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# *In vivo* characterisation of a catalase-based biosensor for real-time electrochemical monitoring of brain hydrogen peroxide in freely-moving animals†

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A catalase-based microelectrochemical biosensor developed for real-time neurochemical monitoring of hydrogen peroxide ($H_2O_2$) was characterised in freely-moving rats. The *in situ* sensitivity of the sensor was assessed by the direct delivery of $H_2O_2$ to the local environment of the implanted sensor and by the chemical manipulation of the endogenous concentration of $H_2O_2$. Inhibitors of $H_2O_2$ enzymatic degradation were utilised including sodium azide (SA) and mercaptosuccinate (MCS). SA and MCS primarily inhibit catalase and glutathione peroxidase (GPx) respectively and the application of each resulted in a significant increase in the $H_2O_2$ signal. The selectivity of the sensor was verified by the absence of a change in the signal in response to the peripheral administration of ascorbic acid (AA) compared to controls. Evidence of a disparity between brain $H_2O_2$ signalling in the freely-moving animal with respect to the anaesthetised subject was also observed. The enzymatic component of the paired $H_2O_2$ sensor was found to be stable over a continuous monitoring period of 12 days, thereby demonstrating the suitability of this sensor for the long-term chronic detection of brain $H_2O_2$.

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$H_2O_2$ is often considered to be a toxic molecule in the neuronal environment. Normally, potentially harmful levels of reactive oxygen species (ROS) such as $H_2O_2$ are maintained at an innocuous level in the brain by the opposing protective anti-oxidant network. If $H_2O_2$ and other ROS are produced beyond a certain threshold, which exceeds the protective capabilities of the anti-oxidant network, deleterious processes occur such as damage to cell structures, DNA, lipids and proteins.[1,2]

The resulting disruptive state which manifests, due to an imbalance between ROS production and regulation, is referred to as oxidative stress. Oxidative stress development has been implicated in the pathophysiology of neurodegenerative conditions such as Parkinson's disease and Alzheimer's disease.[3–6] $H_2O_2$ is a relatively stable ROS in the neuronal environment,[7] therefore it possibly represents a suitable marker for the detection of neurodegenerative disease initiation and progression mediated by oxidative stress.

Conversely, $H_2O_2$ has also recently been shown to play a vital protective role as an intracellular messenger at normal physiological levels.[8–10] It has been reported that $H_2O_2$ can modulate synaptic transmission and neuroplasticity in the rodent brain.[9] $H_2O_2$ has been indicated to act as a neuromodulator *via* the modulation of dopamine (DA) release in *ex vivo* brain slices.[11–13] Specifically, Rice *et al.* have demonstrated that endogenous $H_2O_2$ modulates DA release by activation of K+ sensitive adenosine triphosphate (ATP) channels in striatal and substantia nigra neurons.[9,11] Indeed, a neuroprotective role for $H_2O_2$ has been proposed in incidences of ischaemic stroke.[14]

Fluorescent/bioluminescent and MRI-based methods have been used previously to detect $H_2O_2$ *in vivo*.[15–18] However, none of these reports demonstrate the real-time detection of $H_2O_2$ in the intact living brain and quantitative measurements of brain $H_2O_2$ are not feasible using these methods.

Most brain $H_2O_2$ detection methods rely on the use of microdialysis (MD) which suffers from poor temporal and spatial resolution.[19–21] The estimated basal concentration of brain $H_2O_2$ in rats, determined from MD studies, is between 25 and 50 μM.[22] However, the time delay and invasiveness of the MD technique may have a detrimental impact on the determined substrate level.[21,23,24] Electrochemical methods have an advantageous distinction in that they provide real-time measurements of neurochemical species of interest and are relatively less invasive.[25]

Kulagina *et al.* have previously described the electrochemical detection of $H_2O_2$ in the living brain.[26] However, these studies were conducted in anaesthetised subjects and the sensor utilised requires a mediator and an additional method to remove interference from ascorbic acid (AA); which is not required with

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the $H_2O_2$ sensor presented here. Sombers *et al.* have described the electrochemical detection of brain $H_2O_2$ using fast scan cyclic voltammetry (FSCV) and carbon-fiber microelectrodes (CFE's) in the anaesthetised animal.[27,28] However, these publications do not demonstrate long-term recordings of brain $H_2O_2$ as it is an acute non-recovery experimental condition. In addition the effect of anaesthesia may significantly impact the determined basal $H_2O_2$ level.[29,30]

A scarcity of information is thus available surrounding the basal concentration of brain $H_2O_2$ in the freely-moving animal from electrochemical recordings. Due to recent evidence which has highlighted the action of $H_2O_2$ as a neuromodulator,[9] in addition to the role of $H_2O_2$ as an oxidative stress marker,[1] the real-time direct detection of brain $H_2O_2$ in the freely-moving animal would provide a highly novel analytical means of further understanding its neurochemical role *in vivo*. A variety of biosensors such as those that target brain glucose,[31,32] glutamate[33] and choline[34–36] rely on the enzymatic production of $H_2O_2$ at the senor surface. Therefore, an understanding of the basal concentration of $H_2O_2$ in the neuronal environment is an important consideration with respect to the measurement of these species in the brain using first generation biosensors.

We have previously successfully demonstrated the detection of $H_2O_2$ in the *in vitro* environment with a catalase-based dual platinum (Pt) electrode design.[37,38] This paired $H_2O_2$ design consisted of incorporated permselective layers of poly-*o*-phenylenediamine (PPD) and Nafion® which provided the optimum sensitivity towards the target substrate and minimal interference from a variety of endogenous species, including AA and DA over physiologically relevant concentration ranges. Moreover, the highly suitable physiological performance factors of our $H_2O_2$ sensor for the purpose brain $H_2O_2$ monitoring have recently been shown, including an appropriately low limit of detection and fast response time.[39]

In the present study we report the *in vivo* characterisation and validation of this device in freely-moving animals.

## Results & discussion

### Control experiments

The *in vivo* characterisation of the dual $H_2O_2$ catalase-based sensor design was achieved by investigating the key performance factors of the paired $H_2O_2$ sensor under the following parameters. The response of the sensor to the target analyte, *i.e.* $H_2O_2$ (sensitivity). The ability of the sensor to reject interference from endogenous species, *i.e.* AA (selectivity) and the suitability of the sensor for the purpose of long term recordings (stability). The CONTROL sensor acts as a sentinel *in vivo* and serves to control for and eliminate any possible contribution from non-peroxide signals to the overall paired $H_2O_2$ sensor response.[39] For each *in vivo* experimental investigation the overall paired subtracted current response of the $H_2O_2$ sensor ($I_{PEROX} - I_{CONTROL}$) is presented unless stated otherwise. If a false positive, was noted at the CONTROL electrode, which would infer contribution from a non-peroxide signal to the overall dual $H_2O_2$ sensor response, the results from that subject were not included in the cohort presented here.

All experiments were carried out in the striatum due to the spatial constraints of the MD probe/dual sensor construct. The examination of striatal $H_2O_2$ changes is also important in terms of investigating disease mechanisms which largely affect this area.[4,40] The pathological hallmark of Parkinson's disease is DA cell degeneration in an area of the midbrain known as the substantia nigra pars compacta (SNc) leading to a loss of striatal DA.[4,40] The influence of oxidative stress on the degeneration of neurons in this brain area could be facilitated by monitoring striatal $H_2O_2$ throughout disease progression in an animal model of the disease.

The vehicle used for all local delivery of chemical/pharmacological agents, delivered *via* the reverse microdialysis (RD) method,[41] was artificial cerebrospinal fluid (aCSF). Therefore, control experiments examined the effect of the local perfusion of aCSF on the $H_2O_2$ sensor response. A typical example of the observed change in signal is presented in Fig. 1.

Perfusion of aCSF causes compounds present in the tissue surrounding the MD probe including $H_2O_2$, to diffuse across their concentration gradient through the MD membrane and into the dialysate. Continuous removal of this dialysate by constant perfusion of aCSF, maintains the concentration gradient which leads to a gradual decrease in current detected by the implanted $H_2O_2$ sensor which reaches a minimum plateau level.

On cessation of perfusion, the current gradually returns towards pre-perfusion baseline levels, as the undisturbed concentration of $H_2O_2$ amongst other analytes, is re-established in the tissue surrounding the implant.[31,42]

The local delivery of aCSF through an MD probe, results in an immediate decrease in current ($n = 6$, 4/4) recorded by the paired $H_2O_2$ sensor; which is derived by a subtraction method (see Data acquisition and statistical analysis). A clear change from a baseline of 0.92 ± 0.16 nA to a plateau minimum of 0.58 ± 0.08 nA ($P = 0.0376$* (paired-*t*)) after 11.3 ± 0.9 min was observed. A return to a baseline level is apparent after 30.7 ± 6.9 min. This corresponds to a $H_2O_2$ concentration change of 0.71 ± 0.25 μM based on the *in vitro* sensitivity of the $H_2O_2$ sensor.[38,39]

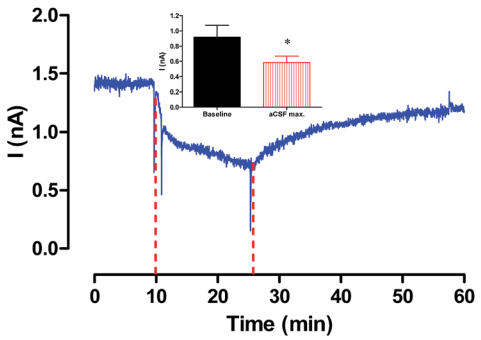


Fig. 1 Typical example of the effect of local perfusion (RD) of aCSF, on the signal recorded from a dual catalase-based $H_2O_2$ sensor, implanted in the striatum of a freely-moving rat. Dashed lines indicate the duration of perfusion. Inset; average current prior to perfusion (baseline) and at maximum ($P < 0.05$* (paired-*t*) $n = 6$, 4/4). Pre *vs.* post-perfusion baseline ($P > 0.05$, unpaired-*t*).

 

Our paired $H_2O_2$ sensor will only respond to a change in $H_2O_2$ levels and not other species present in the extracellular fluid (ECF), as the interference characteristics of the PEROX and CONTROL sensors are matched and the CONTROL sensor removes any contribution from non-peroxide sources.[37,39] The overall decrease in response detected by the paired $H_2O_2$ sensor on perfusion of aCSF reflects the removal of pre-perfusion baseline levels of $H_2O_2$ from the ECF surrounding the implant which subsequently returns to a pre-perfusion baseline level following cessation of perfusion. No significant difference ($P = 0.5424$ (unpaired-*t*)) was observed between the pre-perfusion baseline and the post-perfusion baseline therefore a local perfusion itself (*i.e.* aCSF) has no long-term effect beyond approximately 30 min (30.8 ± 6.9 min) on the $H_2O_2$ sensor response.

A MD probe of 4 mm dialysis membrane length perfused at 2.0 μL $min^{-1}$ has an average approximate analyte *in vivo* recovery rate of 45% for the charged species glucose and lactate.[43,44] As $H_2O_2$ is uncharged and freely diffusible this figure may in fact be an under estimation for this substance.[22] If we assume negligible contribution from analyte clearance, temperature and take into consideration the effect of membrane length[45,46] used here on recovery (*i.e.* 2 mm) we can infer an approximate *in vivo* recovery rate of 22.5%. Similar to previous investigations,[34] based on our recorded concentration change (0.7 μM) and an estimated recovery rate of 22.5% this would suggest an estimated basal ECF concentration of 3.1 μM.

This value is somewhat lower than previous estimates from microdialysis experiments (25–50 μM),[22] however upper limits of intracellular $H_2O_2$ concentrations from 100 nM to 1 μM have been estimated and a level between 15 and 150 μM to facilitate cellular signalling without oxidative damage has been proposed.[9,14,47]

### Selectivity investigations

The catalase based dual $H_2O_2$ sensor is capable of rejecting interference from a wide range of electroactive species present in the ECF of the brain.[37–39] In order to investigate the selectivity of the $H_2O_2$ sensor in the *in vivo* environment, the principal electrochemically active interferent species in the brain, ascorbic acid (AA) was diluted in saline systemically administered and the response of the implanted $H_2O_2$ sensor was monitored (Fig. 2).

There was no significant difference between the maximum response of the dual $H_2O_2$ biosensor (−0.05 ± 0.06 nA max.; 11.4 ± 0.1 min, $n = 2$, 2/2) following AA administration when compared to saline (0.07 ± 0.06 nA max.; 11.4 ± 0.1 min, $n = 3$, 3/3) as a control (Fig. 2, inset). The effect of systemically introduced AA on the ECF concentration of this substance has previously been monitored *in vivo* using carbon paste electrodes (CPE's) operating at +250 mV.[48] Peripherally administered AA increases the CPE sensor signal dramatically to a maximum level approximately 11 min after injection followed by a gradual decrease towards pre-injection baseline levels.[34,48] Under the same conditions (Fig. 2) it is clear that the $H_2O_2$ sensor is selective in the physiological environment due to the absence of the aforementioned amperometric AA signal and a negligible variation in the $H_2O_2$ sensor response.

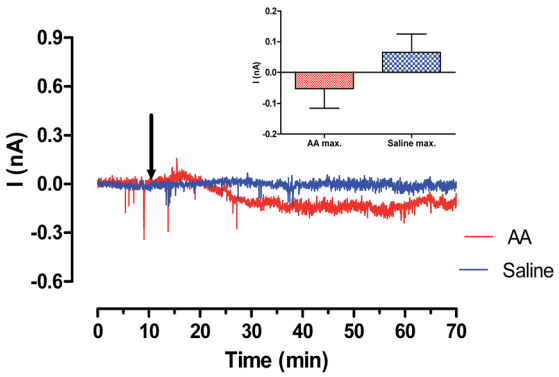


Fig. 2 Typical example of the effect of peripheral (i.p.) administration of AA (0.5 mg $kg^{-1}$) and saline (2 mL $kg^{-1}$) on the signal recorded from a dual $H_2O_2$ sensor, implanted in the striatum of a freely moving rat. Pre *vs.* post injection baseline ($P > 0.05$, unpaired-*t*). Inset; average maximum current following systemic administration of AA and saline ($P > 0.05$ (unpaired-*t*)).

The individual components of the paired $H_2O_2$ sensor *i.e.* the PEROX sensor and CONTROL sensor do not show a significant difference in signal, in comparison to pre-injection baseline levels, due to the systemic administration of AA (PEROX, $P = 0.0566$ (paired-*t*)) and (CONTROL, $P = 0.1592$ (paired-*t*)). In addition, the PEROX sensor signal is well correlated to that of the CONTROL sensor with no significant difference ($P = 0.4156$ (unpaired-*t*)) observed between the maximum response of these sensor designs in response to systemically administered AA.

### Sensitivity investigations, local delivery of $H_2O_2$ (freely-moving animal)

In order to investigate the sensitivity of the dual $H_2O_2$ biosensor to the target analyte *in vivo*, a concentration range of $H_2O_2$ prepared in aCSF (25, 100, 200, 500 and 1000 μM) was added to the perfusate in a randomised order (Fig. 3). With respect to control experiments (aCSF) no significant change in the sensor response can be seen on introduction of $H_2O_2$ to the perfusate up to a concentration of 1000 μM ($P = 0.4111$, one-way ANOVA). Moreover, for each $H_2O_2$ concentration locally introduced to the sensor no significant variation ($P > 0.05$) in the implanted sensor response was noted with respect to pre-perfusion baseline levels (see Table 1).

It has been estimated from previous investigations that the concentration of $H_2O_2$ in the brain is between 25 and 50 μM,[22] therefore one may expect an increase in the sensor response upon exposure to a level of $H_2O_2$ which exceeds this estimate, *i.e.* in excess of 50 μM. However, the data presented in Fig. 3 is contradictory to this expectation. More specifically, although not significant ($P > 0.05$) a small relative increase in the $H_2O_2$ sensor response was detected, with respect to the perfusion of aCSF, following the local addition of 25–1000 μM $H_2O_2$ (Fig. 3). However, as demonstrated by the maximum current change recorded by the implanted $H_2O_2$ sensor (Table 1), following each addition of $H_2O_2$ (25–1000 μM) no significant increase ($P >$

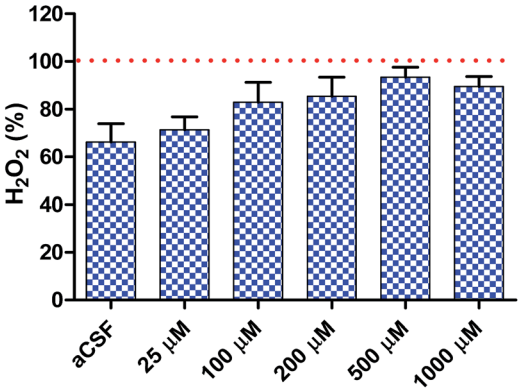


Fig. 3 The average percentage change from baseline (100%) following the local perfusion of various dilutions of $H_2O_2$ compared with the local administration of aCSF, as recorded by the $H_2O_2$ sensor, implanted in the striatum of freely-moving Wistar rats ($P > 0.05$ (one-way ANOVA) $n = 3$–6, 3/3–4/4). Pre *vs.* post-perfusion baseline ($P > 0.05$, unpaired-*t*, see Table 1).

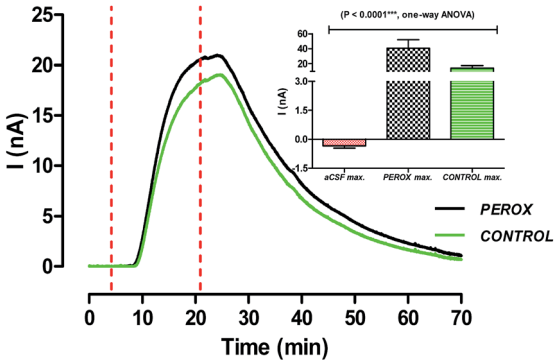


Fig. 4 Typical example of the effect of local perfusion (RD) of acetaminophen (10 mM), on the signal recorded from PEROX and CONTROL sensors, implanted in the striatum of a freely-moving rat. Dashed lines indicate the duration of perfusion. Pre *vs.* post-perfusion baseline ($P > 0.05$, unpaired-*t*). Inset; average maximum current under control conditions (aCSF) and following perfusion with acetaminophen ($P < 0.0001$***, one-way ANOVA, $n = 3$, 3/3).

0.05) above pre-perfusion baseline level was recorded during the perfusion of each concentration of $H_2O_2$. In fact, a decrease was recorded by the implanted $H_2O_2$ sensor in each case following the local addition of the substrate (25–1000 μM $H_2O_2$). This was an unexpected response given that the concentration of substrate added to the perfusate should closely match or exceed the previously estimated ECF concentration of endogenous brain $H_2O_2$ (25–50 μM) and therefore lead to no obvious change in current or an increase in current respectively, as recorded by the implanted sensor.

In order to unequivocally demonstrate that the $H_2O_2$ sensor is functional while implanted in brain tissue an electroactive challenge was undertaken. Acetaminophen (paracetamol), a known electroactive species[49,50] with can be oxidized at a similar potential to $H_2O_2$ was delivered to implanted PEROX and CONTROL sensors in the same manner as $H_2O_2$. As can be seen in Fig. 4 the local delivery of paracetamol (10 mM) through an MD probe, results in an immediate increase in current recorded by PEROX and CONTROL sensors ($n = 3$, 3/3), to a plateau maximum of 43.13 ± 11.77 nA (PEROX) and 16.09 ± 3.08 (CONTROL), ($P = 0.0895$, unpaired-*t*) after 9.2 ± 2.5 min (PEROX) and 12.7 ± 1.3 min (CONTROL), ($P = 0.2837$, unpaired-*t*) with a return to a pre-perfusion baseline level 48.6 ± 0.6 min (PEROX) and 42.1 ± 5.6 min (CONTROL), ($P = 0.3065$, unpaired-*t*) subsequently.

The maximum response recorded here (Fig. 4) following perfusion of 10 mM paracetamol is significantly different to that seen on perfusion of aCSF when compared against each sensor type ($P < 0.0001$***, one-way ANOVA) Fig. 4, inset. Therefore, the implanted electrochemical sensor responds to an electroactive challenge and is functional in brain tissue.

The limit of detection of our PEROX sensor is below 1 μM (ref. 38 and 39) indicating that our PEROX sensor can reliably detect low levels of $H_2O_2$ which is expected to be the case in the brain.[7] Our sensor demonstrates a characteristic fast response time *in vitro*,[39] therefore the detection of this potentially short lived species in the brain should be facilitated.

It has previously been demonstrated, that redox reactions of various electroactive species *in vitro* may depend on the aqueous media conditions utilised to facilitate experiments. A variation in the sensitivity of carbon fiber electrodes to DA and related metabolites has been demonstrated *in vitro* between different aqueous media including HEPES-buffered saline, PBS and aCSF.[51,52]

In our experiments any possible detrimental impact the composition of aCSF has on the viability of $H_2O_2$ delivered to

Table 1 Summary of paired $H_2O_2$ sensor results obtained following addition of aCSF and various concentrations of $H_2O_2$ diluted in aCSF (25, 100, 200, 500 and 1000 μM) to the perfusate in freely-moving animals (data pertaining to Fig. 3). *P* = unpaired-*t*-test

| Analyte | Max current change (nA) | Max response (min) | Return to baseline (min) | Pre-perfusion baseline (nA) | Post-perfusion baseline (nA) | *n* | *P*, max *vs.* control (aCSF) | *P*, pre *vs.* post-perfusion baseline |
|---|---|---|---|---|---|---|---|---|
| aCSF | −0.34 ± 0.12 | 11.3 ± 0.9 | 30.8 ± 6.9 | 0.92 ± 0.16 | 0.78 ± 0.14 | 6, 4/4 | n/a | 0.5424 |
| 25 μM $H_2O_2$ | −0.28 ± 0.09 | 10.2 ± 1.2 | 44.4 ± 4.1 | 0.93 ± 0.18 | 0.84 ± 0.17 | 4, 3/3 | 0.7585 | 0.7207 |
| 100 μM $H_2O_2$ | −0.15 ± 0.11 | 10.5 ± 0.5 | 26.2 ± 2.4 | 0.73 ± 0.22 | 0.63 ± 0.15 | 3, 3/3 | 0.3666 | 0.7234 |
| 200 μM $H_2O_2$ | −0.21 ± 0.09 | 12.3 ± 0.2 | 37.0 ± 2.1 | 1.62 ± 0.42 | 1.42 ± 0.37 | 4, 3/3 | 0.4611 | 0.7425 |
| 500 μM $H_2O_2$ | −0.07 ± 0.04 | 11.3 ± 0.7 | 68.9 ± 27.4 | 0.91 ± 0.16 | 0.93 ± 0.18 | 3, 3/3 | 0.1745 | 0.9478 |
| 1000 μM $H_2O_2$ | −0.09 ± 0.04 | 12.2 ± 0.2 | 39.2 ± 9.8 | 0.83 ± 0.12 | 0.78 ± 0.12 | 3, 3/3 | 0.2109 | 0.7927 |

the striatum using this vehicle has been excluded based on *in vitro* investigations (data not shown).[38]

The delivery of $H_2O_2$ to the implanted catalase-based $H_2O_2$ sensor may be restricted by recovery issues associated with the MD/RD technique.[34,41] Under maximal conditions where a slow flow rate and relatively long MD membrane are utilised the recovery ratio has been estimated to be only 70%.[53] Therefore, with the RD technique not all of the substrate added to the perfusate will reach the tissue surrounding the implanted sensor (potentially as low as 30%). Thus this inherent recovery issue may result in a lower than expected recorded response by the implanted sensor.

However, even with recovery ratio restrictions the highest concentration of $H_2O_2$ delivered to the implanted dual sensor in these experiments *i.e.* 1000 μM should vastly exceed the ECF concentration which is estimated to exist at a much lower range (25–50 μM).[22]

The absence of an increase detected by the implanted dual $H_2O_2$ sensor above pre-perfusion baseline levels (Fig. 3) may then be due to the effect of an inherent mechanism of the *in vivo* environment. It is well understood that an intricate network of neuronal antioxidant mechanisms exist to regulate the level of $H_2O_2$/ROS in the brain. Homeostasis of the balance between ROS concentration and peroxidase activity in the brain is essential and must be maintained to prevent oxidative damage and thus maintain cellular integrity.[9,54,55]

It is possible that any effect of the local addition of $H_2O_2$ on the ECF concentration of this substrate is dampened by this vast network of control mechanisms. Our experiments indicate that the effect of local addition of $H_2O_2$, of a concentration higher than that estimated to be present in the ECF, can be negated by the action of the opposing antioxidant network.

To the best of our knowledge, this is the first time the effect of exogenous application of $H_2O_2$ on the ECF level of this substrate has been examined using electrochemical methods in the freely-moving animal. Previous research has highlighted a detectable response by an implanted electrochemical $H_2O_2$ sensor in response to the local addition of $H_2O_2$, however this research was conducted in the anaesthetised animal and a relatively higher concentration of $H_2O_2$ was utilised (1 M).[28]

Our data provides novel evidence, which would support previous assumptions, that the robust neuronal antioxidant network in the freely moving subject can tolerate the presence of a relatively high concentration of $H_2O_2$.

### Altered enzymatic degradation, catalase inhibition (sodium azide)

As previously discussed a vast network of antioxidant mechanisms regulate the level of $H_2O_2$/ROS in the brain.[9,54,55] Chief among these opposing antioxidant systems are the peroxidase enzymes which consist primarily of catalase and glutathione peroxidase (GPx).[9,56] Systemic administration of sodium azide (SA) has previously been used in *in vivo* experiments and inhibits the function of catalase in the brain.[57–59]

As catalase is an enzyme which degrades $H_2O_2$ into water and oxygen, impairment in the activity of catalase in the brain should lead to an increase in $H_2O_2$ levels. In this experiment (Fig. 5, middle), SA was administered to each subject (20 mg $kg^{-1}$ s.c.) approximately 30 minutes prior to the local administration of 1000 μM $H_2O_2$ to examine the effect of catalase inhibition on the subsequent local delivery of $H_2O_2$ to the implanted sensor.

In the absence of pre-treatment with SA the local addition of 1000 μM $H_2O_2$ (Fig. 5, top) does not impact upon the $H_2O_2$ sensor response ($n$ = 3, 3/3). No significant difference was

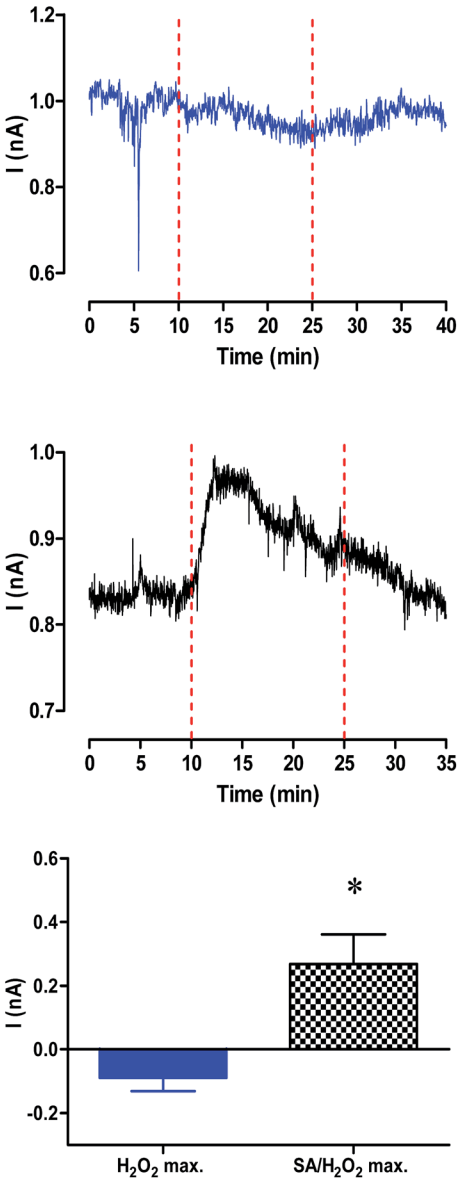


Fig. 5 Typical examples of the effect of various treatments, on the signal recorded from $H_2O_2$ sensors, implanted in the striatum of freely-moving rats. Top; Local perfusion of 1000 μM $H_2O_2$ under control conditions (no pre-treatment). Pre *vs.* post-perfusion baseline ($P$ > 0.05, unpaired-$t$). Middle; 1000 μM $H_2O_2$ local perfusion following peripheral injection of the catalase inhibitor SA (20 mg $kg^{-1}$ s.c.). Pre *vs.* post-perfusion baseline ($P$ > 0.05, unpaired-$t$). Bottom; Average maximum current following perfusion of 1000 μM $H_2O_2$ under control conditions and at maximum following pre-treatment with SA ($P$ < 0.05* (unpaired-$t$) $n$ = 3, 3/3).

observed between the pre-perfusion baseline 0.83 ± 0.12 nA and the maximum deviation in the sensor signal (Fig. 4, top; 0.74 ± 0.11 nA, $P = 0.1614$ (paired-*t*)).

In contrast to the previous finding (Fig. 4, top) pre-treatment with SA (20 mg $kg^{-1}$ s.c.) leads to a marked increase in response detected by the implanted sensor in response to the local addition of 1000 μM $H_2O_2$. As can be seen in Fig. 5 (middle) following pre-treatment with SA; the local delivery of $H_2O_2$ through an MD probe, results in an immediate increase in current ($n = 3$, 3/3) recorded by the $H_2O_2$ sensor, from a baseline of 0.8 ± 0.44 nA to a plateau maximum of 1.07 ± 0.35 nA after 3.3 ± 1.2 min with a return to a pre-perfusion baseline level 31.2 ± 3.4 min subsequently. The maximum response recorded here (Fig. 4, middle) following perfusion of 1000 μM $H_2O_2$ with SA pretreatment is significantly different to that seen in the absence of SA pre-treatment ($P = 0.0238$* (unpaired-*t*) Fig. 5, bottom).

SA has other *in vivo* targets in addition to catalase, *e.g.* cytochrome oxidase, which may influence ROS control and production.[59] However, the effect of SA pre-treatment on the $H_2O_2$ sensor response (0.02 ± 0.04 nA; 7.6 ± 5.2 min, $n = 3$, 3/3) before the perfusion of $H_2O_2$ itself (~30 min prior) is minimal, not significantly different to pre-injection baseline levels ($P = 0.7565$ (paired-*t*)) and not significantly different ($P = 0.5396$ (unpaired-*t*)) in comparison to the negligible effect ($P = 0.3904$ (paired-*t*)) of saline injection (0.07 ± 0.06 nA max.; 7.2 ± 0.3 min, $n = 3$, 3/3).

This SA pre-treatment and subsequent $H_2O_2$ administration paradigm does not affect the response of the CONTROL sensor itself, *i.e.* the catalase adhered to the active surface of this electrode design. No significant difference ($P = 0.8835$ (paired-*t*)) was observed between the pre-perfusion baseline (1.60 ± 0.52 nA $n = 3$, 3/3) of the CONTROL sensor in comparison to the maximum deviation (1.56 ± 0.33 nA $n = 3$, 3/3) in the CONTROL sensor signal recorded throughout the perfusion of 1000 μM $H_2O_2$ following SA pre-treatment, thus eliminating any possible non-peroxide contribution to the $H_2O_2$ sensor signal.

It is quite clear from the evidence presented in Fig. 5 that catalase inhibition *via* the peripheral administration of SA, prior to the local addition of this concentration of $H_2O_2$, is sufficient to elevate the ECF concentration of this substrate and thus lead to an increase in response detected by the $H_2O_2$ sensor.

### Local delivery of mercaptosuccinate (glutathione peroxidase inhibition)

Mercaptosuccinate (MCS) is a glutathione peroxidase (GPx) inhibitor which has previously been implemented to elevate the concentration of $H_2O_2$ in cultured brain cells.[60] Glutathione redox homeostasis maintains the primary anti-oxidant defence systems in the central nervous system and GPx is the primary enzyme responsible for degradation of neuronal ROS/$H_2O_2$.[56] Disruption to the function of GPx in the brain of the freely-moving animal should therefore lead to increased neuronal $H_2O_2$ production as seen previously.[11,28] In addition, while MCS is an electroactive species it is not detectable by the $H_2O_2$ sensor.[39]

The local perfusion of MCS will disrupt $H_2O_2$ removal and hence lead to an increase in $H_2O_2$ levels in the brain and consequently an elevated concentration of $H_2O_2$ in the ECF surrounding the implanted sensor. Indeed, it has previously been demonstrated that exposure of *ex vivo* brain slices to 1–100 mM MCS is sufficient to inhibit GPx and lead to an elevated level of neuronal $H_2O_2$ and this parameter has also been determined by voltammetric methods in anaesthetised rodents using CFE's.[9,28]

The local addition of 1000 μM MCS ($n = 3$, 2/2) to the implanted sensor was sufficient to induce an elevation in endogenous $H_2O_2$ levels as indicated by the clear increase recorded by the dual catalase-based $H_2O_2$ biosensor (Fig. 6). A significant difference was observed between the pre-perfusion baseline (1.35 ± 0.25 nA) and the maximum change in the $H_2O_2$ sensor signal (1.65 ± 0.31 nA, $P = 0.0414$* (paired-*t*)) which represents a maximum change of 0.30 ± 0.06 nA occurring 7.1 ± 1.3 min from the start of the perfusion and which returned to pre-perfusion baseline levels 42.2 ± 9.7 min following cessation of the local delivery of MCS. Moreover, no significant difference ($P = 0.9001$ (paired-*t*)) was observed between the pre-perfusion baseline (0.74 ± 0.14 nA $n = 3$, 2/2) of the CONTROL sensor in comparison to the maximum deviation (0.76 ± 0.24 nA $n = 3$, 2/2) in the CONTROL sensor signal, thereby eliminating any non-peroxide contribution to the $H_2O_2$ sensor signal.

It is clear that altered enzymatic degradation *via* disruption to the function of catalase and GPx is necessary to increase ECF levels of $H_2O_2$. By manipulation of endogenous $H_2O_2$ levels we have been able to detect a change in the level of this substance in the brain using our implanted $H_2O_2$ sensor in the freely-moving animal (Fig. 5 and 6).

This collective real-time data suggests and lends support to previous findings that neuronal $H_2O_2$ levels are tightly controlled under normal circumstances.[5,55] We suggest that in conditions of disease states where antioxidant and controlling mechanisms are compromised $H_2O_2$ levels are relatively

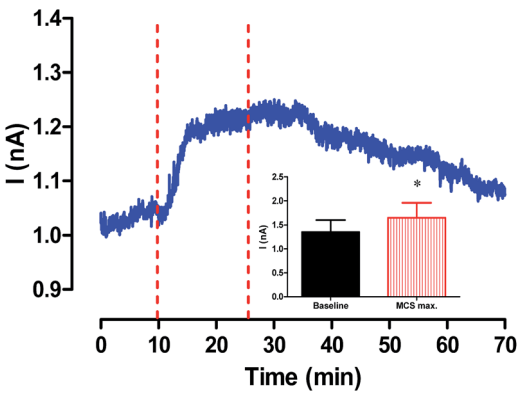


Fig. 6 Typical example of the effect of local perfusion of the GPx inhibitor MCS; (1000 μM), on the signal recorded from a $H_2O_2$ sensor, implanted in the striatum of a freely moving rat. Inset; average current prior to perfusion (baseline) and at maximum ($P < 0.05$*, (paired-*t*) $n = 3$, 2/2). Pre *vs.* post-perfusion baseline ($P > 0.05$, unpaired-*t*).

 

elevated as evidenced by the result of manipulating enzymatic degradation of brain $H_2O_2$.

### Local delivery of $H_2O_2$ (anaesthetised animal)

In light of the previous evidence and existing research, we decided to investigate circumstances where the level of $H_2O_2$ locally introduced greatly exceeds estimated physiological levels of this substance in the ECF.[14,22,47] The maximum concentration that had been tested in the freely-moving animal (*i.e.* 1000 μM, Fig. 5, top) was not sufficient to cause an increase in the $H_2O_2$ sensor response. Moreover, when a similar range was tested (50–1000 μM $H_2O_2$) in the acute setting a relative increase in the paired sensor response was observed, but a variation at the CONTROL sensor was consistent throughout (data not shown).[38] To induce a meaningful increase at the PEROX sensor in the absence of a change at the CONTROL sensor, it was deemed necessary to introduce a much higher concentration of $H_2O_2$. In order to introduce a relatively higher and potentially toxic concentration of $H_2O_2$ (10 mM), while avoiding potential complications and/or adverse or detrimental effects to the freely-moving awake animal, we used anaesthetised animals and carried out an acute non-recovery experimental protocol.

The local perfusion of aCSF in the acute preparation (Fig. 7, top) results in a decrease in current ($-0.05 \pm 0.02$ $n = 4$, 4/4; 6.9 $\pm$ 1.6 min) detected by the implanted dual $H_2O_2$ sensor which is similar to the trend observed in the freely-moving animal where a decrease in sensor signal is apparent due to the aforementioned reasons (Fig. 1). This recorded value (Fig. 7, top) corresponds to a calculated concentration change of $0.10 \pm 0.04$ μM based on the *in vitro* sensitivity of the $H_2O_2$ sensor.[38] Using our estimated *in vivo* recovery rate (22.5%) the approximate basal concentration of brain $H_2O_2$ in the anaesthetised animal is 0.44 μM. This value is lower than that observed in the freely-moving scenario where an estimated ECF concentration of 3.1 μM was determined. The disparity in basal $H_2O_2$ levels in the brain observed between the acute and freely-moving setting may be attributable to the effect of anaesthesia.[29]

In the acute preparation a relatively high concentration of $H_2O_2$ diluted in aCSF was required to induce a substantial change at the peroxide sensing element of this paired design (10 mM, see Fig. 7). A large increase in target substrate concentration was detected by the implanted dual $H_2O_2$ sensor (Fig. 7, middle). Exogenous application of 10 mM through the MD probe in the acute preparation (Fig. 7, middle), results in an immediate increase in response ($0.77 \pm 0.33$ nA $n = 4$, 3/3; 9.3 $\pm$ 1.4 min) detected by the implanted dual $H_2O_2$ sensor. The maximum change in current observed following the administration of 10 mM $H_2O_2$ was significantly different to that observed upon perfusion of aCSF (Fig. 7, top) in the acute preparation ($P = 0.0499$* (unpaired-*t*); Fig. 7, bottom).

This evidence supports previous investigations conducted in the acute preparation, which have demonstrated an increase in $H_2O_2$ levels following local addition at a level which greatly exceeds the estimated ECF concentration of this substrate.[28] The evidence presented in Fig. 7 demonstrates clearly that our

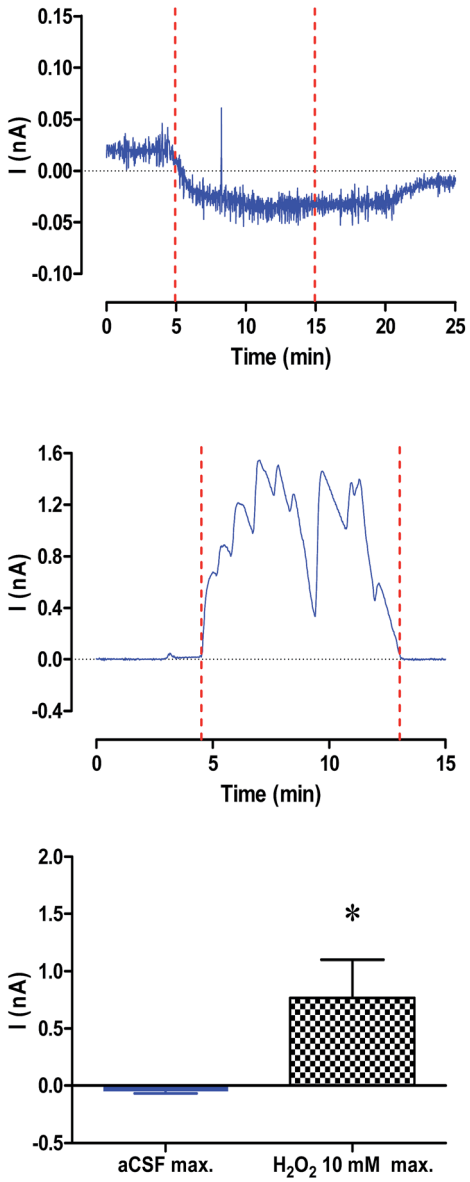


Fig. 7 Typical examples of the effect of various treatments, on the signal recorded from paired $H_2O_2$ sensors, implanted in the striatum of anaesthetised rats. Top; Local perfusion of aCSF. Pre *vs.* post-perfusion baseline ($P > 0.05$, unpaired-*t*). Middle; Local perfusion of 10 mM $H_2O_2$. Pre *vs.* post-perfusion baseline ($P > 0.05$, unpaired-*t*). Bottom; Average maximum current following perfusion of a CSF $n = 4$, 4/4 compared against maximum following 10 mM $H_2O_2$ administration $n = 4$, 3/3 ($P < 0.05$*, unpaired-*t*).

$H_2O_2$ sensor responds to an induced change in the target substrate in the neuronal environment.

A contrasting $H_2O_2$ response was observed here in comparison to that observed in the freely-moving scenario. A clear increase above baseline levels of $H_2O_2$ was detected by the $H_2O_2$ sensor following perfusion of 10 mM $H_2O_2$ in the acute experimental set-up (Fig. 7, middle). Although a higher concentration was applied in the acute scenario (*i.e.* 10 mM) the disparity in the results between the acute and freely-moving animal data (Fig. 3) may also be influenced by the constant use of anaesthetic in the acute procedure (see below).

A disparity between the average baseline current of the $H_2O_2$ sensor was apparent in the acute preparation ($-0.27 \pm 0.04$ nA, $n = 3$, 3/3) when compared to the freely-moving scenario $1.97 \pm 0.23$ nA, $n = 5$, 5/5 ($P = 0.0003$*** (unpaired-$t$)). In the anaesthetised animal the pre-perfusion baseline of the CONTROL and PEROX sensor are in very close proximity to each other and share similar baseline current values which explains the negative paired baseline response of the dual catalase-based $H_2O_2$ sensor ($-0.27 \pm 0.04$ nA, $n = 3$, 3/3). This observation contrasts with that observed in the freely-moving animal, where the baseline of the PEROX sensor is higher than the CONTROL sensor in the freely-moving animal thereby resulting in a positive average subtracted value ($1.97 \pm 0.23$ nA, $n = 5$, 5/5).

A relatively limited amount of time is available to conduct an acute experiment due to the nature of the experimental conditions. It is possible that in the acute set-up the capacitance of the $H_2O_2$ sensor does not have sufficient time to dissipate which may influence the calculated baseline. This parameter was investigated by calculating the average time facilitated between the application of the potential to the implanted sensor prior to commencement of perfusions in the acute preparation ($100.7 \pm 11.9$ min, $n = 3$, 3/3) and calculating a new average baseline from freely-moving $H_2O_2$ data at the same time point. A discrepancy in baseline levels between the acute and freely-moving preparations was still apparent following a similar time-frame ($\sim$100 min, $n = 5$, 5/5) from the application of the potential to the working electrodes ($P = 0.0487$* (unpaired-$t$)).

Anaesthesia has been shown to cause an alteration of various mechanisms and levels of neurotransmitters in the central nervous system (CNS).[29,30,61] A limited amount of information exists surrounding the effect of volatile anaesthetics such as isoflurane on electroactive species such as $H_2O_2$ in the brain. Anaesthesia may influence the function of the antioxidant network in the brain. In which case exogenously applied $H_2O_2$ may not be efficiently removed resulting in an increased response detected by the implanted $H_2O_2$ sensor. Anaesthesia causes systemic CNS depression and hence a decrease in neuronal activity.[61] The production of $H_2O_2$ is linked to increased neuronal activity due to an increased level of $O_2$ consumption which generates $H_2O_2$.[9] The effect of anaesthesia may therefore explain the reduction between the estimated basal concentration of brain $H_2O_2$ determined in the freely-moving animal (3.1 μM) when compared to the acute scenario (0.44 μM).

### Stability investigations

A decrease in the sensitivity of a biosensor can often be observed following contact with a biological sample. Typically a variable 20–50% decrease may occur following implantation due predominantly to fouling of the sensor by proteins and other biomolecules present within the tissue.[33,35] The normal reaction of the body to the presence of a foreign object may also negatively affect the sensitivity of an implanted sensor. However, the brain is an immunoprivileged organ to an extent[62,63] and immune reactions are relatively suppressed in comparison to the periphery and long-term implants are relatively more tolerated.

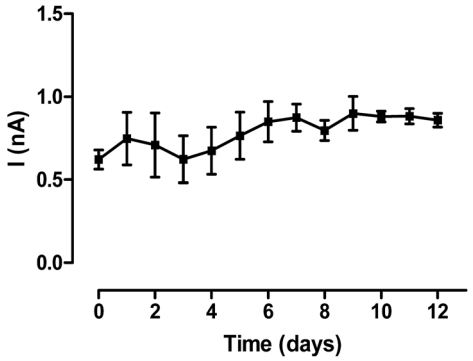


Fig. 8 Average baseline *in vivo* data for a consecutive period of 12 days following implantation ($P > 0.05$, one-way ANOVA), collected from a cohort of 7 animals. Data was recorded by catalase modified sensors implanted in the striatum of freely moving rats.

The mean baseline current of the enzymatic component of the $H_2O_2$ sensor *i.e.* the catalase based sensor (CONTROL) was examined over a consecutive period of twelve days in the freely-moving animal (Fig. 8). Recordings were taken following an initial period of *ca.* 24 h recovery from surgery and an additional 24 h following the application of the potential to the sensors (*i.e.* 48 h following implantation). This process effectively eliminates non-faradaic capacitance contributions to the sensor signal and contributions from disruption to the blood brain barrier and other immediate disturbances to the tissue in the initial period following implantation.

Each baseline value was calculated from the same one hour period (5–6 am) prior to commencement of experimental procedures for each day of implantation. A one-way ANOVA analysis of variance revealed that the baseline current showed no significant variation ($P = 0.9185$) over the stated duration of implantation. These results verify the stability of this sensor for the purpose of obtaining long-term *in vivo* electrochemical recordings.

## Conclusions

Significant signal changes were observed at the $H_2O_2$ sensor following the local administration of aCSF and MCS. Catalase inhibition *via* pre-treatment with SA facilitated an increase in the response of the $H_2O_2$ sensor following the exogenous application of 1000 μM $H_2O_2$ in the freely-moving animal. The local administration of 10 mM $H_2O_2$ under anaesthesia led to an immediate increase in response detected by the catalase-based dual $H_2O_2$ sensor. Such results confirm the sensitivity of the $H_2O_2$ sensor to the target substrate in the neuronal environment. No interference was observed in the sensor response upon administration of high levels of AA in the *in vivo* environment. Moreover, the response of the enzymatic component of the dual $H_2O_2$ sensor design remained stable over a continuous period of 12 days.

 

The local application of $H_2O_2$ up to a concentration of 1000 μM in the freely-moving scenario was not sufficient to elevate brain $H_2O_2$ levels, as determined by the lack of a change in response of the implanted $H_2O_2$ sensor. Our research suggests that inherent controlling mechanisms of the neuronal environment are sufficient to handle the exogenous application of $H_2O_2$ within the range tested here in the freely-moving animal. The highly effective anti-oxidant network present in the brain serves as a mop-up mechanism which removes potentially harmful reactive oxygen species such as $H_2O_2$. The results outlined here, lend support to the hypothesis that brain $H_2O_2$ is tightly controlled by the anti-oxidant network *in situ*.[1,5,55]

Brain $H_2O_2$ concentration estimates determined using our electrochemical methods in the anaesthetised animal (0.44 μM) and in the freely-moving scenario (3.1 μM) demonstrate a disparity in the baseline level of this substance. A decrease in neuronal activity corresponds to a decreased demand for regional cerebral blood flow and hence a matched reduction in oxygen levels.[64] Under anaesthesia the decreased demand for and consumption of $O_2$ is due to an induced drop in neuronal activity.[61] This process should reflect a relative change in brain $H_2O_2$ production as $H_2O_2$ is derived indirectly from the mitochondrial reduction of $O_2$.[9] Our research also questions the ability of the antioxidant network to suppress the effect of exogenous application of $H_2O_2$ under conditions of anaesthesia in the acute preparation.

As previously discussed, a variety of first generation biosensors rely on the enzymatic production of $H_2O_2$ at the active surface of the sensor.[33–36] Local perfusion data presented here indicates that endogenous $H_2O_2$ is tightly regulated under normal circumstances. Indeed, the estimated basal concentration of brain $H_2O_2$ in the freely-moving animal (3.1 μM) is quite low with respect to previous estimates.[22] Therefore, endogenous $H_2O_2$ is unlikely to be an interferent for first generation biosensors in terms of neurochemical monitoring. However, such interference would need to be validated for each device given the variety of electrode types, surface modifications and enzyme immobilisation techniques used.

The main performance factors of the $H_2O_2$ sensor *i.e.* sensitivity, selectivity and stability have been verified in the *in vivo* environment. Therefore, the real-time detection of $H_2O_2$ in normal cellular signalling and as an oxidative stress marker in neurodegenerative conditions such as Parkinson's disease, may be facilitated by using this $H_2O_2$ sensor design.

# Methods

### Chemical and solutions

All chemicals used were of analytical reagent grade or higher quality and purchased from Sigma Chemical Co. (Dublin, Ireland). All *in vitro* experiments were conducted in phosphate buffered saline (PBS), pH 7.4; NaCl (0.15 M), NaOH (0.04 M) and $NaH_2PO_4$ (0.04 M) which was prepared in doubly distilled deionised water ($ddH_2O$). Local *in vivo* administrations of a range of $H_2O_2$ concentrations (25, 100, 200, 500, 1000 μM and 10 mM), mercaptosuccinate (MCS, 1000 μM) and acetaminophen (10 mM) were prepared in artificial cerebrospinal fluid (aCSF) which contained NaCl (0.15 M), KCl (0.004 M), $CaCl_2$ (0.0016 M) and $MgCl_2$ (0.021 M) made up in $ddH_2O$. For systemic administrations sodium azide (SA, 20 mg $kg^{-1}$)[58] and (AA, 0.5 mg $kg^{-1}$) were prepared in normal saline (0.9%) and administered by peripheral injection, subcutaneous (s.c.) and intraperitoneal (i.p.) injection respectively, at a volume of 2 mL $kg^{-1}$. The chosen administered dose of both AA and SA was based on previous investigations[34,58,59,65] and had no adverse effect on the animal.

### $H_2O_2$ sensor preparation

For $H_2O_2$ sensor preparation a pair of 1 mm Pt (Pt/Ir) (90%/10%) wire (125 μm bare diameter, 175 μm coated diameter (5T); Advent Research Materials, Suffolk, UK) cylinder electrodes ($Pt_c$) were modified by a procedure described elsewhere in extensive detail.[37–39] Briefly, two polymer modified (Nafion® and PPD) $Pt_c$ amperometric electrodes were prepared, one with catalase adhered (CONTROL) and the other without (PEROX). The $H_2O_2$ sensitivity of this biosensor design was derived by subtracting the CONTROL sensor current response from that recorded at the PEROX sensor.

### Data acquisition and statistical analysis

As previously stated the $H_2O_2$ sensitivity of the paired biosensor design is derived by subtracting the CONTROL sensor current response from that recorded at the PEROX sensor. The overall subtracted response of the paired $H_2O_2$ sensor has previously been provided in extensive detail.[39]

For each change in experimental parameter presented herein the overall paired subtracted current response of the $H_2O_2$ sensor is discussed ($I_{PEROX} - I_{CONTROL}$) unless stated otherwise.

All averaged data is represented as a mean ± standard error, *n* refers to the number of determinations (perfusions, *i.e.* repeat determinations) followed by the number of subjects/sensors. All analysis was performed using the commercial package Prism (version 6; GraphPad Software, Inc., CA, USA). Two-tailed unpaired or paired *t*-tests (stated in text) and one-way ANOVAs with Bonferroni's *post hoc* test were performed where appropriate (specified in text). All statistical analysis was performed using Prism and values of $P < 0.05$ were considered to indicate statistical significance.

### Instrumentation and software

*In vitro* calibrations were performed ($H_2O_2$: 0–550 μM; AA 0–1000 μM) in a standard three-electrode glass electrochemical cell containing 20 mL PBS solution as per a previously described procedure.[38] 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) was utilised without exception for all electrochemical experiments *in vivo*/*in vitro* using a low-noise potentiostat (4 channel Biostat, ACM instruments, UK). Data acquisition was performed with either a notebook PC (*in vitro*) or Mac® (*in vivo*), a PowerLab interface system (ADInstruments Ltd, Oxford, UK) and LabChart® for Windows and Mac® (Version 6, ADInstruments Ltd).

### Surgical protocol

Combined MD probe/dual $H_2O_2$ sensors were implanted in the left/right striatum (randomised) as per a modified version of a previously described procedure.[21] The precise surgical protocol used in the experiments listed here, is described in detail and provided separately here as ESI.†

Briefly, each working electrode (*i.e.* PEROX and CONTROL) was positioned accurately, with the use of a microscope, at either side of an MD probe (MD-2200, 510 μM cannula diameter BASi, West Lafayette, IN, USA). A total of two working electrodes (*i.e.* one PEROX electrode and one CONTROL electrode) were fixed in place on each individual MD probe. A more thorough description of the MD probe/dual $H_2O_2$ sensor construct is provided as ESI.†

Animals were anaesthetised using isoflurane (4% in air for induction, 1.5–3.0% for maintenance; IsoFlo®, Abbott, U.K.) and placed in a stereotaxic frame (Kopf) and kept on a heating pad during the surgical procedure to prevent hyperthermia. The specific coordinates for implantation with the skull levelled between bregma and lambda were as follows; A/P (1.0 mm), M/L (2.5 mm) from bregma and D/V (−5 mm) from dura. Further verification of striatal MD probe/sensor implantation was facilitated by *post mortem* examination of brain tissue.

A reference electrode and auxiliary electrode were constructed in-house (200 μm bare diameter, 270 μm coated diameter (8T), Advent Research Materials, Suffolk, UK). The reference electrode was placed in the cortex and the auxiliary electrode was attached to a surgical support screw as has reported previously[21] and further described here as ESI.† The potential of the reference electrode utilised *in vivo* is comparable to that seen by the SCE used in *in vitro* experiments.[21]

The electrode/MD probe construct was fixed in place using surgical screws (Fine Science Tools GmbH) and dental cement (Dentalon®, Heraeus-Kulzer, Germany). The animal was removed from the stereotaxic frame and placed in an incubator (27 °C) until fully recovered. Post-operative analgesia was provided in the form of an injection (1 mL $kg^{-1}$, s.c.) of Buprecare® (AnimalCare Ltd, UK) which was administered after recovery from surgery. The subject was then closely monitored and left to recover for several hours in a thermostatically controlled cage (Thermacage MkII, Datesand Limited, Manchester, UK).

Animals were allowed to recuperate for a minimum of 24 h following surgery prior to connection with experimental instrumentation. Subjects were assessed for good health according to published guidelines immediately after recovery from anaesthesia and prior to the commencement of experimental procedures each day. All experiments conducted on animals were approved by Maynooth University research ethics committee and performed under license (B100/2205) in accordance with the European Communities Regulations 2002 (Irish Statutory Instrument 566/2002 Amendment of Cruelty to Animals Act 1876).

### Experimental conditions *in vivo*

Male Wistar rats (Charles River UK Ltd., Kent, UK) weighing between 200 and 300 g were singly housed using Raturn® sampling cage systems (BASi, West Lafayette, IN, USA) in a temperature (17–23 °C), humidity and light-controlled (12 h light/12 h dark; lights on at 07:00) environment with access to food and water *ad libitum*. All experiments were carried out with the animal in its home bowl. The implanted sensors were connected directly to the potentiostat (4 channel Biostat, ACM instruments, UK) *via* a six-pin Teflon® socket (Plastics-One, Roanoke, VA, USA) using a flexible screened six-core cable (Plastics-One, Roanoke, VA, USA). 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 commenced in order to ensure that the background currents for the electrodes were completely stabilised. A low-pass digital filter (50 Hz cut-off) was used to eliminate mains AC noise and all data was recorded at either 4 or 10 Hz. A Univentor 801 syringe pump was used for perfusion of substances through the implanted MD probe.

### Local administrations

All local administrations were delivered to the striatum using the RD method.[41] Substances of interest were perfused through the MD probe *in vivo* for a period of approximately 15 minutes (freely-moving subjects) and 10 minutes (anaesthetised subjects) at a rate of 2.0 μL $min^{-1}$.

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