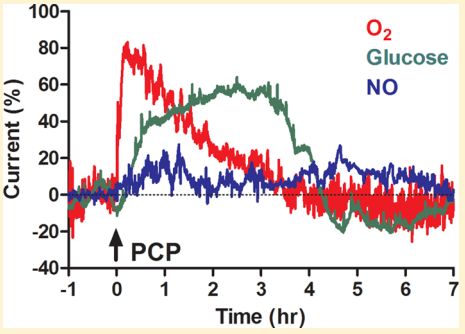
ABSTRACT: Glucose, O_2, and nitric oxide (NO) were monitored in real time in the prefrontal cortex of freely moving animals using microelectrochemical sensors following phencyclidine (PCP) administration. Injection of saline controls produced a decrease in glucose and increases in both O_2 and NO. These changes were short-lived and typical of injection stress, lasting ca. 30 s for glucose and between 2 and 6 min for O_2 and NO, respectively. Subchronic PCP (10 mg/kg) resulted in increased motor activity and increases in all three analytes lasting several hours: O_2 and glucose were uncoupled with O_2 increasing rapidly following injection reaching a maximum of 70% (ca. 62 μM) after ca. 15 min and then slowly returning to baseline over a period of ca. 3 h. The time course of changes in glucose and NO were similar; both signals increased gradually over the first hour post injection reaching maxima of 55% (ca. 982 μM) and 8% (ca. 31 nM), respectively, and remaining elevated to within 1 h of returning to baseline levels (after ca. 5 and 7 h, respectively). While supporting increased utilization of glucose and O_2 and suggesting overcompensating supply mechanisms, this neurochemical data indicates a hyperfrontal effect following acute PCP administration which is potentially mediated by NO. It also confirms that long-term in vivo electrochemical sensors and data offer a real-time biochemical perspective of the underlying mechanisms.



KEYWORDS: *Hypofrontality, brain, electrochemical sensors, real-time monitoring, metabolism, schizophrenia*

Functional imaging studies in schizophrenic patients have suggested reduced frontal lobe activity, that is, reduced glucose utilization and decreased regional cerebral blood flow (rCBF),[1−4] especially during cognitive tasks involving the prefrontal cortex such as working memory or verbal fluency.[5,6] This hypofrontality was first report by Ingvar and Franzén in 1974[1] and has been highly controversial since then with conflicting findings being reported in the literature right up to the present day.[5−7] While neuroimaging measures of metabolism and rCBF traditionally reflect the average activity across an anatomically defined "region of interest", or more recently identification of clusters of significant activation using voxel-by-voxel comparisons across the entire brain,[4] they do not provide direct in situ neurochemical information on the potential molecular underpinnings of hypofrontality. In addition, the technique, while ideally suitable to cognitive testing in humans, is not amenable to such testing in rodents due to the need to anaesthetize the experimental subjects.

The aim of the current study, therefore, was to investigate whether these specific issues could be addressed using long-term in vivo electrochemistry. LIVE involves the detection of substances in brain extracellular fluid (ECF) using electrochemistry with implanted amperometric sensors. By implanting a microelectrode (sensor) in a specific brain region, applying a suitable potential profile and recording the resulting Faradaic current, changes in the concentration of a variety of ECF species can be monitored. This allows investigations of the functions of specific chemicals in neuronal signaling, drug actions, and well-defined behaviors. It offers excellent spatial (e.g., 10−200 μm) and temporal (millisecond) resolutions, and a major advantage of long-term stability (continuous monitoring in vivo over several weeks).[8−10]

We have previously reported the in vitro[11−17] and in vivo[13,15,17−19] characterization of several sensors (e.g., glucose, O_2 and nitric oxide (NO)) developed to study brain energy metabolism. Operational characteristics such as sensitivity, selectivity, response time, limit of detection, etc., were characterized in detail for each device and their target substrate. The sensors were then subsequently used to study brain function in different application (e.g., pharmacological and behavioral) studies.[20−24] We have also shown that the measured tissue O_2 signal can serve both as an index of changes in blood flow, [25] and of the magnitude of the blood oxygenation level dependent (BOLD) fMRI response, thus providing a reliable awake animal surrogate of human fMRI experimentation, and an effective translational tool which can

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better enable the comparison of preclinical and clinical research.[26] In the present study we have explored the effects of hypofrontality induced using phencyclidine (PCP), a noncompetitive inhibitor of the NMDA receptor, on extracellular glucose, O_2 and NO monitored in real-time in freely moving animals using our previously developed LIVE sensors.

RESULTS AND DISCUSSION

LIVE Neurochemical Monitoring. LIVE sensors enable continuous real-time monitoring of a variety of neurochemicals in freely moving behaving animals where both gross and fine details can be recorded. For example, typical prolonged recordings of tissue O_2 during day and night are shown in Figure 1. Observed changes can be rapid, occurring over

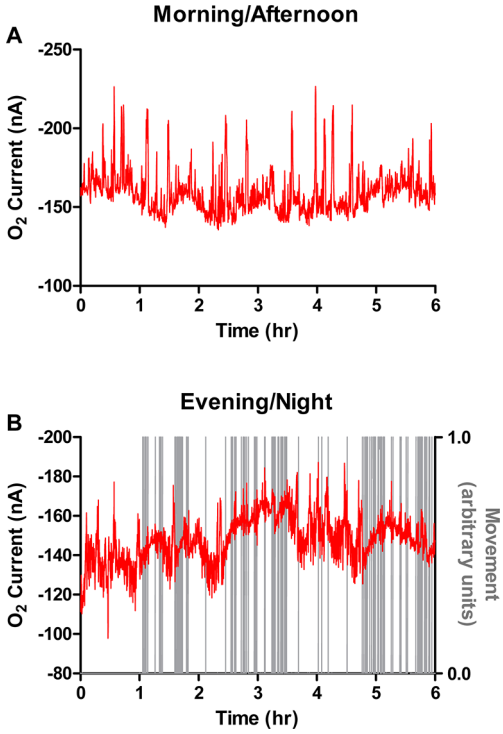

Figure 1. Typical examples of continuous real-time in vivo recordings of brain tissue O_2 during six-hour periods covering morning/afternoon (A, 10 am to 4 pm) and evening/night (B, 6 pm to 12 am, gray lines represent simultaneously monitored motor activity).

periods ranging from seconds to minutes, or more prolonged, lasting one or more hours. The former tends to be associated with physiological phenomena such as grooming, feeding, and even sleep (see Figure 2), while the latter occurs mainly with periods of intense activity (see Figure 1B). For O_2, both are reflective of the fact that the measured real-time $[O_2]$ is the dynamic balance between supply and utilization. Such changes in signal (nA) can be converted to units of pressure (mmHg, often used to represent pO_2,[15,27] using post in vivo calibration data and literature reported concentration and pressure data associated with the air-saturated $[O_2]$ at 37 °C:[28] the maximum increases of 22 nA (grooming, Figure 2A), 26 nA (feeding/drinking, Figure 2B) and 76 nA (sleep, Figure 2C) correspond to ca. 25 μM O_2, 29 μM O_2 and 84 μM O_2, or 18, 24, and 63 mmHg, respectively.

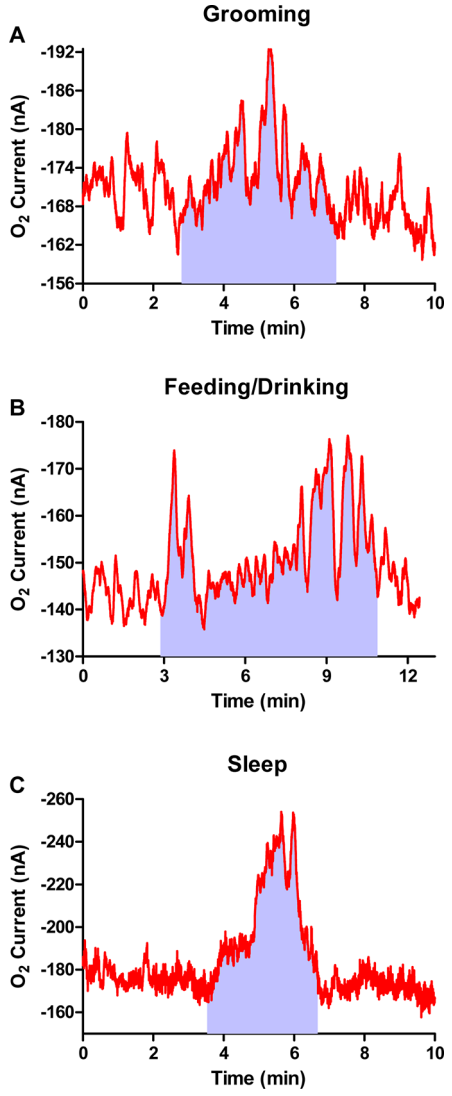

Figure 2. Typical examples of changes (shaded areas) in baseline in vivo brain tissue O_2 signals associated with naturally occurring grooming (A), feeding/drinking (B), and sleep (C). All data recorded during morning/afternoon periods (10 am to 4 pm).

The recent finding that brain tissue O_2 monitored using a microelectrochemical sensor in behaving animals can act as a surrogate for BOLD fMRI responses in humans[26,29,30] opens the possibility of studying, in animal models, controversial hypotheses or paradigms where data has primarily come from clinical studies. Hypofrontality is one such example. Ingvar and Franzén reported rCBF measurements in their original paper and found a hypofrontal rCBF distribution pattern at rest in chronic deteriorated schizophrenics.[1] Since then, while resting hypofrontality has been replicated in a number of studies,[31−33] there have also been many conflicting reports.[34,35] This may in part be explained by the potential for substantial variance in the resting state from individual to individual because of the heterogeneity of patient samples.[36,37] Also, some reports suggest that the ability of schizophrenic patients to recruit the prefrontal cortex decreases when they were stimulated with a frontal cognitive challenge.[36,38] However, like resting hypofrontality, this task-related or activation hypofrontality has not been consistently replicated.[39] While the debate has primarily concentrated on issues such as lack of standard resting

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baselines, application of a divergent array of cognitive tasks, clinical presentation and medication status, there is also the possibility that the different rCBF measuring techniques (e.g., ^{133}Xenon inhalation, single photon emission tomography (SPECT), positron emission tomography (PET) and fMRI) used may be contributing to the confounding results. Motion restriction and the potentially stressful injection of radioisotopes are characteristics of these techniques and may themselves alter rCBF in both the resting and activation states. Such issues have recently been addressed by Hoshi et al. who used near-infrared time-resolved spectroscopy (NIRS) to study resting and activation hypofrontality in a cohort of schizophrenic patients and age-matched controls.[37] Interestingly, their results indicated that a key factor in determining a positive or negative outcome was duration of illness with resting hypofrontality being characterized as a chronically developed feature of the disease potentially associated with anatomical and/or functional changes in frontal microcirculation. Indeed, this would be in agreement with the original report of Ingvar and Franzén.[1] While NIRS is a technique which is relatively insensitive to head motion and does not require potentially stressful injections, it is not readily applicable to behaving animals. In addition, while it does enable the observation of dynamic changes in rCBF, it does not facilitate measurement of other metabolic parameters such as glucose and O_2.

Effect of Saline. As PCP was administered systemically we first performed control experiments involving normal saline (NaCl 0.9%) injections. The signals for all three analytes exhibited short-lived changes associated with the injection stress. Glucose decreased by 0.02 ± 0.01 nA from a baseline of 0.43 ± 0.09 nA (P = 0.0160), representing a maximum decrease of 5% (ca. 13 μM) after 39 ± 3 s (n = 4). The signal returned to baseline levels after 93 ± 5 s (n = 4). The O_2 reduction current increased to a maximum of −168.5 ± 17.1 nA (32% (ca. 38 μM), P = 0.0030) after 33 ± 7 s and returned to baseline levels (−127.1 ± 14.6 nA) after 111 ± 12 s (n = 4). A similar increase was observed in NO which reached a maximum of 4.0 ± 0.8 pA (2% (ca. 2.5 nM), P = 0.0077) after 2.2 ± 0.4 min and returned to baseline (266 ± 34 pA) after 5.9 ± 1.3 min (n = 5). There was no significant difference in the baseline signals before and after the brief injection effects with the baseline values remaining stable for up to 60 min post injection: P = 0.0696 (glucose), P = 0.2396 (O_2), and P = 0.0504 (NO). A typical example of saline induced changes in the NO signal is shown in Figure 3A. Similar brief injection effects for saline administration have also been observed for glucose,[40] O_2[40] and NO[18] monitored using LIVE sensors implanted in the striatum. The stress of the injection stimulates neuronal activation,[41] increasing rCBF and thus O_2, and decreasing glucose through an increase in neuronal glucose utilization.

Effect of PCP. Typical data for glucose and O_2 recorded over several hours using sensors implanted in the prefrontal cortex following administration of PCP (10 mg/kg) is shown in Figure 4. Simultaneously recorded motor activity is also shown and highlights the expected locomotor hyperactivity associated with this dose of PCP.[42,43] There was an initial brief decrease in glucose following injection which lasted several (ca. 18) minutes and was concomitant with the start of the increase in motor activity. This was followed by a gradual increase to a maximum of 4.21 ± 0.28 nA after 72.8 ± 16.5 min, representing an increase of 55 ± 7% (ca. 982 μM) from a baseline level of 2.75 ± 0.20 nA (P < 0.0001, n = 15). The signal then remained elevated for several hours before returning to a preinjection

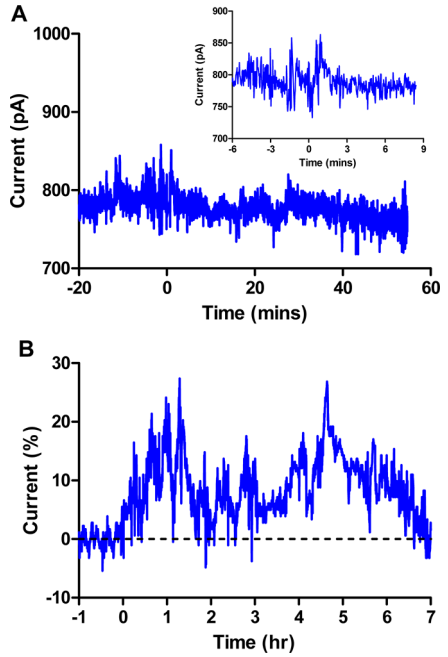

Figure 3. (A) Typical example of the effect of a saline injection on nitric oxide monitored in the prefrontal cortex of a freely moving rat. Inset: Close-up of the injection region. (B) A typical example of the effect of the administration of PCP (10 mg/kg) on nitric oxide. Time zero indicates the point of injection.

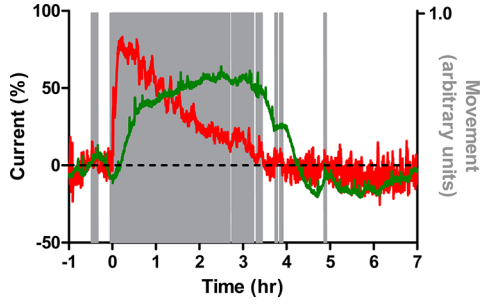

Figure 4. Typical examples of the effects of the administration of PCP (10 mg/kg) on glucose (green) and O_2 (red) monitored simultaneously in the PFC of a freely moving rat. Time zero indicates the point of injection. The gray lines represent motor activity (movement) recorded using a passive infrared detector.

baseline of 2.63 ± 0.14 nA (P = 0.2981) 275.0 ± 34.1 min after injection. Oxygen immediately began to rise following injection reaching a maximum of −164.1 ± 4.4 nA after 16.1 ± 2.7 min, representing an increase of 70 ± 4% (ca. 62 μM) from a baseline level of −97.1 ± 2.3 nA, (P < 0.0001, n = 15). The signal then began to decrease reaching a preinjection baseline of −96.9 ± 2.3 nA (P = 0.2981) 198.7 ± 13.9 min after injection. NO levels also increased following injection (see Figure 3B) reaching a maximum of 401 ± 60 pA 45 ± 6 min post injection, representing an increase of 8 ± 2% (ca. 31 nM) from a baseline level of 375 ± 56 pA (P = 0.0447, n = 13). In a similar manner to glucose the signal then remained elevated for several hours before rapidly returning to a preinjection baseline of 379 ± 95 pA (P = 0.3085) 470 ± 82 min after injection. The total change in signal for each analyte in response to both saline and PCP was calculated as the area under the curve: Glucose −0.06 ± 0.01 nA × min (saline, n = 4), 275.0 ± 34.1 nA × min (PCP, n = 15); O_2 −278.1 ± 23.4 nA × min (saline, n = 4), 6792.3 ±

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374.7 nA × min (PCP, n = 15); NO −0.045 ± 0.008 nA x min (saline, n = 5), 23.4 ± 1.4 nA × min (PCP, n = 5). These results clearly show that LIVE sensors can provide continuous real-time metabolic data in an animal model of psychosis. Not only that, they also enable simultaneous recording of multiple data sets facilitating a direct comparison and investigation of their inter-relationship.

The observed increase in O_2 suggests an initial rapid increase in rCBF,[40] and given that the changes were coincident with increased locomotor activity it is likely to be due to increased delivery to cope with the increased energy demand. The slower increase in glucose is also more likely to reflect increased supply rather than a decrease in metabolic activity, and hence glucose utilization as observed in resting state hypofrontality. Since the O_2 signal represents the balance between supply from the vasculature and utilization, the subsequent declining signal suggests a decrease in supply rather than increased utilization, given the continuous locomotor hyperactivity and the fact that the glucose signal remains elevated during this declining period. The uncoupling and the difference in the time scales for the changes in glucose and O_2 is something we have observed previously with anesthetics[40] and during mild neuronal activation induced by a 5 min period of stimulation (tail pinch).[22] While some of the supplied glucose may be coming from the increased blood flow it is likely that there is a significant contribution from another source or intermediate compartment. Evidence suggests that this is likely to involve astrocytes, which now appear to play a central role in the delivery of metabolic substrates.[44,45]

One of the current theories of hypofrontality suggests that a hyper-glutamatergic state of the PFC corresponds to hypo-activation which is caused by a disruption of glutamatergic signaling of the NMDA receptor complex.[5] This acute NMDA receptor antagonist model is in apparent contradiction to the profound increases in cortical excitatory activity observed following NMDA receptor blockade, that is, hyperfrontality, or increases in glucose utilization and blood flow in the PFC.[46−49] However, recent animal studies using techniques directly analogous to those used in patients have demonstrated that repeated or chronic treatment with PCP evokes neuroadaptive processes more relevant to schizophrenia than acute treatment and that these processes manifest as hypofrontality.[50] The glucose and O_2 data presented herein support a hyperfronal effect following subchronic treatment. The NO data suggests a potential role for this gaseous second messenger of the NMDA receptor in mediating this process. Indeed, it has previously been implicated in the mechanism of PCP psychosis[23,51,52] as well as cognitive dysfunction.[53,54] It has also recently been reported that the hyperactive state of glutamatergic neurons in the PFC may be mediated through inhibition of GABAergic interneurons by NO.[5] One must also remember that NO interacts with the dopaminergic as well as the serotonergic systems, thereby linking glutamatergic to monoaminergic signaling. In fact disruption in the latter suggests that hypofrontality is a delayed consequence of PCP administration; repeated PCP exposure has been reported to induce a selective reduction in basal and stress-evoked dopamine utilization in the PFC, and rats previously subchronically treated with PCP were found to be impaired in a spatial working memory task.[55] Interestingly, these dopaminergic and cognitive deficits were observed following withdrawal from PCP, and as such were reported to be due to drug-induced neurochemical changes rather than direct drug effects.

CONCLUSIONS

This study presents real-time neurochemical measurements of glucose, O_2, and NO recorded using microelectrochemical sensors in an animal model of schizophrenia known to induce hypofrontaility. Injection of saline produced small short-lived changes in all three analytes while PCP resulted in locomotor hyperactivity and increased signals lasting several hours. The time course of changes in glucose and NO were similar; both signals increased gradually over the first hour post injection and remained elevated to within an hour of returning to baseline levels (after ca. 5 and 7 h, respectively). Oxygen on the other hand increased rapidly following injection reaching a maximum after ca. 15 min and then slowly returning to baseline over a period of ca. 3 h. While the presented results provide direct in situ real-time neurochemical and behavioral information on the effects of PCP administration validating the application of LIVE sensors in such studies, they do not support a hypofrontal effect for subchronic doses and reinforce the notion of disturbed prefrontal NO neurotransmission playing a role in schizophrenia. Future work will investigate whether a metabolic hypofunction exits under chronic conditions and if post chronic PCP administration is a better model for schizophrenia, as has been suggested for dopamine.

METHODS

Chemicals and Solutions. Saline solutions (0.9%) were prepared by dissolving 0.9 g of NaCl in 100 mL of doubly distilled water. Phencyclidine hydrochloride (PCP, Sigma Chemicals, St. Louis, MO) was dissolved in saline and administered via systemic (intraperitoneal, glucose and O_2; subcutaneous, NO) injection. A dose of 10 mg/kg in a volume of 2 mL was used based on previously published studies.[43,55] All standard chemicals (NaCl, NaH_2PO_4, and NaOH) were used as received and purchased from Sigma-Aldrich Ireland Ltd. (Dublin).

Sensor Preparation. Sensors for O_2 (carbon paste disk electrodes; 8T Teflon-coated Ag wire, 200-μm bare diameter, 270-μm coated diameter, Advent Research Materials, Suffolk, U.K.), glucose (5T Teflon-coated Pt disk electrodes modified with poly(o-phenylenediamine) and glucose oxidase; 125 μm bare diameter, 160 μm coated diameter, Advent Research Materials), and NO (5T Teflon-coated Pt disk electrodes modified with Nafion) were prepared following previously reported protocols.[15,18,19] In vitro calibrations were performed over physiologically relevant concentrations (glucose, 0−10 mM; O_2, 0−240 μM; NO, 0−1 μM) in a standard three-electrode glass electrochemical cell containing 15 mL of phosphate buffer saline (PBS) solution, pH 7.4 (0.15 M NaCl, 0.04 M NaH_2PO_4, and 0.04 M NaOH). A saturated calomel electrode (SCE) was used as the reference electrode, and a Pt wire served as the auxiliary electrode.

Instrumentation and Software. Constant potential amperometry (CPA; −650 mV (O_2), +700 mV (glucose), and +900 mV (NO)) was performed in all electrochemical experiments using a low-noise custom designed potentiostat (Biostat IV, ACM Instruments, Cumbria, U.K.). Data acquisition was performed with a notebook PC, a PowerLab interface system (ADInstruments Ltd., Oxford, U.K.), and LabChart for Windows software (ADInstruments Ltd.).

All data are presented as mean ± standard error (SEM). Data is reported as baselines, maximum/peak responses (currents) and durations (time). To facilitate ease of comparison area under curve (AUC) analysis was performed to quantify the observed changes in the sensor signals. For PCP the AUC was calculated by integrating the current for each analyte over the period during which it deviated from the baseline. For saline the AUC was calculated for a fixed period of 10 min. For presentation (in Figures 3 and 4), data was normalized to the respective baseline levels for each sensor. All analysis was performed using Microsoft Excel 2007 and the commercial packages Prism (version 5.01) and InStat (GraphPad Software Inc., La Jolla, CA). The statistical significance of differences observed was calculated using Student's t tests (two-tailed paired or unpaired observations where

appropriate). Values of $P < 0.05$ were considered to indicate statistical significance.

Surgical Procedures. Male Wistar rats weighing 200−300 g were anesthetized with the volatile anesthetic isoflurane (4% in air for induction, 1.5−3.0% for maintenance; IsoFlo, Abbott, U.K.) using a Univentor 400 Anaesthesia Unit (AgnTho's AB, Sweden). Once surgical anesthesia was established, animals were placed in a stereotaxic frame and the sensors implanted following a previously described procedure.[22] Coordinates for the prefrontal cortex with the skull leveled between bregma and lambda were as follows: A/P + 3.2, M/L ± 0.8 from bregma, and D/V −4.2 from dura. Sensors were implanted bilaterally in each animal in either glucose/O_2 or NO/NO combinations. A reference electrode (8T Ag wire, 200 μm bare diameter; Advent Research Materials) was placed in the cortex and an auxiliary electrode (8T Ag wire) attached to one of the support screws (see below). The reference potential provided by the Ag wire in brain tissue is very similar to that of the SCE. The electrodes were fixed to the skull with dental screws and dental acrylate (Associated Dental Products Ltd., Swindon, U.K.). Postoperative analgesia was provided in the form of a single injection (0.1 mg/kg, s.c.) of Vetergesic (buprenorphine hydrochloride, Reckitt and Colman Pharmaceuticals, Hull, U.K.) given immediately following the surgery. Animals were allowed to recuperate for 24 h after surgery and were assessed for good health according to published guidelines[56] immediately after recovery from anesthesia and at the beginning of each day. This work was carried out under license in accordance with the European Communities Regulations 2002 (Irish Statutory Instrument 566/2002, Amendment of Cruelty to Animals Act 1876).

Experimental Conditions in Vivo. Rats were housed in large plastic bowls, in a windowless room under a 12 h light, 12 h dark cycle, lights coming on at 8 a.m., with free access to water. Food was available ad libitum. All experiments were carried out with the animal in its home bowl. Implanted sensors were connected to the potentiostat after the 24 h recuperation period, through a six-pin Teflon socket (MS363, Plastics One, Roanoke, VA), and a flexible screened six core cable (363−363 6TCM, Plastics One) which was mounted through a swivel (SL6C, Plastics One) above the rat's head. This arrangement allowed free movement of the animal which remained continuously connected to the instrumentation. After application of the appropriate applied potential each animal was given a further 24 h before experiments were begun in order to ensure that the background currents for the electrodes were completely stabilized. A low-pass digital filter (50 Hz cutoff) was used to eliminate mains AC noise and all data was recorded at 4 Hz.

Animal motor activity (movement) was recorded using a passive infrared (PIR) detector (Gardscan QX PIR, Gardiner Technology, Queensway, Rochdale, OL11 1TQ, UK) positioned directly over the home bowl and connected to a dedicated channel in the data acquisition system (PowerLab/LabChart). Movement originating in the field of view of the sensor activates the "alarm" function of the detector thereby sending a count (voltage trigger) to the PowerLab which appears as a 0/1 (off/on) spike in the LabChart trace. This facilitates the direct correlation of movement data with the in vivo data from the implanted sensors.

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Notes

The authors declare no competing financial interest.

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