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Simultaneous measurement of cholinergic tone and neuronal network dynamics *in vivo* in the rat brain using a novel choline oxidase based electrochemical biosensor

Ricardo M. Santos [a,c], João Laranjinha [a,b], Rui M. Barbosa [a,b,*], Anton Sirota [c,*]

[a] Center for Neuroscience and Cell Biology, University of Coimbra, 3004-517 Coimbra, Portugal
[b] Faculty of Pharmacy, University of Coimbra, Health Campus, Azinhaga de Santa Comba, 3000-548 Coimbra, Portugal
[c] Bernstein Center for Computational Neuroscience and Munich Cluster for Systems Neurology (SyNergy), Ludwig-Maximilians Universität München, Grosshaderner Strasse 2, 82152 Planegg-Martinsried, Germany

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ABSTRACT

Acetylcholine (ACh) modulates neuronal network activities implicated in cognition, including theta and gamma oscillations but the mechanisms remain poorly understood. Joint measurements of cholinergic activity and neuronal network dynamics with high spatio-temporal resolution are critical to understand ACh neuromodulation. However, current electrochemical biosensors are not optimized to measure nanomolar cholinergic signals across small regions like hippocampal sub-layers.

Here, we report a novel oxidase-based electrochemical biosensor that matches these constraints. The approach is based on measurement of H_2O_2 generated by choline oxidase (ChOx) in the presence of choline (Ch). The microelectrode design consists of a twisted pair of 50 μm diameter Pt/Ir wires (sensor and sentinel), which is scalable, provides high spatial resolution and optimizes common mode rejection. Microelectrode coating with ChOx in chitosan cross-linked with benzoquinone is simple, mechanically robust and provides high sensitivity (324 ± 46 nA μM^{-1} cm^{-2}), a limit of detection of 16 nM and a t_{50} response time of 1.4 s.

Local field potential (LFP)-related currents dominate high-frequency component of electrochemical recordings *in vivo*. We significantly improved signal-to-noise-ratio compared to traditional sentinel subtraction by a novel frequency domain common mode rejection procedure that accounts for differential phase and amplitude of LFP-related currents on the two channels.

We demonstrate measurements of spontaneous nanomolar Ch fluctuations, on top of which micromolar Ch increases occurred during periods of theta activity in anesthetized rats. Measurements were not affected by physiological O_2 changes, in agreement with the low biosensor Km for O_2 (2.6 μM). Design and performance of the novel biosensor opens the way for multisite recordings of spontaneous cholinergic dynamics in behaving animals.

1. Introduction

Acetylcholine (ACh) is a neuromodulator strongly implicated in learning and cognition (Deiana et al., 2011; Hasselmo and Sarter, 2011; Micheau and Marighetto, 2011; Picciotto et al., 2012). In the hippocampus, cholinergic afferent activity modulates multiple aspects of neuronal network function implicated in cognition via modulation of internal dynamics, including theta, gamma oscillations and sharp-waves (Buzsáki, 2002; Pignatelli et al., 2011; Vandecasteele et al., 2014). However, the complexity of cholinergic neuromodulation, partly owing to the ubiquitous expression of ACh receptors in multiple interacting neuron types and to the apparently diffuse patterns of cholinergic innervation in hippocampal and cortical regions has rendered the precise mechanisms of ACh action poorly understood (Buzsáki, 2002; Pignatelli et al., 2011; Teles–Grilo Ruivo and Mellor, 2013). Nevertheless, it has become increasingly recognized that the interaction between the spatio-temporal profile of ACh signals and the neurophysiological and behavioral context in which they occur is critical to shape ACh effects (Leão et al., 2012; Lovett–Barron et al., 2014; Muñoz and Rudy, 2014; Parikh et al., 2007). Therefore, sensitive measurements of cholinergic activity with high spatio-temporal resolution

* Corresponding authors.
E-mail addresses: rbarbosa@ff.uc.pt (R.M. Barbosa), sirota@biologie.uni-muenchen.de (A. Sirota).

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coupled to simultaneous assessment of neuronal network dynamics and behavior are critical to understand ACh neuromodulation *in vivo*.

Fast electrochemical techniques, when associated with microelectrodes are particularly suitable for monitoring neuromodulators in the brain extracellular space owing to the high sensitivity, selectivity and spatio-temporal resolution that can be achieved with minimal tissue damage (Lama et al., 2012). Additionally, electrochemical recordings carry local field potential (LFP)-related currents (Zhang et al., 2009), thus offering the unique possibility to measure changes in neuromodulator tone together with ongoing neuronal network activity (inferred from LFP features) using a single sensor.

Different types of enzyme-based electrochemical microbiosensors have been used to study cholinergic transmission by measuring extracellular changes in choline (Ch), a product of ACh hydrolysis by acetylcholinesterase (Burmeister et al., 2003; Garguilo and Michael, 1996, 1993; Xin and Wightman, 1997). However, most of them were constrained by high limits of detection (LODs in the micromolar range) and slow responses (decades of seconds). A successful approach relied on immobilization of choline oxidase (ChOx) on the surface of ceramic-based platinum microelectrode arrays (MEAs). The enzyme catalyzes Ch oxidation in the presence of O_2, generating H_2O_2, which can be detected by electrochemical oxidation on the electrode surface. These sensors exhibited sub-micromolar LODs and response times of few seconds (Burmeister et al., 2003; Parikh et al., 2007, 2004; Zhang et al., 2010). Each Pt site is individually addressable allowing the use of specific sensing chemistry, which is a major advantage of MEAs. Comparison between recordings from sentinel sites (coated with a matrix lacking ChOx) and Choline-measuring (ChOx) sites allows identification of current profiles not related with Ch dynamics (e.g. fluctuations of electroactive interferents like ascorbate or LFP-related currents). Thus, common mode rejection using a sentinel channel greatly improves selectivity and signal-to-noise of biosensor recordings.

The use of MEA-based Ch biosensors *in vivo* supported the reliability of measuring extracellular Ch dynamics as an index of local ACh release, provided that acetylcholinesterase activity is stable (Parikh and Sarter, 2013; Parikh et al., 2004). Insights into the link between ACh, neuronal network activity and behavior have been provided with much better spatio-temporal resolution than in previous microdyalisis studies. For instance, choline increases in the nanomolar range were measured during theta periods in the CA1 sub-region of the hippocampus which lagged theta onset by several seconds (Zhang et al., 2010). In freely behaving rats, micromolar Ch transients lasting few seconds were recorded in the pre-frontal cortex when animals attended to a cue. These signals occurred on top of slower Ch fluctuations whose slope predicted animal's success in cue detection (Parikh et al., 2007). Thus, choline signals can apparently span multiple time scales and amplitudes ranging from seconds to minutes and from low nanomolar to micromolar, respectively. Characterizing the different types of Ch dynamics and their associated neurophysiological and behavioral context is therefore of great relevance to understand ACh neuromodulation.

However, the measurement of spontaneous nanomolar Ch fluctuations across small heterogeneous regions like hippocampal sub-layers is technically challenging and requires further optimization of the biosensor design. In particular: (a) the size of the recording sites should be small enough to allow resolution of small brain regions like hippocampal sub-layers; (b) enzyme immobilization should be efficient in order to keep good sensitivity at small electrode surfaces; (c) given that in most cortical and dorsal-hippocampal regions the gradient of LFP profiles is much steeper across the dorso-ventral axis, the ChOx and sentinel sites should be located at the same depth in order to optimize common LFP mode rejection using sentinel channel; (d) since complex impedance spectra of ChOx and sentinel sites are, in general, expected to be different across wide range of frequencies, common mode rejection procedure has to be performed in the complex form in the frequency domain, which should improve signal-to-noise ratio compared to conventional sentinel subtraction; and (e) chronic measurement of Ch dynamics across multiple regions and layers in freely moving animals requires flexible and minimally invasive design of the electrode that can be scaled and individually driven to the target location, similar to conventional stereotrode electrodes for extracellular recordings (McNaughton et al., 1983).

An additional concern, related to the use of oxidase-based biosensors *in vivo* is their O_2 dependence. Measurements of extracellular O_2 concentration in the neocortex and hippocampus have shown a broad distribution of O_2 basal levels around a mean of 35–50 μM but with a considerable number of observations at lower levels, down to 10 μM (Murr et al., 1994; Nair et al., 1987). The extracellular O_2 concentration is also dynamically regulated according to changes in metabolic demand imposed by neuronal activity (Lourenço et al., 2014; Masamoto and Tanishita, 2009). Thus, negligible O_2 dependence over the range of concentrations described above is a critical requirement for a biosensing application in the brain, particularly when fluctuations in analyte concentration are very small (nanomolar range). Data on Ch biosensors O_2 dependence is scarce, but some results obtained with MEA-based biosensors do not exclude a potential interference when O_2 levels are low (Burmeister et al., 2003).

In this work we developed a new choline microbiosensor design for the measurement of spontaneous fluctuations of cholinergic tone, taking into account the limitations described above. The microelectrodes consisted of two side-by-side disk shaped 50 μm diameter Pt/Ir wires. The microdisks provide high spatial resolution for measurements within the hippocampus and the orientation of the wires, positioned at the same depth, optimizes sentinel channel subtraction *in vivo*.

Choline oxidase was immobilized in a chitosan matrix using *p*-benzoquinone as crosslinker. This novel coating procedure is robust and fast (biosensors can be used 1 h after preparation) and provides very good sensitivity considering the small size of the recording sites.

In anesthetized rats, microbiosensor measurements of Ch and LFP-related currents were carried out in the *stratum oriens* of the CA1 field of hippocampus. Large Ch increases were observed during periods of theta oscillations, which superimposed on the smaller spontaneous nanomolar Ch fluctuations. *In vitro* and *in vivo* results indicate that the biosensor response is not affected by physiological O_2 changes. The data supports the reliability of the microbiosensors to jointly measure spontaneous cholinergic activity and neuronal network dynamics in the brain.

2. Methods

2.1. Chemicals and solutions

All chemicals were of analytical grade and were used as received. Dibasic sodium phosphate hepta-hydrate, monobasic potassium phosphate, sodium chloride and potassium chloride were purchased from Carl Roth. Hydrogen peroxide (30%), choline chloride, sodium L-ascorbate (AA), uric acid (UA), dopamine (DA), chitosan (low molecular weight), choline oxidase (EC 1.1.3.17) from *Alcaligenes sp.*, bovine serum albumin (BSA), *p*-benzoquinone, Nafion® perfluorinated resin 5% in lower aliphatic alcohols and water (15–20%) and urethane were obtained from Sigma-Aldrich.