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Ceramic-based multisite microelectrode arrays for simultaneous measures of choline and acetylcholine in CNS

Jason J. Burmeister [a], Francois Pomerleau [a], Peter Huettl [a], Clelland R. Gash [b], Catherine E. Werner [a], John P. Bruno [b], Greg A. Gerhardt [a,*]

[a] *Departments of Anatomy & Neurobiology and Neurology, Center for Microelectrode Technology, and the Morris K. Udall Parkinson's Disease Research Center of Excellence, University of Kentucky Chandler Medical Center, Lexington, KY, USA*
[b] *Departments of Psychology and Neuroscience, Ohio State University, Columbus, OH, USA*

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Abstract

A ceramic-based microelectrode array (MEA) with enzyme coatings for the accurate measurement of acetylcholine (ACh) in brain tissues is presented. Novel design features allow for self-referencing recordings for improved limits of detection and highly selective measurements of ACh and choline (Ch), simultaneously. Design and fabrication features also result in minimal tissue damage during implantation and improved enzyme coatings due to isolated recording sites. In these studies we have used a recombinant human acetylcholinesterase enzyme coating, which has better reproducibility than other commercially available enzymes. The precisely patterned recording site dimensions, low limit of detection (0.2 μM) and fast response time (∼1 s) allow for second-by-second measurements of ACh and Ch in brain tissues. An electropolymerized meta-phenylenediamine (mPD) layer was used to exclude interfering substances from being recorded at the platinum recording sites. Our studies support that the mPD layer was stable for over 24 h under *in vitro* and *in vivo* recording conditions. In addition, our work supports that the current configuration of the MEAs produces a robust design, which is suited for measures of ACh and Ch in rat brain.

Keywords: Microelectrode; Array; Phenylenediamine; Acetylcholine; Choline

1. Introduction

Although acetylcholine (ACh) is a key neurotransmitter in the central nervous system its robust regulation in the mammalian brain has made it difficult to quantify *in situ*. Endogenous acetylcholinesterase (AChE) quickly converts ACh to Ch in the brain. Some reports use microdialysis sampling and an enzyme reactor to remove choline prior to acetylcholine quantification by various methods including liquid chromatography (Herzog et al., 2003; You et al., 2003), online electrochemical sensors (Niwa et al., 1998; Guerrieri et al., 2006), and flow injection analysis with chemiluminometric detection (Kiba et al., 2003). Although excellent detection limits and selectivity can be achieved with the aforementioned methods, due to their temporal limitations, they are unable to monitor second-by-second events *in vivo*. AChE/choline oxidase (ChOx)-based microelectrode arrays (MEAs) implanted into brain tissues provide faster response times and finer spatial resolutions required for accurate second-by-second measurements of ACh and Ch in heterogeneous brain tissues (Burmeister et al., 2005; Karube et al., 1993; Tamiya and Karube, 1992; Navera et al., 1991; Eppelsheim and Hampp, 1994; Doretti et al., 2000; Lin et al., 2004; Mitchell, 2004; Parikh et al., 2004; Cui et al., 2001; Garguilo and Michael, 1996).

In prior studies we have reported a Ch recording microelectrode, which exhibits excellent sensitivity and provides an indirect marker of ACh release (Burmeister et al., 2003; Parikh et al., 2004). In order to directly and accurately measure ACh, a dual enzyme (AChE/ChOx) approach must be employed. To detect ACh using enzyme coated MEAs, ACh must first be converted to choline using AChE. Choline is then converted to hydrogen peroxide (H_2O_2) via ChOx. The

* Corresponding author at: Department of Anatomy and Neurobiology, University of Kentucky Chandler Medical Center, Room 306 Davis Mills Building, Lexington, KY 40536-0098 USA. Tel.: +1 859 323 4531; fax: +1 859 257 5310.
E-mail address: gregg@uky.edu (G.A. Gerhardt).

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reporter molecule generated, H_2O_2, can then be oxidized at a platinum microelectrode by means of constant potential amperometry. Because other sources of Ch may contribute to the signal quantified as ACh, Ch must be monitored simultaneously with recording sites located close to the ACh measuring sites. An adjacent enzyme-coated recording site with ChOx only is used for this purpose. Accurate ACh measurements are further complicated if the microelectrodes are responding to other interfering neurochemicals such as dopamine (DA), norepinephrine (NE) or ascorbic acid (AA), which need to be minimized by a selective layer such as phenylenediamine (Friedemann et al., 1996). Thus, the selectivity of MEAs is essential if multiple recording sites are to be compared for quantification for the selective determination of ACh.

Previous attempts to quantify ACh using microelectrodes involved use of two separate hand-made wire-type microelectrodes placed independently for measurements of Ch and ACh (Mitchell, 2004). The recording properties of hand-made microelectrodes are variable due to the nature of their assembly. The properties of these microelectrodes can differ greatly depending on the person assembling them. Mass-fabricated MEAs are much more uniform in their design and production (Burmeister et al., 2002, 2003). When comparing signals from multiple microelectrode recording sites it is essential that the microelectrodes have matched recording properties. In addition, for use *in vivo,* the microelectrodes must be placed in close proximity (<100 μm) to minimize brain regional differences in the concentrations of ACh and Ch. MEAs are ideally suited for signal subtraction because matched recording sites can be fabricated in a set geometrical configuration for optimal *in vivo* recordings. Multiple hand-fabricated microelectrodes do not afford the routine ability for precise placement close enough for accurate *in vivo* measurements of ACh.

Recent studies from our group have used 4-site ceramic-based MEAs designed for *in vivo* brain recordings. These have been used with great success in measuring glutamate (Glu) and Ch *in vivo* (Burmeister et al., 2002, 2003; Burmeister and Gerhardt, 2003; Parikh et al., 2004; Day et al., 2006). We recently reported the ability of the ACh-sensitive MEAs to simultaneously detect ACh and Ch release in prefrontal cortex of anesthetized and freely moving rats (Bruno et al., 2006a,b). This was achieved by employing matched recording site pairs located within 100 μm of each other, with appropriate coatings and self-referencing recording methods. In this study we include a more detailed and well characterized procedure for the fabrication of the MEAs for simultaneous measures of ACh and Ch *in vitro* and *in vivo.* We now report the use of a recombinant human acetylcholinesterase (hAChE) enzyme, which has high purity and results in more reproducible recordings of ACh. Another purpose of the present manuscript was to extend our initial report by determining the response time of the ACh-sensitive MEA and to perform a comparison between Nafion and mPD as barriers to enhance selectivity for these measures. *In vivo* recordings of ACh and Ch signals are also included to show its performance in brain tissue.

2. Methods

2.1. Chemicals

All chemicals were used as received unless otherwise stated. DA, NE, H_2O_2, Ch, potassium chloride, ACh, sodium chloride, glutaraldehyde (25% in water), bovine serum albumin (BSA), monosodium phosphate, disodium phosphate, recombinant hAChE expressed in HEK 293 cells, and ChOx from *Anthrobacter globiformis* were obtained from Sigma. The meta-phenylenediamine dihydrochloride (mPD), AA, Nafion® (5% in aliphatic alcohols and water) were obtained from Aldrich. Glutaraldehyde was stored at −20 °C and mPD was stored in a desiccator at 25 °C.

2.2. Microelectrode assembly

MEAs were assembled as previously described in conjunction with Thin Film Technologies and Hybrid Circuits (Burmeister et al., 2002; Burmeister et al., 2000; Bruno et al., 2006a,b; Gerhardt and Burmeister, 2000; Salvatore et al., 2003). Briefly, a ceramic substrate was patterned with Ti then Pt and insulated with polyimide using photolithographic procedures. Individual MEAs were cut from the substrate using a diamond saw. These MEAs were attached by wire bonding and epoxy to a printed circuit board 'paddle' for connection to equipment for testing.

2.3. Coatings

2.3.1. Enzyme coatings

Enzymes were coated prior to plating with mPD. All recording sites were coated 3 times with approximately 0.1 μl of a mixture containing 0.2 units/μl ChOx, 0.9% BSA and 0.11% glutaraldehyde then they were allowed to cure at 4 °C for at least 48 h. ACh detecting recording sites were coated 3 times with 0.83 units/μl hAChE, 0.83% BSA and 0.10% glutaraldehyde. The enzyme thickness was approximately 5–10 microns (unpublished data). Ch detecting recording sites were coated 3 times with a mixture of 1% BSA and 0.13% glutaraldehyde to simulate the diffusion layer thickness of the ACh measuring sites. Enzyme-coated microelectrodes were allowed to cure at 4 °C for at least 24 h before further coating or use.

2.3.2. Meta-Phenylenediamine

A 5 mM meta-Phenylenediamine (mPD) solution was prepared in 0.05 M PBS (0.9% NaCl, pH 7.4, 25 °C) that had been previously purged with N_2 gas to displace dissolved O_2 to limit auto-oxidation of mPD. Electropolymerization of mPD onto the microelectrode recording sites was accomplished by employing cyclic voltammetry between +0.2 and +0.7 V versus an Ag/AgCl reference electrode in the unstirred mPD solution using a scan rate of 50 mV/s. The microelectrodes were coated until they showed sufficient selectivity over AA and DA (between 20 and 30 min plating time).

2.4. Calibration

The microelectrode tips were soaked in 0.05 M PBS at 25 °C for at least 20 min prior to calibration. The electrodes were calibrated in stirred 0.05 M PBS (37 °C, pH 7.4) at a constant potential of +0.7 V versus Ag/AgCl reference electrode. The change in measured current was recorded as aliquots of 20 mM test solutions (2 mM for DA) were added to the PBS solution yielding the following concentrations: AA [250 μM], ACh [20–60 μM], Ch [20 μM], and DA [2 μM]. The FAST16 recording system and software (Quanteon, Nicholasville, KY, USA) was used for all electrochemical measurements. Selectivity ratios were calculated for AA, Ch and DA. If the selectivity was negative (no change) or very high, the following selectivity ratios were used to adjust ratios for calculation purposes: AA 500, Ch 500, and DA 200.

2.5. Response time

Microelectrode response time was measured using a custom flow stream system. The flow stream consisted of a syringe pump (Razel, A-99), with a 60 ml syringe, an HPLC sample injector (Beckman, 210A) having a 500 μl sample loop, a polyethylene reservoir and polyethylene tubing (1.14 mm i.d.). The MEA tip was placed at the end of the tubing and a Ag/AgCl reference electrode was placed in the reservoir. The reservoir and approximately 15 cm of PE tubing were immersed in a temperature-controlled water bath held at 37 °C. H_2O_2 (8.8 μM, 300 μl) or ACh (40 μM, 300 μl) from the sample loop was injected into the flow stream at a rate of 2.17 ml/min. A bubble was injected between the sample and flow stream to decrease mixing of the sample with the PBS carrier yielding a rapid step in concentration. The microelectrode response time was calculated as the time for the response to rise from 5% to 90% of the maximum amplitude for the concentration step.

2.6. In vivo measures

Animals were cared for and handled in our Association for Assessment and Accreditation of Laboratory Animal Care International (AAALACI)-approved animal resource center at the University of Kentucky. They were used following specific protocols approved by our Institutional Animal Care and Use Committee. Male Fischer 344 rats (3–6 months old) were prepared for *in vivo* experimentation as previously described (Bruno et al., 2006a,b; Burmeister et al., 2002; Friedemann and Gerhardt, 1992; Cass et al., 1993). Micropipettes (i.d. ~15 μm) formed from single barrel glass capillaries (1.0 mm × 0.58 mm, 6″, A-M Systems, Inc., Everett, WA) were attached to the microelectrode PCB holder with a spacing of 50–100 μm between the tip of the micropipette and the center of the MEA recording sites. These were used to locally apply drug solutions by pressure ejection (KCl: 70 mM KCl, 2.5 mM $CaCl_2$, 75 mM NaCl, pH 7.4; or ACh: 10 mM in 0.9% saline, pH 7.4) into brain tissue of urethane anesthetized rats (1.25 g/kg, ip). A miniature (200 μm diameter) Ag/AgCl reference electrode was implanted into brain parenchyma that was remote from the recording areas. MEAs with attached micropipettes were slowly lowered into the striatum of the rat brain using stereotaxic coordinates (ML −2.5, AP +1.1, DV −4.0 to −7.0 mm) versus bregma calculated using the rat brain atlas of Paxinos and Watson (1986). A computer controlled FAST16 recording system (Quanteon, LLC) was used to simultaneously record and quantify data from the MEA recording sites.

3. Results

3.1. Microelectrode array

A previously described ceramic-based 4-site MEA was used in this study (Burmeister et al., 2002; Burmeister et al., 2000; Bruno et al., 2006a,b; Gerhardt and Burmeister, 2000; Salvatore et al., 2003). The array consists of 2 pairs of 333 μm × 15 μm Pt recording sites with 30 μm spacing between adjacent pairs and a 100 μm distance between the paired ends of the MEA. The fixed geometry ensures consistent spacing between recording sites for optimal signal comparison and subtraction. The planar design is advantageous when comparing signals from recording sites because it allows for the precise placement of the ACh/Ch and Ch only detecting sites of the MEA. This proximity affords the ability to reliably and accurately subtract out any Ch signal not attributable to ACh while still recording from a region in close enough proximity to the ACh site to monitor similar brain tissue.

The current MEAs have a polyimide insulating layer that was increased from 2 to 4 μm to better isolate each recording site. In addition to being more durable and a better insulator, the thicker polyimide layer provides a deeper 'well' for enzyme adhesion. The small blunt tip allows the recording sites to be closer to the microelectrode tip (100 μm) for recording from deeper brain structures. This small blunt tip design penetrates into brain tissue with less damage than sharper designs that can cut into tissue instead of pushing aside and moving past brain tissue. This MEA design has been implanted for up to 3 months *in vivo* for electrochemical recordings (Rutherford et al., 2007). Although during these tests the glutamate oxidase coated MEAs lost their response to Glu after 2 weeks the microelectrode array did respond to locally injected H_2O_2 up to 3 months post-implantation.

The sensitivity and selectivity of a number of MEAs configured for ACh and Ch measures are summarized in Table 1.

Table 1

Analytical properties	Single electrode value ± S.E.M.	Subtracted value ± S.E.M.
Detection limit (μM)	0.12 ± 0.02	0.18 ± 0.02
Linearity—R^2 (0–80 μM)	0.998 ± 0.001	0.998 ± 0.001
Sample size—*N*	16	16
Slope (pA/μM)	4.7 ± 0.3	
Selectivity (ACh:Interferent)		
Ascorbic acid	254 ± 41	496 ± 4
Choline	0.90 ± 0.03	500 ± 0
Dopamine	101 ± 26	113 ± 25

On average, the present design is capable of detecting <0.2 μM changes or resting levels of ACh (limit of detection based on signal-to-noise of current = 3). The MEAs exhibit excellent linearity over a concentration range of 0–60 μM ACh, which is well within the normal range seen in rat brain (Bruno et al., 2006a,b). The self referencing measures result in selectivities of ~500: 1 for ACh versus AA and Ch. In addition, selectivity against DA is >100:1. We have found that electrodes that are selective over DA are also selective over other monoamines such as NE. Thus, DA is used as a test molecule for all monoamines. All of these performance parameters exceed previously reported miniature or microelectrode technologies for measures of ACh and/or Ch.

Of particular note for this current MEA design is the use of the hACh enzyme. We have found that other commercially available ACh enzymes, such as that derived from the electric eel (Sigma), were often variable in purity. 'Initial attempts to analyze the AChE from electric eel showed impurities from the purification process and/or degradation products from the enzyme. The exact chemical makeup of the impurities is not known. Impurities can yield inconsistent results when the enzymes are immobilized. Others have reported poor results when using AChE from electric eel (Giuliano et al., 2007). If such enzymes were used the reproducibility of the results, when they worked, was not comparable to those seen in Table 1. We are in the process of testing more commercially available ACh enzymes as well as AChE purification schemes for the detection of ACh.

3.2. Oxygen dependence of the ACh and Ch MEAS

Fig. 3A shows the microelectrode response to ACh as the O_2 concentration is varied. The O_2 dependence of the ACh microelectrodes is essentially the same as that of previously reported Ch microelectrodes (Burmeister et al., 2003). This is consistent with the fact that AChE does not consume O_2 when converting ACh to Ch. When detecting 25 mM ACh the MEAs retained $85 \pm 8\%$ ($n = 4$) of their response to ACh when the O_2 tension was reduced from the air-saturated calibration concentration of 200 to 50 μM, which is the normal brain O_2 concentration (Dixon et al., 2002; Lowry et al., 1998; Hu et al., 1994). Thus, there is little oxygen dependence of the present MEA configuration.

3.3. Response time

Flow stream analysis was used to compare the response time of MEAs that were coated with mPD to Nafion® with high temperature curing. No significant response time slowing for H_2O_2 detection was observed when the MEAs were coated with Nafion® or mPD. The average 90% response time to 8.8 μM H_2O_2 for the mPD coated microelectrode was 0.119 ± 0.008 s ($n = 12$) while the response time for Nafion® coated microelectrodes was 0.117 ± 0.006 s ($n = 16$). An un-coated microelectrode yielded a 90% response in 0.111 ± 0.007 s ($n = 20$). Thus, neither Nafion® nor mPD significantly slowed the response time for H_2O_2. Enzyme-coated ACh microelectrodes with mPD had 90% response times of 1.162 ± 0.037 s ($n = 10$) to 40 μM ACh and 0.335 ± 0.007 s ($n = 10$) to 8.8 μM H_2O_2. This suggests that enzyme kinetics were responsible for the majority of the slower response of the enzyme coated microelectrodes. However, the enzyme layer itself does act as a diffusion barrier to H_2O_2 detection contributing to some of the delayed signal response. Our strategy in the future is to use thinner and fewer enzyme layers to minimize microelectrode response times due to increased diffusion time. Fig. 3B shows flow stream data for a bare electrode responding to 8.8 μM H_2O_2 (black trace) and an enzyme coated microelectrode responding to 40 μM ACh (red trace).

We have chosen enzyme thicknesses that yield excellent sensitivity. We do not know the complete relationship between enzyme thickness and response time. Experience shows that microelectrode sensitivity is lower and response time is faster for very thin coatings. Sensitivity increases with enzyme thickness up to a point then levels off; however, response time becomes longer as a thicker diffusion barrier is applied to the microelectrode. The enzyme thicknesses in the present study were chosen because they yield acceptable sensitivity and response time. The relationship between enzyme thickness and response time however needs further study. For this purpose an automated enzyme coater is being developed to precisely apply enzymes in a controlled thickness.

Interestingly we have found that the selectivity over DA and AA is greatly diminished if enzymes were applied after the mPD was plated onto the electrode surface. The glutaraldehyde cross-linking process seems to be detrimental to the mPD layer function, maybe by increasing pore sizes. Best results were obtained when enzymes were applied before plating with mPD. As others have reported, benefits of plating mPD after enzyme application include fast response, improved sensitivity due to peroxide being generated close to the electrode surface, recycling of peroxide back to oxygen, and improved stability of the polymerized layer (Chen et al., 2002; Matsumoto et al., 2002). The enzymatic activity is not affected by mPD electropolymerization. In addition, we have found that the most consistent results were obtained if the enzymes were allowed to cure on the recording sites for at least 24 h before plating with mPD. This ensures that the enzymes will not dissipate from the MEA surface when put into solution.

3.4. Enzyme coatings

Fig. 1 shows the function of the various layers for detection of ACh. The ACh microelectrodes detect ACh by using immobilized AChE to convert ACh to Ch. Immobilized ChOx converts the produced Ch to H_2O_2, which can be quantified at a platinum electrode when polarized at a potential of +0.7 V versus Ag/AgCl. Most remaining potential interferents are blocked by the selective layers. A second recording site is used to quantify Ch using ChOx alone. This electrode is sensitive to the H_2O_2 generated from non-ACh attributable Ch degradation and whatever electroactive interferents not blocked by the selective layers. Ideally the response from the Ch-detecting site may be subtracted from the ACh-detecting site yielding only the ACh signal.

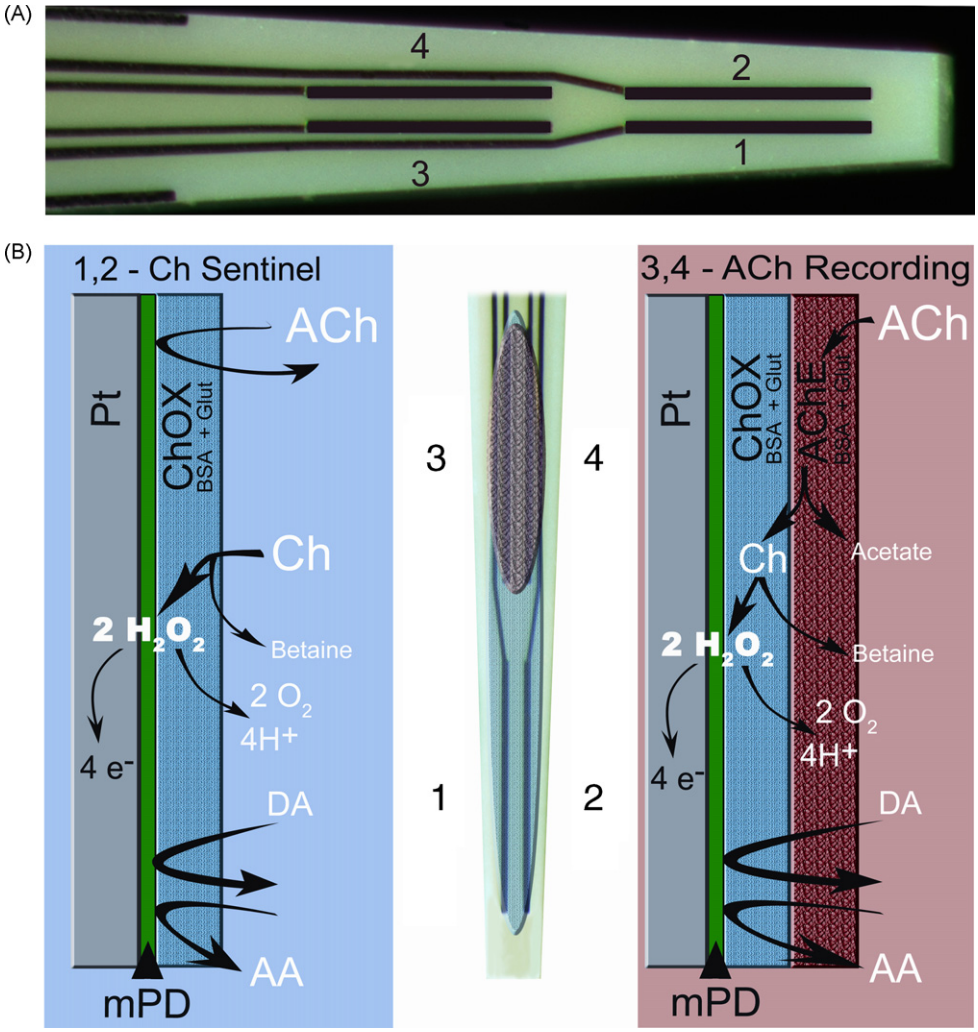

Fig. 1. (A) Photograph of the microelectrode tip. (B) Schematic diagram showing the function of the various layers for detection of ACh and Ch on the MEA. The ovals on the recording sites indicate the placement of the ChOx and hAChE enzyme layers. All four recording sites are coated with COx. Only sites 3 and 4 are additionally coated with hAChE. The microelectrode array consists of four 333 × 15 micron Pt recording sites. Note the blunt tip, which penetrates into brain tissue with minimal damage.

In order for ACh MEA self-referencing recordings to work properly, prerecording calibrations with Ch were used to normalize the ACh-detecting and Ch-detecting recording sites. In our previous reports DA was used to normalize Glu and Ch microelectrodes for accurate DA removal (Bruno et al., 2006a,b; Burmeister et al., 2005; Burmeister et al., 2003; Burmeister et al., 2002; Burmeister and Gerhardt, 2001). Small differences in Ch sensitivity are corrected for accurate Ch signal removal and ACh quantification.

Signal subtraction or 'self-referencing' is important for removal of endogenous Ch from the ACh signal (Burmeister and Gerhardt, 2001). This is complicated by the fact that depending on placement and MEA spatial orientation the recording sites on a single MEA may be in functionally different locations in the brain. For accurate signal subtraction the recording sites should be exposed to the same source of neurochemicals. Although the heterogeneity of the CNS can result in differences in the ACh-detecting and Ch-detecting sites, the present work presents a viable ACh-detecting MEA when properly employed.

Fig. 2 shows the microelectrode response to additions of stock solutions during calibration. The majority of AA and essentially all DA are blocked by the mPD layer. Table 1 lists the analytical properties of the ACh/Ch microelectrodes. The selectivity over AA was 496:1 ± 4 ($n = 16$) and DA 113:1 ± 25 ($n = 16$). The ACh limit of detection was less than 0.2 μM in both single and self-referencing modes, showing that low detection limits and high selectivity can be achieved using electropolymerized mPD as the interferent rejection layer Fig. 3.

3.5. *In vivo* Measures

Fig. 4 shows the ACh microelectrode response to *in vivo* ACh applications (10 mM, 125 nl) in rat striatum. The peaks measured on the ACh recording sites were larger than those on the Ch sites. The bottom trace shows the self-referencing signal functioning as designed to subtract background interferents, in particular the Ch signal not attributable to ACh degradation by AChE at the recording site.

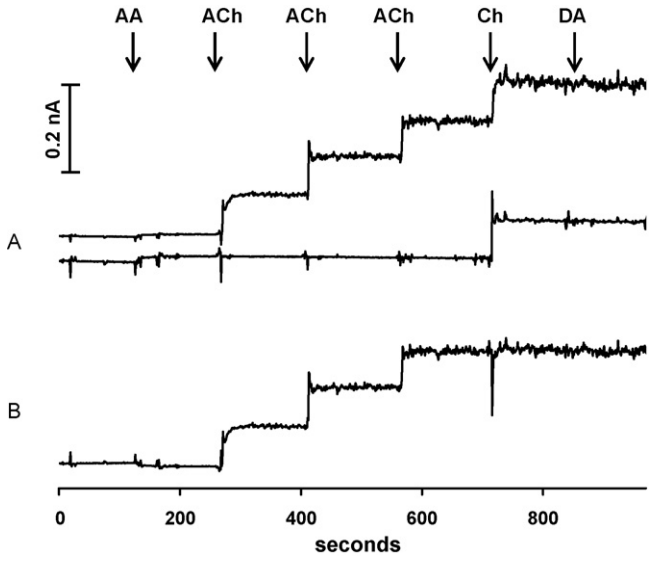

Fig. 2. Time plots showing microelectrode current responses to additions of stock solutions during calibration. (A) Upper trace is the signal from the ACh ± Ch electrode and the lower trace is the Ch electrode. Additions of analytes and interferents occurred at the arrows: AA, ACh, Ch, NE and DA. The majority of AA and essentially all DA simulated interferent responses were blocked by the polyphenylenediamine layer. (B) The Ch signal can be removed by self-referencing or subtraction.

Fig. 5 shows ACh microelectrode responses to repeated KCl applications (70 mM, 450 nl) in rat striatum at various depths (coordinates: ML ± 2.5, AP ± 1.1, DV −4.5, −5.5 and −7.0 mm). Again, the peaks on the ACh electrode were larger than those on the Ch electrode. There were no dilution artifacts due to AA or longer lasting dome-shaped signals attributable to DA or NE. Again the self-referencing function shows the subtracted ACh signal. More *in vivo* measures can be found elsewhere (Bruno et al., 2006a,b).

4. Discussion

The polyphenylenediamine coatings have been previously used to eliminate interfering substances from being detected at a variety of microelectrodes (Mitchell, 2004; Leegsma-Vogt et al., 2004; Yang et al., 2004; Law et al., 2003; Yang et al., 2002; McMahon et al., 2004; Lowry et al., 1998; Ryan et al., 1997; Friedemann et al., 1996). When it is electropolymerized onto a microelectrode surface, meta-phenylenediamine forms a matrix with molecular pores that can exclude larger organic molecules like DA and AA while allowing smaller molecules like H_2O_2 and O_2 to pass through to the microelectrode surface. While mPD provides an ideal interferent rejection layer for acute recording conditions, its long-term stability requires further study. Locally high H_2O_2 concentrations may contribute to mPD degradation. Presently for long term recordings (chronic implants) lasting more than 72 h, we recommend Nafion® alone or in conjunction with mPD as the exclusion layer to avoid artifacts and misleading results caused by a variety of interfering molecules that can affect the background signal observed (Burmeister et al., 2002; Burmeister and Gerhardt, 2001, 2003; Pomerleau et al., 2003; Burmeister et al., 2003, 2005). To date

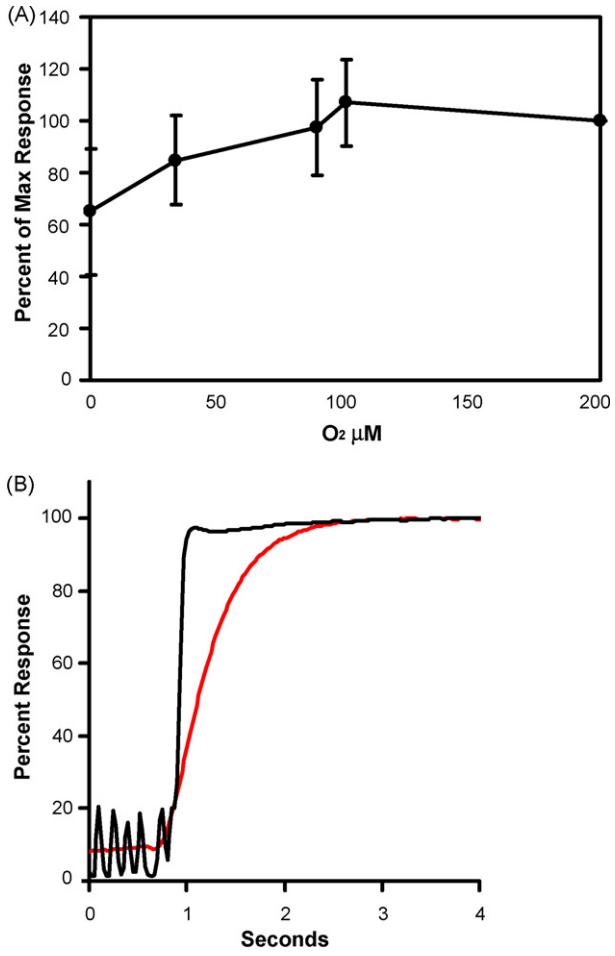

Fig. 3. (A) *In vitro* ACh microelectrode response to 100 µM ACh vs. O_2 concentration in PBS. ($n = 4$, error bars are the standard deviation) (B) Flow stream data of a bare microelectrode responding to 8.8 µM H_2O_2 (red trace) and enzyme coated microelectrode responding to ACh (black trace).

the AChE and ChOx enzyme coatings remain viable for at least 1 week when chronically implanted. Periodic tests are employed to check microelectrode sensitivity and selectivity for long term *in vivo* experiments.

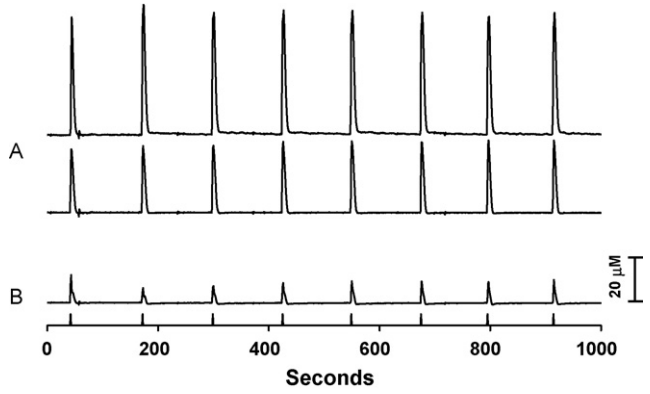

Fig. 4. Recordings by the MEAs in the rat striatum from locally applied ACh. (10 mM pH 7.4 in O.9% saline, 125 nl; coordinates: ML ± 2.5, AP ± 1.1, DV −4.0 mm). (A) Responses from the ACh/Ch sensitive sites (upper trace) and Ch sensitive site (lower trace). (B) ACh only response from self-referencing. Marks above the time axis indicate the times of the local ACh applications.

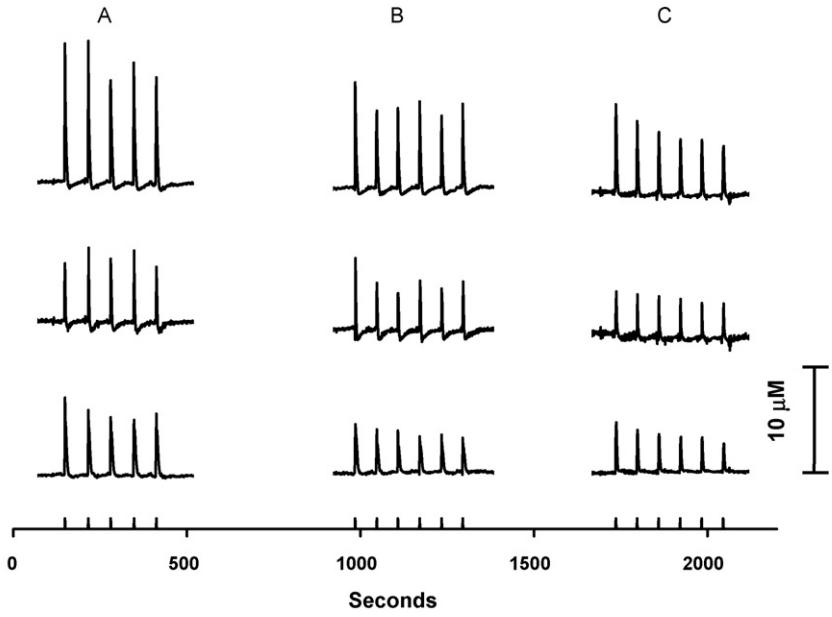

Fig. 5. *In vivo* measures of ACh and Ch produced by local application of KCl (70 mM, 450 nl) in rat striatum (coordinates: ML −2.5, AP ± 1.1) at the following depths: (A) 4.5 mm, (B) 5.5 mm, and (C) 7.0 mm. Upper traces are the ACh/Ch recording site, middle traces are the Ch only recording site and the lower traces are the subtracted result (ACh). Marks above the time axis indicate the times of the KCl applications.

Other ACh microelectrodes have included ascorbate oxidase into the enzyme layer to consume ascorbate near the microelectrode recording surface (Mitchell, 2004; Hu et al., 1994). Ascorbate oxidase was not used here to avoid further O_2 dependence and decreased response time due to the increased coating thickness. Although O_2 is consumed by ChOx as it converts choline into betaine and H_2O_2, some of the O_2 is recovered by the oxidation of H_2O_2 at the electrode surface replacing some of the consumed O_2. The O_2 consumed by ascorbate oxidase, however, is lost from the system as ascorbate is converted to dehydroascorbate and water. Furthermore, ascorbate concentrations are often relatively high, 250–500 μM, compared to *in vivo* O_2 concentrations, ∼50 μM (Dixon et al., 2002; Hu et al., 1994).

An analytical issue in the use of this new type of dual enzyme based microelectrode array involves the practical detection limits for acetylcholine. Prior studies of microdialysis have supported that extracellular levels of choline are high relative to acetylcholine. Under these circumstances, the subtraction approach used by the electrode recording technique yield a very small signal relative to the large choline response. Experimentally we have found in anesthetized, as well as awake animals, that the levels of choline do not far exceed those of acetylcholine using the microelectrode recording technique. Typical ratios of choline/acetylcholine response range from 2/1 to 10/1 (Köppen et al., 1996). We attribute the lower levels of choline measured by the microelectrode to a decrease in the damage produced by the microelectrode as compared to the damaged produced by microdialysis. However, it should be noted that certain pharmacological manipulations, different animal strains and transgenic animals with altered cholinergic function may contribute to conditions where the choline/acetylcholine ratio is high and the capability of the dual electrode recording technique for measuring both acetylcholine and choline will be compromised. Further studies are needed to determine the situations where the dual electrode technology is incapable of recording both choline and acetylcholine in the extracellular space of the brain.

The results from the experiment on KCl-induced ACh release (Fig. 5) indicate that the current detected at the site sensitive to ACh and Ch was significantly greater in magnitude than the current simultaneously measured at the site sensitive to only Ch. Such site-dependent differences in current for the ACh/Ch-sensitive MEA have been previously reported from intracortical ejections of KCl or nicotine in anesthetized rats or in response to a startling auditory stimulus (Bruno et al., 2006a,b). The self-referencing (Bruno et al., 2006a,b; Burmeister and Gerhardt, 2001) technique used in this manuscript allows for the isolation of the current due exclusively to the oxidation of Ch from recently released ACh. A plausible explanation for these observed differences in current is that, under the above stimulating conditions, released ACh diffuses into the extrasynaptic space where it can be hydrolyzed by a combination of endogenous and membrane-bound AChE. The ability of released ACh to enter the extrasynaptic compartment and gain access to the MEA, as well as the established ability to measure ACh using microdialysis in the absence of an inhibitor of AChE (see Herzog et al., 2003; Ichikawa et al., 2002) is consistent with the position that ACh can function in a diffuse, volume-transmission mode (Descarries, 1998; Descarries et al., 1997; Descarries and Mechawar, 2000) as well as the more conventional, spatially restrictive synaptic neurotransmission signaling mode.

5. Conclusions

The present studies demonstrate an improved ACh-sensitive MEA for *in vivo* self-referencing recordings created by employ-

ing precisely patterned recording sites consisting of specific and selective coating layers. ACh and Ch were accurately and simultaneously measured *in vitro* and *in vivo* due to the use of electropolymerized mPD combined with precisely patterned recording sites in close proximity to one another. While mPD is effective at blocking DA on enzyme-coated microelectrodes for at least 24–36 h, long term viability issues require further studies. The AChE and ChOx enzyme coatings remained viable for at least 1 week when chronically implanted. Selective coatings (Nafion®, mPD) yielded rapid response times that were not significantly different for H_2O_2 on uncoated microelectrodes. Thinly applied enzyme layers yield ACh microelectrodes with 1 s response times. The ability to differentially coat adjacent recording sites with and without the specific enzymes creates a functional means to accurately subtract out interfering signals in order to quantify the ACh and Ch signals independently. Self-referencing may be used to quantify and remove the contribution from Ch resulting in a more clearly defined ACh signal. We have recently published a more extensive *in vivo* validation of this methodology (Bruno et al., 2006a,b). The ACh-sensitive MEA represents a sensitive and selective technique for quantifying *in vivo* ACh release with a temporal and spatial resolution that far exceeds conventional methodologies.

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