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*A submission to the Analyst (themed issue In Vivo Analysis)*

**A microelectrochemical biosensor for real-time in vivo  
monitoring of brain extracellular choline**

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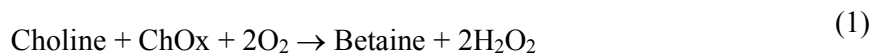
**Abstract**

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7 A first generation Pt-based polymer enzyme composite biosensor developed for real-  
8 time neurochemical monitoring was characterised *in vivo* for sensitive and selective  
9 detection of choline. Confirmation that the sensor responds to changes in extracellular  
10 choline was achieved using local perfusion of choline which resulted in an increase in  
11 current, and the acetylcholinesterase inhibitor neostigmine which produced a  
12 decrease. Interference by electroactive species was tested using systemic  
13 administration of sodium ascorbate which produced a rapid increase in extracellular  
14 levels before gradually returning towards baseline over several hours. There was no  
15 overall change in the response of the biosensor during the same period of recording.  
16 Oxygen interference was examined using pharmacological agents known to change  
17 tissue oxygenation. Chloral hydrate produced an immediate increase in O<sub>2</sub> before  
18 gradually returning to baseline levels over 3 h. The biosensor signal displayed an  
19 initial brief decrease before increasing to a maximum after 1h and returning to  
20 baseline within 2 h. L-NAME caused a decrease in O<sub>2</sub> before returning to baseline  
21 levels after *ca.* 1.5 h. In contrast, the biosensor current increased over the same time  
22 period before slowly returning to baseline levels over several hours. Such differences  
23 in time course and direction suggest that changes in tissue O<sub>2</sub> levels do not affect the  
24 ability of the sensor to monitor choline reliably. Although it was found to rapidly  
25 respond to behavioural activation, examination of baseline *in vivo* data suggests a  
26 stable viable signal for at least 14 days after implantation. Using *in vitro* calibration  
27 data the basal extracellular concentration of choline was estimated to be 6.3 μM.  
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## Introduction

Acetylcholine (ACh) is an important neurotransmitter in both the central and peripheral nervous systems,<sup>1-3</sup> and has been implicated in many critical need late-onset disorders such as Parkinson's and Alzheimer's disease.<sup>4-6</sup> Analytical measurements of ACh *in vivo* have been mainly performed using brain microdialysis,<sup>7, 8</sup> with some reports of monitoring using microelectrochemical biosensors.<sup>9-13</sup> However, the poor spatial and temporal resolution of the former, and the need to use multiple enzymes in the latter, have generally limited their applications. An alternative approach has been to target its precursor and metabolite, choline (Ch);<sup>1, 14, 15</sup> ACh is synthesised in nerve terminals from acetyl coenzyme A and Ch in a reaction catalysed by choline acetyltransferase, while rapid inactivation of acetylcholine by acetylcholine esterase produces Ch. In fact, such rapid fluxes in Ch produced by this process have been validated as a marker of cholinergic (ACh) activity in brain tissue.<sup>1, 15, 16</sup>

There have been several reports of first (H<sub>2</sub>O<sub>2</sub> detecting) and second (mediator detecting) generation choline oxidase-based electrochemical biosensors for monitoring choline in various applications. The overall enzymatic process may be written as Reaction 1, while the current generating electrochemical step is represented by Reaction 2:



Different transducers have been used, including conducting organic salts,<sup>17</sup> carbon fibres,<sup>1</sup> noble metals<sup>9</sup> and ceramic-based multisite microelectrode arrays,<sup>15</sup> with varying degrees of success. We have previously reported the development and characterisation of Pt-based microelectrochemical biosensors for glucose, H<sub>2</sub>O<sub>2</sub> and glutamate based on novel polymer enzyme composite designs.<sup>18-21</sup> Operational characteristics such as sensitivity, selectivity, response time, limit of detection, etc, have been characterised in detail for each device and their target substrate. Some have subsequently been used to study brain function in different application (e.g. pharmacological) studies.<sup>22-25</sup> Based on knowledge from our previous designs we have now modified the coating procedure to allow immobilisation of choline oxidase onto the electrode surface<sup>26</sup> using poly(*o*-phenylenediamine) (PPD) for interference

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3 rejection, and various stabilising agents including methyl methacrylate (MMA),  
4 cellulose acetate, bovine serum albumin (BSA), glutaraldehyde, and  
5 polyethyleneimine (PEI). In this study we have characterised the sensitivity,  
6 selectivity and performance of this new choline microelectrode *in vivo*. The resulting  
7 biosensor compares positively with previously reported devices, allowing rapid,  
8 sensitive and selective neurochemical measures of choline in freely-moving animals,  
9 with the benefit of stable chronic recordings lasting up to two weeks.  
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## 16 **Experimental**

### 17 **Chemicals and solutions**

18 The NaCl (SigmaUltra), NaH<sub>2</sub>PO<sub>4</sub> (Sigma, A.C.S. reagent), NaOH (SigmaUltra), KCl  
19 (SigmaUltra), CaCl<sub>2</sub> (SigmaUltra), MgCl<sub>2</sub> (SigmaUltra), Choline chloride (≥ 97 %),  
20 L-Ascorbic acid (AA; sodium salt, A.C.S. reagent), Bovine serum albumin (BSA,  
21 fraction V from bovine plasma), Glutaraldehyde (Grade 1, 25 %), *o*-  
22 Phenylenediamine (*o*-PD, 1,2-diaminobenzene, ≥ 98 %), Polyethyleneimine (PEI, 80  
23 % ethoxylated), Methyl methacrylate (MMA, 99 %), L-N<sup>G</sup>-Nitroarginine methyl ester  
24 hydrochloride (L-NAME), Neostigmine bromide (≥ 98 %), Cellulose acetate  
25 (Mn ~50,000 g/mol) and the enzyme choline oxidase (ChOx; from *Alcaligenes* sp.,  
26 EC 232-840-0, 1 KU) were supplied by Sigma-Aldrich Ireland Ltd (Dublin). Chloral  
27 hydrate was obtained from BDH Laboratory Supplies (Poole, UK).  
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37 All electrochemical experiments *in vitro* were carried out in a phosphate buffered  
38 saline (PBS) solution, pH 7.4; NaCl (150 mM), NaH<sub>2</sub>PO<sub>4</sub> (40 mM) and NaOH (40  
39 mM). Fresh solutions of choline chloride (0.1 M), *o*-PD monomer (300 mM in N<sub>2</sub>-  
40 saturated PBS), BSA (1 %), cellulose acetate (2 % in 2:1 acetone:ethanol),  
41 glutaraldehyde (0.5 %), PEI (2 %) and ChOx (500 U/mL in PBS) were prepared as  
42 needed. All solutions were either used as supplied, or prepared using doubly distilled  
43 water unless otherwise stated.  
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49 For *in vivo* experiments normal saline (0.9 %) and artificial cerebrospinal fluid  
50 (aCSF; 147 mM NaCl, 4 mM KCl, 1.2 mM CaCl<sub>2</sub>, and 1 mM MgCl<sub>2</sub>) solutions were  
51 prepared as previously reported.<sup>27</sup> Solutions for systemic administrations (AA, 0.5 g  
52 kg<sup>-1</sup>; L-NAME, 30 mg kg<sup>-1</sup>; chloral hydrate, 350 mg kg<sup>-1</sup>) were prepared in normal  
53 saline and administered by intraperitoneal injection. The Neostigmine solution (100  
54 mM) for local perfusion (microdialysis, MD) was prepared in aCSF.  
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### Working electrode preparation

Carbon paste disk electrodes (CPEs) for AA measurements (8T Teflon-coated Ag wire, 200  $\mu\text{m}$  bare diameter, 270  $\mu\text{m}$  coated diameter, Advent Research Materials, Suffolk, U.K.) were prepared following a previously reported protocol.<sup>28</sup> The electrode used for O<sub>2</sub> measurements was a 5T Teflon<sup>®</sup>-coated Pt/Ir (90 %/10 %) disk electrode (125  $\mu\text{m}$  bare diameter, 175  $\mu\text{m}$  coated diameter, Advent Research Materials, Suffolk, UK).<sup>29</sup> The electrodes were approximately 6 cm in length and approximately 3 mm of Teflon<sup>®</sup> insulation was removed from one end of the wire. This was subsequently soldered into a gold clip (Fine Science Tools GmbH, Heidelberg, Germany) to provide rigidity and electrical contact for connection to the potentiostat. The opposite end of the wire acted as the active surface and a disk was freshly cut just before use. Cylinder electrodes for biosensor construction were also prepared from approximately 6 cm lengths of the coated wire. After connection of the gold clip approximately 1.0  $\pm$  0.1 mm of Teflon was then stripped away from the other end using a microscope. The bare wire electrodes were first coated with an electropolymerised PPD layer<sup>29</sup> before initially being dipped (*ca.* 0.5 s) into the MMA and cellulose acetate solutions, and then sequentially dipped into ChOx, BSA, glutaraldehyde and PEI using a dip adsorption method,<sup>21</sup> with each layer being allowed 5 min drying time before repeating the sequence a further nine times. These were allowed to dry at room temperature for a minimum of an hour and stored at 4 °C until calibrated before use. Hereafter we refer to this polymer composite electrode as Pt-PC/ChOx/PC.

### Instrumentation and software

*In vitro* calibrations were performed (choline: 0 – 3 mM; AA 0 – 1,000  $\mu\text{M}$ ; O<sub>2</sub>: 0 – 1,200  $\mu\text{M}$ ) in a standard three-electrode glass electrochemical cell containing 20 mL PBS solution following procedures similar to those previously reported.<sup>28, 30</sup> A saturated calomel electrode (SCE) was used as the reference electrode, and a Pt wire served as the auxiliary electrode. Constant potential amperometry (CPA; +700 mV (choline), +250 mV (AA), -650 mV (O<sub>2</sub>)) was performed in all electrochemical experiments using custom designed low-noise potentiostats: *In vitro* - Biostat IV, ACM Instruments, Cumbria, UK; *In vivo* - Biostat II, Electrochemical and Medical Systems, Newbury, UK. Data acquisition was performed with either a notebook PC

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3 (in vitro) or Mac<sup>®</sup> (in vivo), a PowerLab interface system (ADInstruments Ltd.,  
4 Oxford, UK) and LabChart<sup>®</sup> for Windows and Mac<sup>®</sup> (Version 6, ADInstruments Ltd).  
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6 All data are presented as mean  $\pm$  standard error (SEM), *n* refers to the number of  
7 determinations (repeat measurements) expressed along with the *number of*  
8 *animals/number of sensors*. Data is reported as baselines, maximum/peak responses  
9 (currents) and durations (time). All analysis was performed using the commercial  
10 package Prism (version 6.04; GraphPad Software, Inc., CA, USA). Paired *t*-tests  
11 (two-tailed) and one-way ANOVAs with Bonferroni's post hoc test were performed  
12 as appropriate using Prism. Values of  $P < 0.05$  were considered to indicate statistical  
13 significance.  
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### 20 21 22 **Surgical procedures**

23 Male Wistar rats (200-300 g; Charles River Laboratories International, Inc., UK)  
24 were anaesthetised with the volatile anaesthetic isoflurane (4 % in air for induction,  
25 1.5-3.0 % for maintenance; IsoFlo<sup>®</sup>, Abbott, UK) using a Univentor 400 Anaesthesia  
26 Unit (AgnTho's AB, Sweden). The level of anaesthesia was checked regularly (pedal  
27 withdrawal reflex). Once surgical anaesthesia was established animals were placed in  
28 a stereotaxic frame and the sensors implanted following a previously described  
29 procedure.<sup>25</sup> Coordinates for the striatum with the skull levelled between bregma and  
30 lambda, were: A/P + 1.0, M/L  $\pm$  2.5 from bregma, and D/V -6.0 from dura. Sensors  
31 were implanted bilaterally in either Pt-PC/ChOx/PC<sub>choline</sub>|Pt-PC/ChOx/PC<sub>choline</sub>-  
32 MD<sub>probe</sub>, Pt-PC/ChOx/PC<sub>choline</sub>|PtO<sub>2</sub>, CPE<sub>AA</sub>|CPE<sub>AA</sub> or Pt-PC/ChOx/PC<sub>choline</sub>|Pt-  
33 PC/ChOx/PC<sub>choline</sub> combinations. A reference electrode (8T Ag wire, 200  $\mu$ m bare  
34 diameter; Advent Research Materials) was placed in the cortex and an auxiliary  
35 electrode (8T Ag wire) attached to one of the support screws. The reference potential  
36 provided by the Ag wire in brain tissue is very similar to that of the SCE. All  
37 microdialysis probes were MBR-4 (BASi, West Lafayette, IN, USA). The  
38 electrodes/probes were fixed to the skull with dental screws (Fine Science Tools  
39 GmbH) and dental acrylate (Dentalon<sup>®</sup> Plus, Heraeus-Kulzer, Germany). All animals  
40 were given saline (0.9 %) and analgesia (Buprecare<sup>®</sup>, AnimalCare Ltd., UK) and  
41 allowed to recover in a thermostatically controlled cage (Thermacage MkII, Datasand  
42 Limited, Manchester, UK) for several hours.  
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3 Animals were allowed to recuperate for 24 h after surgery before being connected  
4 to the instrumentation. They were assessed for good health according to published  
5 guidelines<sup>31, 32</sup> immediately after recovery from anesthesia and at the beginning of  
6 each day. This work was carried out under license in accordance with the European  
7 Communities Regulations 2002 (Irish Statutory Instrument 566/2002 - Amendment of  
8 Cruelty to Animals Act 1876).  
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### 13 14 15 **Experimental conditions *in vivo***

16 Twenty four hours following recovery, animals were singly housed in Ratum<sup>®</sup>  
17 sampling cage systems (BASi, West Lafayette, IN, USA) in a temperature-controlled  
18 facility with a 12 hour light/dark cycle (lights on at 07:00) with access *ad libitum* to  
19 food and water. All experiments were performed in the animal's home bowl. The  
20 implanted sensors from each animal were connected directly to the potentiostat via the  
21 six-pin Teflon<sup>®</sup> socket (MS363, Plastics One, Roanoke, VA, USA) using a flexible  
22 screened six core cable (363-363 6TCM, Plastics One). This arrangement allowed  
23 free movement of the animal which remained continuously connected to the  
24 instrumentation. After application of the appropriate applied potential each animal  
25 was given a further 24 h before experiments were begun in order to ensure that the  
26 background currents for the electrodes were completely stabilised. A low-pass digital  
27 filter (50 Hz cut-off) was used to eliminate mains AC noise and all data was recorded  
28 at either 1k or 4 Hz. A UniSwitch syringe selector (BASi) was used during the  
29 microdialysis experiments to switch between solutions for perfusion into the probe.  
30 Animal motor activity (movement) was observed visually and recorded using the *Add*  
31 *Comment* command in the LabChart<sup>®</sup> software.  
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### 45 **Results and discussion**

#### 46 ***Control experiments***

47 We chose the striatum for all experiments as it has the highest density of cholinergic  
48 neurons and the highest tissue content of acetylcholine in the brain.<sup>33</sup> Characterisation  
49 of the Pt-PC/ChOx/PC biosensor signal *in vivo* was achieved by testing the response  
50 of the sensor to a broadly defined set of criteria, including verifying that the sensor  
51 responds to the target analyte with good sensitivity, is free or has minimal interference  
52 from endogenous species, and displays good stability under acute or chronic  
53 conditions depending on the required application.<sup>28, 34, 35</sup> Chemical/pharmacological  
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agents used in these tests are administered either systemically or locally, and as such we first performed control experiments involving injection of normal saline (NaCl 0.9%) and local perfusion of aCSF.

Typical examples of the observed changes in signal are shown in Fig. 1. Saline resulted in a short-lived increase in current ( $n = 10, 4/8$ ) of  $0.05 \pm 0.01$  nA ( $P < 0.005$ ) from a baseline of  $1.5 \pm 0.3$  nA, representing a maximum increase of  $5.7 \pm 2.0$  % and a concentration change of  $0.12 \pm 0.04$   $\mu$ M (based on *in vitro* calibration data) after  $27 \pm 9$  s. The signal returned to resting baseline levels ( $1.5 \pm 0.3$  nA,  $P > 0.15$ ) after  $66 \pm 20$  s. However, if the injection was followed by a period of locomotor activity the return to baseline was typically  $32 \pm 3$  min ( $n = 7, 4/5$ ; see *Stability* below). Similar short-lived injection stress effects for saline administration have also been observed for glucose,<sup>24</sup> O<sub>2</sub><sup>24</sup> and NO<sup>35</sup> monitored using sensors implanted in the striatum.

Local perfusion of artificial cerebrospinal fluid (aCSF) through a co-implanted microdialysis probe resulted in an immediate decrease in current ( $n = 13, 5/9$ ) from a baseline of  $4.0 \pm 0.5$  nA to a plateau minimum of  $2.8 \pm 0.3$  nA ( $P < 0.001$ ) after  $40 \pm 6$  min. This represents a percentage decrease of  $29 \pm 3$  % from the pre-perfusion baseline, and a concentration change of  $2.8 \pm 0.8$   $\mu$ M. On cessation of perfusion the signal returned to baseline levels after  $46 \pm 11$  min. Perfusion of aCSF results in removal of choline from the local environment of the sensor causing a decrease in current. We have previously observed this for both glucose<sup>27</sup> and NO.<sup>35</sup> Concentric microdialysis probes with 4 mm membrane lengths perfused at  $2.0$   $\mu$ L  $\text{min}^{-1}$  typically have analyte *in vivo* recovery rates of 30 - 60 % (e.g. glucose 39 %<sup>36</sup> and lactate 56 %).<sup>37</sup> Assuming an average rate of 45 %, minimum changes in relative recovery associated with analyte clearance, and using the concentration change (2.8  $\mu$ M) calculated from *in vitro* calibrations, this would suggest an approximate basal ECF concentration of 6.3  $\mu$ M. This is in good agreement with values estimated by other groups using both sensors (6.6  $\mu$ M - striatum<sup>14</sup> and 4.9  $\mu$ M - frontoparietal cortex<sup>38</sup>) and microdialysis (4.1  $\mu$ M - hippocampus<sup>39</sup>).

### ***Choline response studies***

In order to demonstrate that the biosensor responds to changes in extracellular choline levels a choline solution (800  $\mu$ M) was perfused locally. There was an immediate increase in signal following commencement of perfusion and a subsequent return to baseline post administration (Fig. 2). The current ( $n = 2, 2/2$ ) reached a maximum of

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3 3.8 ± 1.4 nA, (9.1 ± 1.3 %) after 14 ± 4 min, and had returned to baseline (3.4 ± 1.2  
4 nA) after 18 ± 5 min. Similar type increases have been reported for ceramic-based  
5 microelectrode array biosensors for local injections of choline solutions (100 μM –  
6 100 mM) through co-implanted micropipettes.<sup>14, 38, 40</sup>  
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10 In addition, we used the acetylcholinesterase inhibitor neostigmine to block the  
11 hydrolysis of acetylcholine to choline. Initial perfusion of aCSF resulted in a baseline  
12 current of 8.7 ± 3.0 nA (*n* = 3, 2/2), which decreased to a minimum of 8.2 ± 2.8 nA (*P*  
13 < 0.09) after 34 ± 5 min upon switching to neostigmine (Fig. 3 top). This corresponds  
14 to a concentration change of 1.4 ± 0.6 μM. Subsequent perfusion with aCSF resulted  
15 in a return to baseline levels (8.8 ± 3.2 nA, *P* > 0.49). Similar effects of neostigmine  
16 on the response of choline biosensors have been reported previously, demonstrating  
17 the contribution of acetylcholine hydrolysis to the recorded choline signal<sup>14</sup> and  
18 confirming that the latter can be used as a proxy for changes in acetylcholine. Data  
19 recorded from a sensor implanted bilaterally with no probe attached showed no effect  
20 of either aCSF or drug perfusion (Fig. 3 bottom).  
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### 29 **Interference**

30 The *in vivo* application of first generation biosensors (i.e. those incorporating a  
31 flavoenzyme (e.g. choline oxidase) as the biorecognition element<sup>41</sup>) in neurochemical  
32 monitoring can potentially be compromised by interference from endogenous species.  
33 Ascorbic acid (AA) is regarded as the principal electroactive interferent<sup>1, 42</sup> as it has a  
34 high basal level (*ca.* 300–500 μM) and a continuously changing extracellular  
35 concentration.<sup>43</sup> As molecular O<sub>2</sub> is required to produce the signal generating H<sub>2</sub>O<sub>2</sub>  
36 (Reaction 2, see *Introduction*) a clear limitation of the enzymatic reaction (Reaction  
37 1) is that the signal may be prone to interference from the effects of changes in O<sub>2</sub>,  
38 undermining its use to reflect choline concentrations unambiguously.  
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46 In order to determine if AA interferes with choline detection using a Pt-  
47 PC/ChOx/PC biosensor we examined the effect of systemic AA administration (0.5  
48 g/kg) on the biosensor response (Fig. 4). The time course of the changes in AA is best  
49 monitored using a carbon paste electrode and constant potential amperometry at +250  
50 mV;<sup>28</sup> the signal increases immediately reaching a maximum 11 ± 3 min (*n* = 5, 3/5)  
51 after injection before gradually decreasing towards baseline levels over several hours  
52 (Fig. 4, top). There was no overall change in the response of the biosensor (*n* = 9, 4/7)  
53 during a similar period of recording (Fig. 4, bottom): 2.9 ± 0.6 nA (pre-injection  
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3 baseline);  $3.0 \pm 0.7$  nA (11 min,  $P > 0.55$ );  $3.1 \pm 0.7$  nA (60 min,  $P > 0.08$ ), clearly  
4 demonstrating that the observed choline signal is not affected by changes in AA *in*  
5 *vivo*.  
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8 *In vivo* O<sub>2</sub> interference was examined using pharmacological agents known to  
9 change cerebral blood flow and thus tissue oxygenation. Chloral hydrate is a non-  
10 volatile anaesthetic agent which causes central nervous system (CNS) depression  
11 through its metabolite trichloroethanol.<sup>44</sup> We have previously shown that an  
12 anaesthetic dose (350 mg/kg) causes parallel increases in both regional cerebral blood  
13 flow (rCBF) and O<sub>2</sub>, and as such is an ideal agent for examining the O<sub>2</sub> dependence of  
14 a first generation biosensor exposed to increased physiological O<sub>2</sub> levels. The latter  
15 was monitored using a Pt disc electrode and constant potential amperometry at -650  
16 mV. The signal ( $n = 4$ , 3/3) increased following the expected pattern reaching a  
17 maximum after  $22 \pm 4$  min and then gradually returning to baseline over a 3 h ( $188 \pm$   
18 6 min) period (Fig. 5, top):  $-45 \pm 14$  nA, (pre-injection baseline);  $-78 \pm 25$  nA  
19 (maximum,  $P < 0.07$ );  $-46 \pm 14$  nA (188 min,  $P > 0.51$ ).  
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23 The time course of the signal changes observed at the Pt-PC/ChOx/PC biosensor  
24 was different to that found for O<sub>2</sub> (Fig. 5, top). The current ( $n = 7$ , 4/4) displayed an  
25 initial brief decrease over a  $10 \pm 4$  min period, followed by an increase to a maximum  
26 after *ca.*  $61 \pm 17$  min before decreasing to levels below the pre-injection baseline at  
27  $114 \pm 16$  min:  $3.6 \pm 0.4$  nA (pre-injection baseline);  $3.5 \pm 0.4$  nA (decrease,  $P < 0.02$ );  
28  $4.1 \pm 0.5$  nA (maximum,  $P < 0.002$ );  $3.5 \pm 0.4$  nA (114 min,  $P < 0.03$ ). Using *in vitro*  
29 calibration data the maximum increase in the signal corresponds to a choline  
30 concentration change of  $0.8 \pm 0.2$   $\mu$ M, representing a percentage increase of  $14 \pm 2$  %  
31 from the pre-injection baseline. A similar effect (initial decrease followed by increase  
32 and return to baseline) of chloral hydrate ( $400$  mg  $\text{kg}^{-1}$ ) on striatal choline has been  
33 reported by Damsma and Fibiger using microdialysis.<sup>45</sup> However, previous  
34 microdialysis work by Bertorelli *et al.*<sup>46</sup> reported that choline was unaffected by the  
35 same anaesthetic dose. It is difficult to explain this disparity especially as both groups  
36 reported decreased acetylcholine levels during the period of anaesthesia.  
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40 L-N<sup>G</sup>-Nitroarginine methyl ester hydrochloride (L-NAME) is a nitric oxide  
41 synthase (NOS) inhibitor which has previously been shown to decrease cerebral blood  
42 flow *via* vasoconstriction<sup>47</sup> resulting from decreased production of NO. Given the  
43 close correlation between rCBF and oxygenation<sup>48</sup> we used L-NAME to examine the  
44 effect of reduced tissue O<sub>2</sub> levels on the biosensor's response. Injection of L-NAME  
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3 (30 mg kg<sup>-1</sup>,  $n = 3$ , 2/3) caused a decrease in O<sub>2</sub> from a baseline of  $-72 \pm 15$  nA  
4 reaching a minimum of  $-61 \pm 13$  nA ( $P < 0.03$ ) 36 ± 5 min after injection, before  
5 returning to baseline levels ( $-71 \pm 17$  nA, 88 ± 12 min). The current recorded at the  
6 biosensor increased to  $4.8 \pm 0.3$  nA from a baseline of  $4.5 \pm 0.2$  nA ( $P < 0.06$ ) over  
7 the same time period, representing a concentration change of  $0.8 \pm 0.6$  μM, before  
8 slowly returning to baseline levels over several hours.

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11 Taken together, the differences in time course and direction for the O<sub>2</sub> and choline  
12 signals, reflecting physiological phenomena associated with chloral hydrate and L-  
13 NAME, suggest that changes in tissue O<sub>2</sub> levels do not affect the ability of the  
14 biosensor to monitor choline reliably in brain ECF *in vivo*.

### 20 21 **Stability**

22 Contact of biosensors with brain tissue can result in a decrease in sensitivity due to  
23 surface fouling by biological constituents such as lipids and proteins.<sup>49, 50</sup> Such  
24 decreases generally vary between 20 and 50 % and occur over several hours following  
25 implantation. As such we investigated the stability of the Pt-PC/ChOx/PC biosensor  
26 by examining the baseline *in vivo* data for 14 days following implantation (day 0).  
27 This period represents the lifetime of the biosensor as defined by observation of  
28 viable enzyme activity (K.L. Baker, F.B. Bolger and J.P. Lowry, unpublished work).  
29 No significant variation was observed over this period (Fig. 6, top): average baseline  
30 =  $2.8 \pm 0.1$  nA ( $P > 0.99$ , one-way ANOVA with Bonferroni's *post hoc* analysis,  
31  $F_{18,227} = 0.2923$ ; pooled data for 14 days,  $n = 9-26$ , 14/9-26).

32  
33 It is important, however, to point out that during a 24 h period the signal can  
34 exhibit naturally occurring changes from baseline levels. These changes can be rapid,  
35 occurring over several minutes, or more prolonged, lasting one or more hours, and  
36 tend to be associated with physiological phenomena such as grooming, feeding and  
37 locomotor activity. A typical example associated with the latter is shown in Fig. 6  
38 (bottom). Day *et al.*,<sup>51</sup> using microdialysis, reported a positive correlation between  
39 acetylcholine release and locomotor activity in several brain areas including the  
40 striatum, and similar occasionally occurring changes in cholinergic activity measured  
41 using microelectrode biosensors have been reported by other groups.<sup>16</sup>

### 56 57 **Conclusions**

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3 A Pt-based microelectrode was modified with choline oxidase, PPD for interference  
4 rejection, and stabilising agents (MMA, cellulose acetate, BSA, glutaraldehyde, and  
5 PEI), for the sensitive and selective detection of choline in brain extracellular fluid. A  
6  
7 detailed characterisation was carried out in rat striatum under chronic conditions and  
8  
9 significant signal changes were recorded against baseline following local  
10 administration (reverse microdialysis) of choline, and the acetylcholinesterase  
11 inhibitor neostigmine. Systemic administrations of ascorbic acid, chloral hydrate and  
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13 L-NAME suggest minimal interference from physiological levels of potential  
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15 endogenous electroactive interferents, and changing O<sub>2</sub> concentrations. While analysis  
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17 of stability over 14 days indicated no significant change in baseline after implantation,  
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19 the biosensor does respond rapidly to behavioural activation such as locomotor  
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21 activity.  
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### 24 25 **Acknowledgements**

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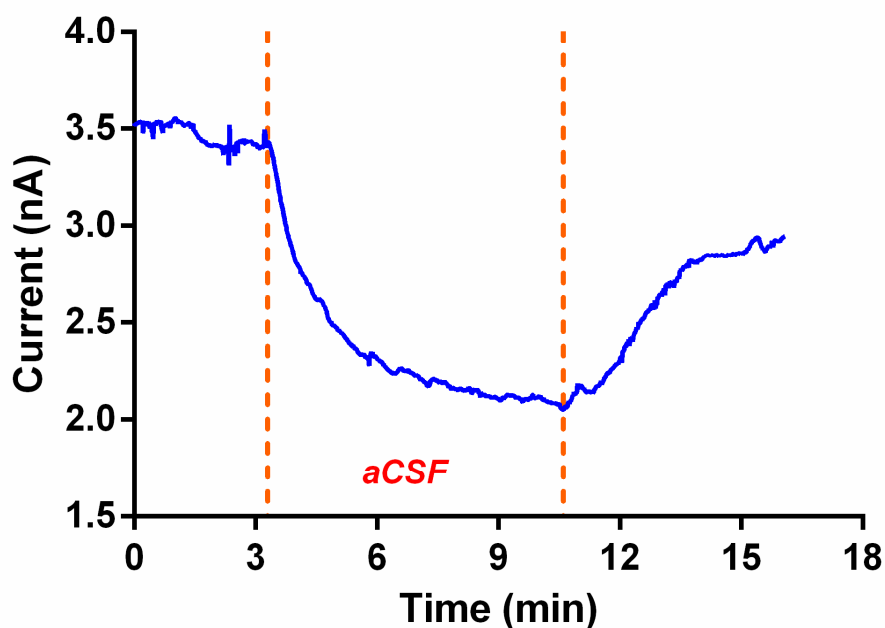
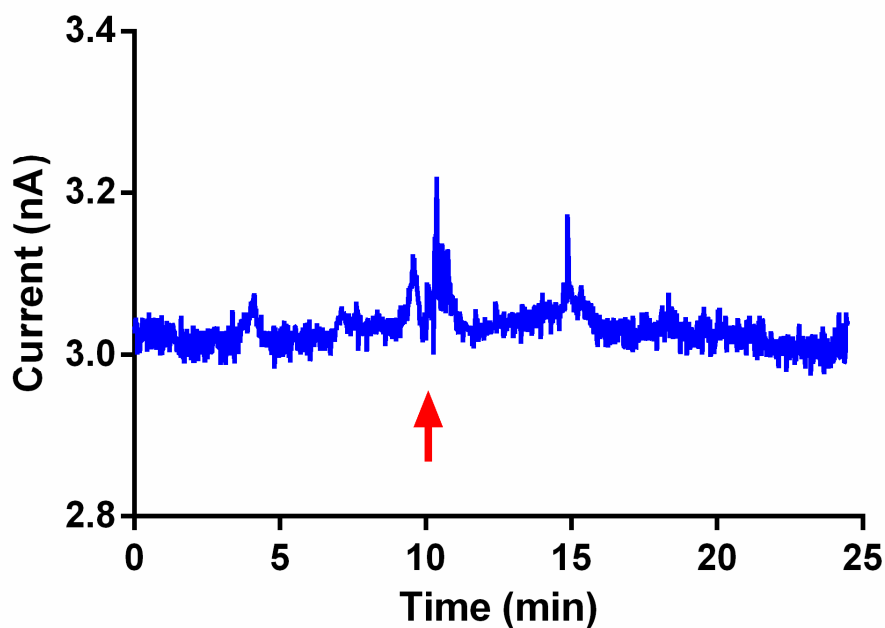
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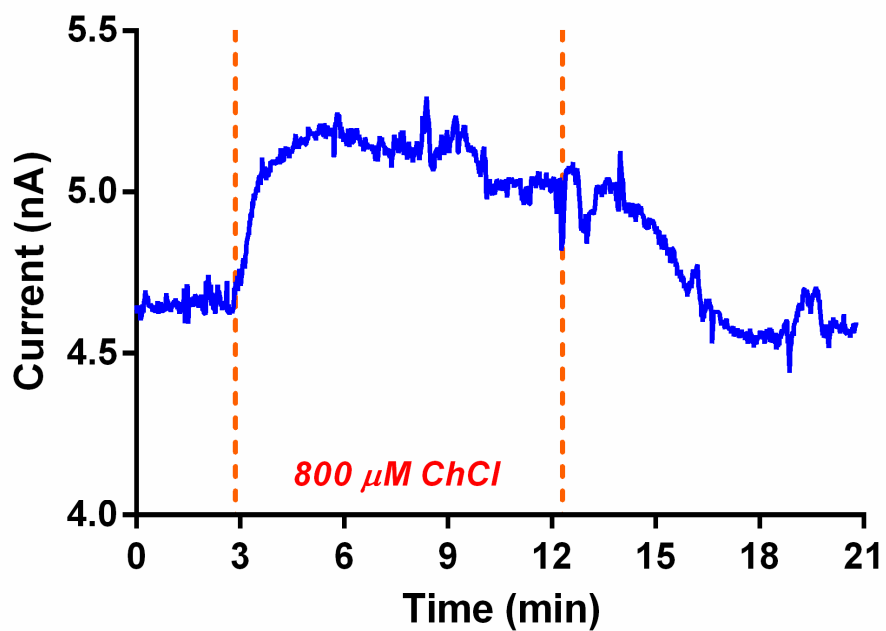


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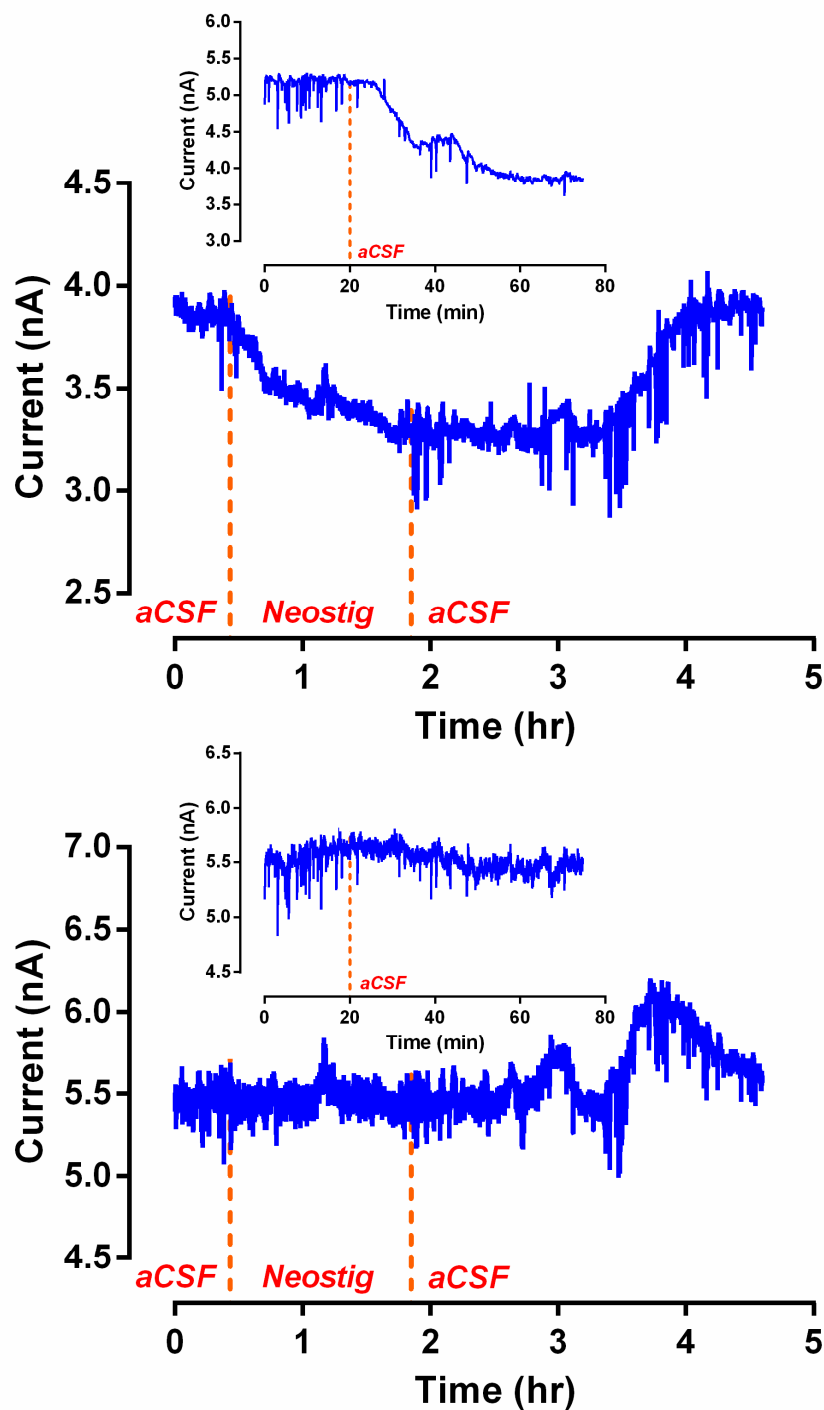




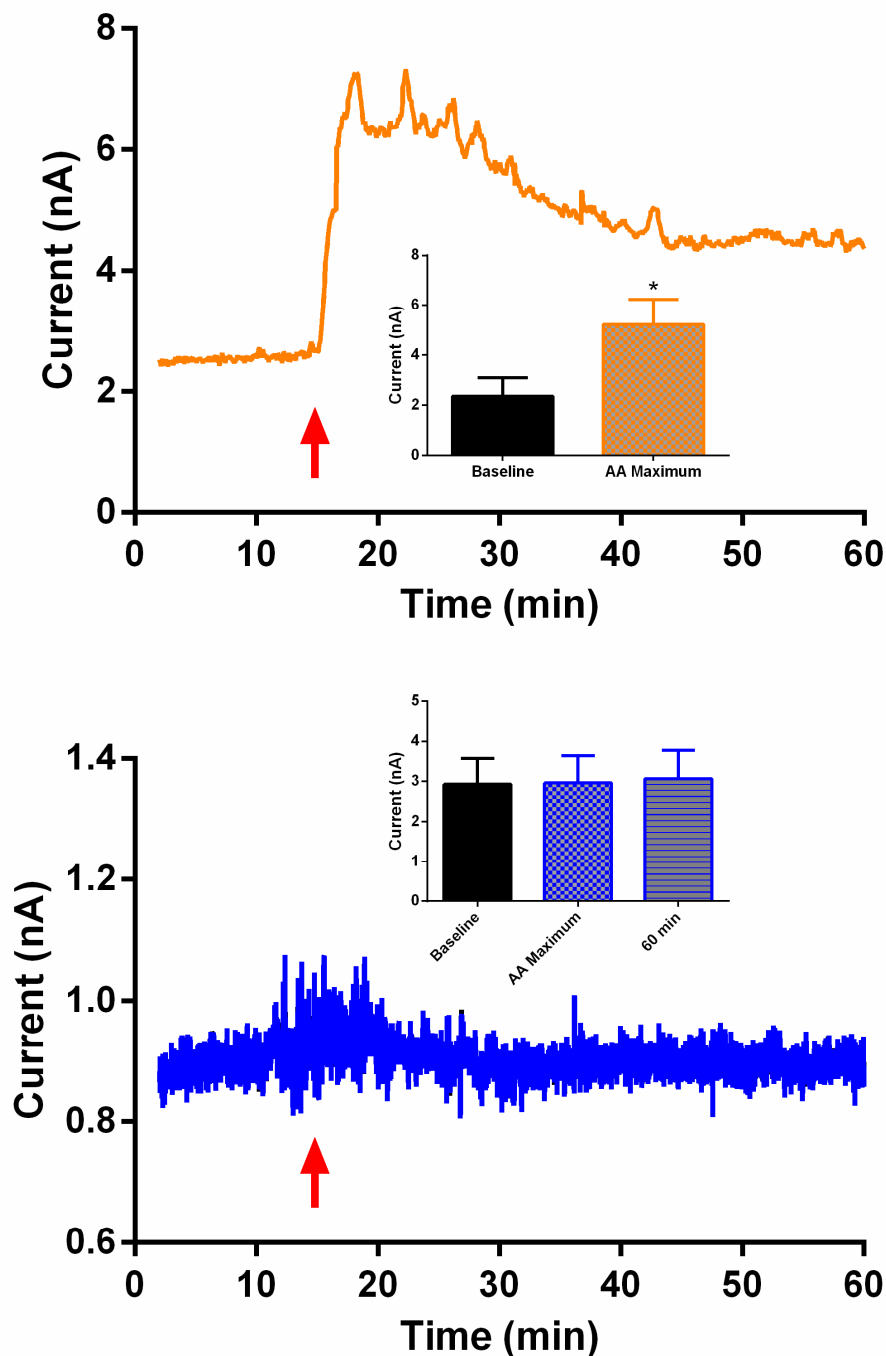
**Fig. 1** Typical examples of the effects of intraperitoneal injection (top) of normal saline (1.0 mL, NaCl 0.9%) and local perfusion (bottom) of aCSF ( $2.0 \mu\text{L min}^{-1}$ ) on the signal recorded from a Pt-PC/ChOx/PC choline biosensor implanted in the striatum of a freely moving rat. Arrow and hashed lines indicate the point of injection and period of perfusion respectively.



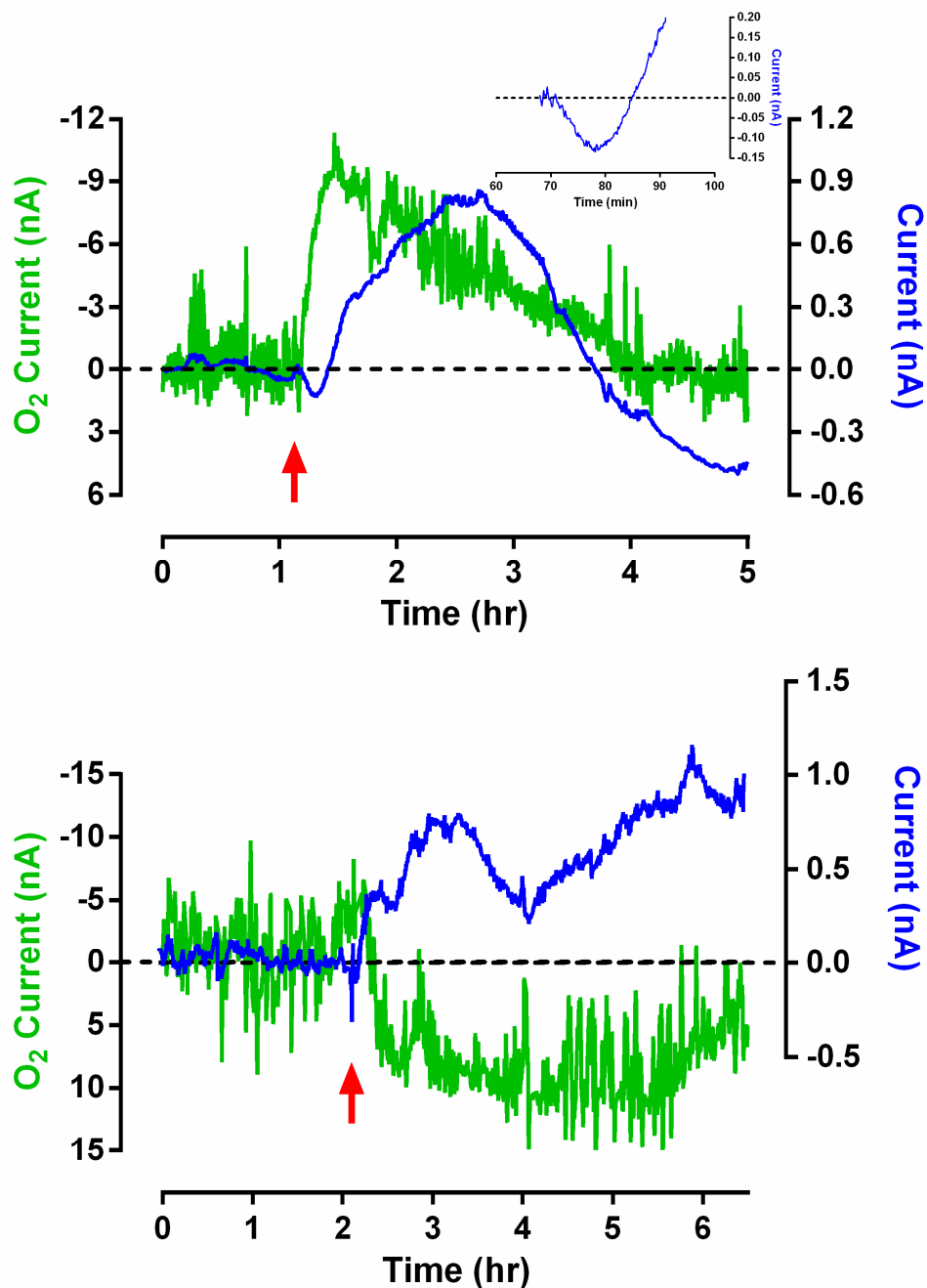
**Fig. 2** A typical example of the effect of local perfusion (reverse microdialysis) of choline chloride ( $800 \mu\text{M}$ ,  $2.0 \mu\text{L min}^{-1}$ ) on the response of an adjacent Pt-PC/ChOx/PC biosensor implanted in the striatum of a freely moving rat.



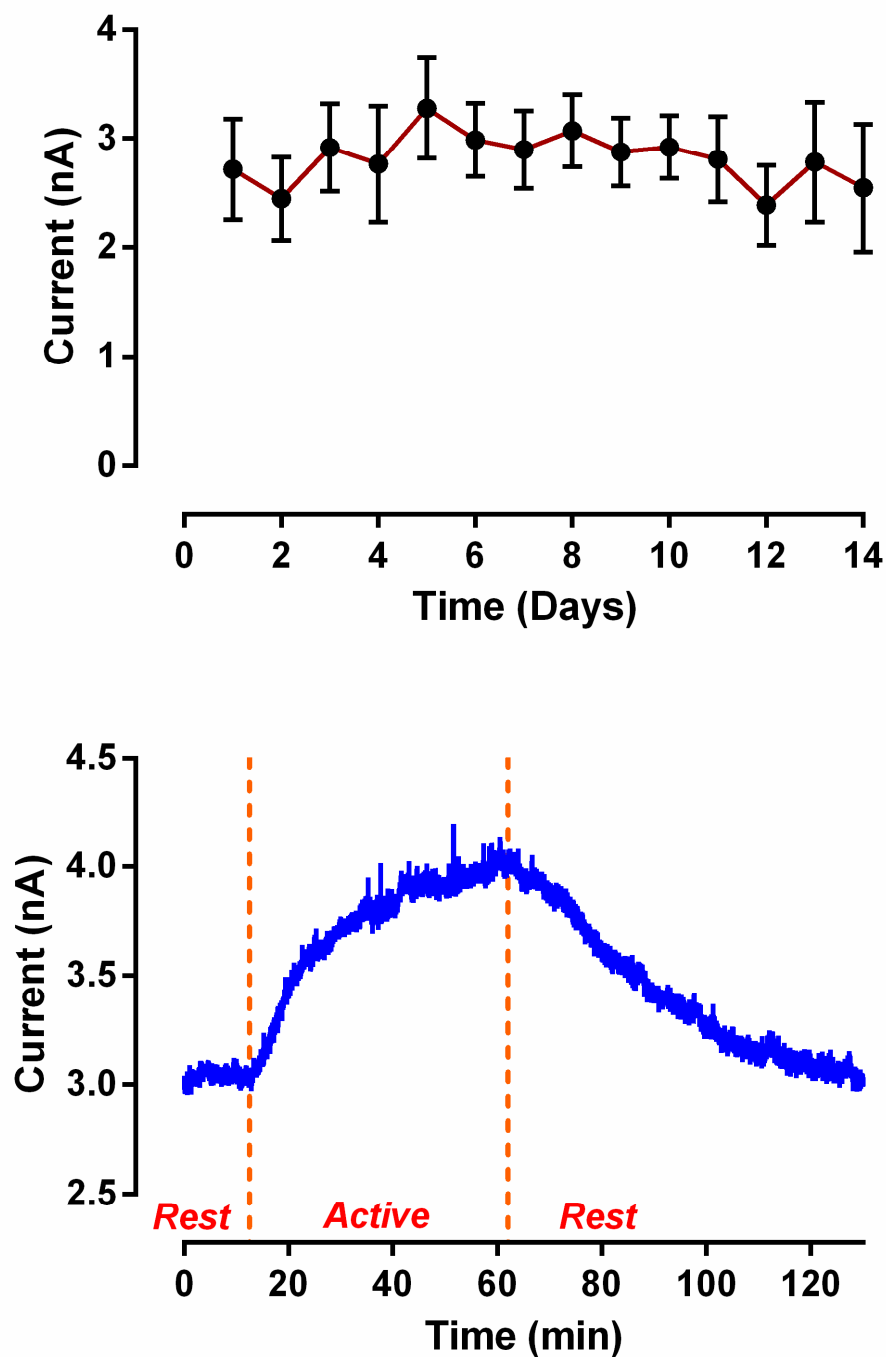
**Fig. 3** Typical examples of the effects of reverse microdialysis perfusion of neostigmine ( $100 \text{ mM}$ ,  $2.0 \mu\text{L min}^{-1}$ ) on the baseline (aCSF perfusion) response of Pt-PC/ChOx/PC biosensors implanted bilaterally with (top; left striatum) and without (bottom; right striatum) a co-implanted microdialysis probe. Hashed lines differentiate the different perfusions. *Insets*: aCSF perfusion prior to neostigmine administration.



**Fig. 4** Typical examples of the effects of intraperitoneal injection of ascorbic acid (AA) on the amperometric response of carbon paste (top) and Pt-PC/ChOx/PC (bottom) sensors implanted in the striatum of freely moving rats. *Inset:* Top - Average AA current before injection (baseline) and at maximum ( $P < 0.05$ ). Bottom - Average choline current before injection (baseline), at AA maximum (11 min) and at 60 min.



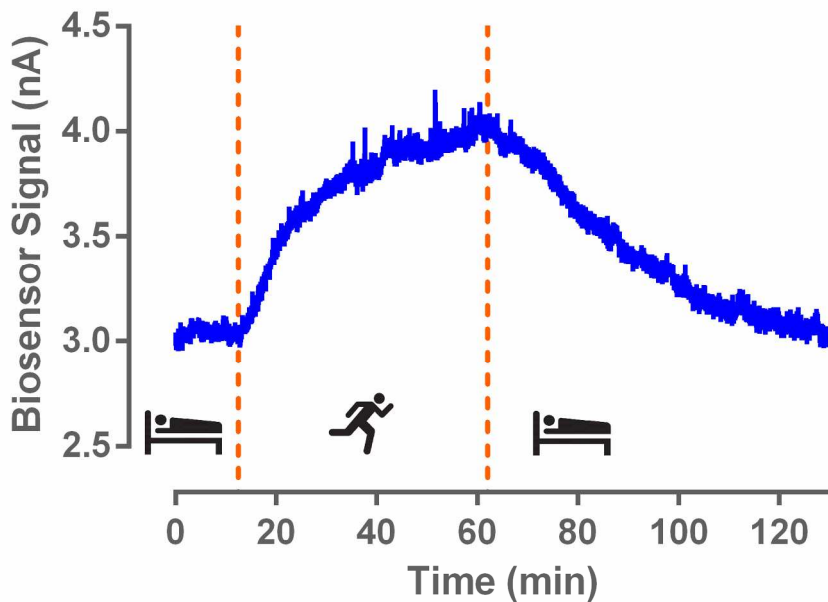
**Fig. 5** Typical examples of the effects of intraperitoneal injection of chloral hydrate ( $350 \text{ mg kg}^{-1}$ ; top) and L-NAME ( $30 \text{ mg kg}^{-1}$ ; bottom) on the O<sub>2</sub> (green) and choline (blue) signals monitored simultaneously *in vivo* with a Pt disc electrode (right striatum) and a Pt-PC/ChOx/PC biosensor (left striatum) respectively. Baselines were normalised to zero to facilitate direct data comparisons. *Inset*: Close-up of initial decrease following administration.



**Fig. 6** Average ( $\pm$  SEM) baseline *in vivo* data (pooled from 14 animals) for Pt-PC/ChOx/PC biosensors ( $n = 9-26$ ) recorded in rat striatum using CPA at +700mV over 14 days (top). All data taken from the same daily one-hour (12 - 1 pm) period. A typical example (bottom) of changes in baseline associated with naturally occurring locomotor (walking/running) activity.

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*Brain Extracellular Choline*



195x152mm (300 x 300 DPI)