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## Detection of evoked acetylcholine release in mouse brain slices

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The study of transmitter interactions in reward and motor pathways in the brain, including the striatum, requires methodology to detect stimulus-driven neurotransmitter release events. Such methods exist for dopamine, and have contributed to the understanding of local and behavioral factors that regulate dopamine release. However, factors that regulate release of another key transmitter in these pathways, acetylcholine (ACh), are unresolved, in part because of limited temporal and spatial resolution of current detection methods. We have optimized a voltammetric method for detection of local stimulus-evoked ACh release using enzyme-coated carbon-fiber microelectrodes and fast-scan cyclic voltammetry. These electrodes are based on the detection of  $\text{H}_2\text{O}_2$  generated by the actions of acetylcholine esterase and choline oxidase, and reliably respond to ACh in a concentration-dependent manner. Methods for enzyme coating were optimized for mechanical stability that allowed for their use in *ex vivo* brain slices. We report here the first quantitative assessment of extracellular ACh concentration after local electrical stimulation in dorsal striatum in slices from control mice. The selective detection of ACh under these conditions was confirmed by showing that the response detected in the control slices was absent in slices from mice bred to lack ACh synthesis in the forebrain. These electrodes represent a new tool to study ACh and ACh–dopamine interactions with micrometer spatial resolution.

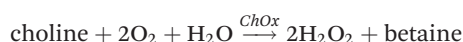
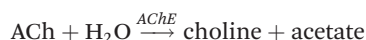
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## Introduction

Electrochemical methods to identify and quantify neurotransmitters were first used for the detection of catecholamines, including dopamine and norepinephrine, following the introduction of electrochemical detection for high-performance liquid chromatography (HPLC-EC)<sup>1</sup> and direct detection using carbon microelectrodes *in vivo*.<sup>2</sup> Detection of neurotransmitters that are not electroactive, like acetylcholine (ACh) has proved to be more challenging. The primary detection method for ACh has been HPLC, with separation of ACh from other analytes, mixing the ACh-containing effluent in a post-column reaction coil with acetylcholinesterase (AChE) and choline oxidase (ChOx), which together produce hydrogen peroxide ( $\text{H}_2\text{O}_2$ ) through the following simplified reaction pathways:



The resulting  $\text{H}_2\text{O}_2$  is electroactive and can be quantified using electrochemical detection to indicate the ACh concentration.<sup>3–5</sup>

This HPLC-EC method can be used to determine tissue content of ACh, but can also be used to assess the extracellular concentration of ACh ( $[\text{ACh}]_o$ ) in the brain *in vivo* when paired with a technique like microdialysis<sup>6,7</sup> that allows monitoring of molecules over time. Microdialysis with off-line HPLC analysis has been used to study changes in extracellular ACh, particularly in the striatum,<sup>8–10</sup> which is a brain region that subserves motor and reward behaviors. In the striatum, the primary source of ACh is from cholinergic interneurons (ChIs), which are tonically active, but dynamically regulated by synaptic input.<sup>11–13</sup>

Two limitations of microdialysis are the requirement for off-line sample analysis and the relatively slow temporal resolution, with typical sampling intervals of 5–20 min.<sup>8–10</sup> These features preclude assessment of dynamic changes in  $[\text{ACh}]_o$  on the timescale afforded by direct detection of electroactive substances like dopamine and norepinephrine at carbon electrodes using chronamperometry<sup>2</sup> or fast-scan cyclic voltammetry (FCV).<sup>14</sup> In particular, FCV has been used to detect rapid spontaneous changes in extracellular dopamine concentration ( $[\text{DA}]_o$ ) *in vivo*<sup>16–18</sup> and evoked dopamine release in *ex vivo* slices,<sup>19,20</sup> that are consistent with phasic firing patterns of dopamine neurons and axons during reward and motor behavior.<sup>21,22</sup> Importantly, stimulated ACh release from striatal ChIs prompts phasic dopamine release;<sup>23,24</sup> thus, factors that alter ACh release will thereby indirectly regulate dopamine

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release, e.g., insulin.<sup>25</sup> These findings reinforce the necessity to characterize stimulus-driven ACh release in order to probe its role in brain reward and motor behavior.

For dopamine detection using FCV with carbon-fiber microelectrodes, a triangular voltage waveform is used, for example from  $-0.7$  V to  $1.3$  V (vs. an Ag/AgCl reference electrode).<sup>20</sup> Dopamine oxidizes within the range of the anodic (oxidizing) sweep, producing a detectable current peak.<sup>15–20</sup> Other neurotransmitters, including ACh and glutamate, are not electroactive and thus cannot be monitored using bare carbon fiber. For both of these, however, enzyme-coated electrodes have been used to convert the neurotransmitter of interest to  $\text{H}_2\text{O}_2$ , usually coupled with amperometric detection.<sup>26,27</sup>

ACh-sensitive carbon-fiber microelectrodes operate using the same principles as ACh detection for HPLC, in that AChE and ChOx are required for generation of electroactive  $\text{H}_2\text{O}_2$ .<sup>26,28–34</sup> Recent improvements include incorporation of bovine serum albumin (BSA) to improve electrode stability,<sup>30,31</sup> and coating with an appropriate polymer to immobilize the mixture at the electrode surface.<sup>29,32,33</sup> The ideal polymer must be stable and resistant to minor physical manipulation, so common practice is the use of a cross-linkable polymer – such as an electrically-polymerized pyrrole or the light-linked polyvinyl alcohol bearing styrylpyridinium (PVA-SbQ).<sup>31–33</sup> Regardless of design, constant-potential amperometry has been the preferred electrochemical method for  $\text{H}_2\text{O}_2$  detection, typically using a Pt electrode base.

Notably,  $\text{H}_2\text{O}_2$  can also be detected at carbon-fiber microelectrodes within the potential range used for FCV.<sup>35</sup> Although carbon-fiber-based ACh sensors have been reported previously,<sup>29,34</sup> these were also used with amperometry for either direct detection of  $\text{H}_2\text{O}_2$  or mediated electron transfer, and were not tested in tissue.

The present study builds on this history of ACh detection and electrode fabrication, with the introduction of an ACh-sensitive carbon-fiber electrode for use with FCV in brain tissue preparations. In contrast to the 30–200  $\mu\text{m}$  diameter carbon fibers used previously,<sup>35,36</sup> those used as the base for the present ACh sensors are only 7  $\mu\text{m}$ . Moreover, the use of FCV for  $\text{H}_2\text{O}_2$  detection provides better signal identification and quantitation than possible with amperometry. The optimized electrode reported here has the mechanical and enzyme stability required for *in situ* recording of endogenous ACh release in brain slices.

## Experimental

### Animal handling

Animal procedures were in accordance with NIH guidelines and approved by the NYU School of Medicine Animal Care and Use Committee. All mice were on a 12 h light:dark cycle, with lights on from 06:30 to 18:30 and were group-housed with *ad libitum* access to mouse chow and water. Young adult male (5–8 weeks) C57Bl6/J mice, and forebrain choline acetyltransferase knock-out (ChAT-KO mice), and littermate controls were

used (see below). All mice were deeply anesthetized with pentobarbital (typically  $120 \text{ mg kg}^{-1}$ , intraperitoneal) before preparation of *ex vivo* brain slices for studies of evoked ACh release; slices were prepared between 10:00 and 12:00.

### Forebrain ChAT knockout mice

Mice with a conditional floxed allele of the ChAT gene *Chat* (ChAT<sup>flox</sup> mice) were crossed with a *Nkx2.1*<sup>Cre</sup> transgenic line to produce mice in which ablation of ACh synthesis is restricted to the forebrain, here referred to as ChAT-KO mice.<sup>36</sup> Cre non-expressing transgenic littermates, *Chat*<sup>flox/flox</sup>, were used as controls. All mice were bred on a C57Bl/6 background and were genotyped within 7 days of birth and after each experiment to confirm genetic identity.

### Ex vivo slice preparation and physiological solutions

Coronal forebrain slices (300  $\mu\text{m}$  thick) were cut on a Leica VT1200S vibrating blade microtome (Leica Microsystems; Bannockburn, IL) in ice-cold HEPES-buffered artificial cerebrospinal fluid (aCSF) containing (in mM): NaCl (120);  $\text{NaHCO}_3$  (20); glucose (10); HEPES acid (6.7); KCl (5); HEPES sodium salt (3.3);  $\text{CaCl}_2$  (2);  $\text{MgSO}_4$  (2), equilibrated with 95%  $\text{O}_2$ /5%  $\text{CO}_2$ . Slices were then maintained in this solution at room temperature for at least 1 h before transfer to the recording chamber. All voltammetry experiments were conducted in a submersion recording chamber at 32 °C that was superfused at  $1.5 \text{ mL min}^{-1}$  with aCSF containing (in mM): NaCl (124); KCl (3.7);  $\text{NaHCO}_3$  (26);  $\text{CaCl}_2$  (2.4);  $\text{MgSO}_4$  (1.3);  $\text{KH}_2\text{PO}_4$  (1.3); and glucose (10), and equilibrated with 95%  $\text{O}_2$ /5%  $\text{CO}_2$ ; slices were allowed to equilibrate in this environment for 30 min before experimentation.

### ACh microelectrode preparation

For each electrode, an unsized 7  $\mu\text{m}$  diameter carbon fiber (Goodfellow Corporation, Berwyn, PA, USA) was inserted into a glass capillary tube, pulled on an electrode puller to create a seal around the carbon fiber, then cut to a length of 50 to 100  $\mu\text{m}$  beyond the end of the glass. To complete the circuit, the carbon fiber within the capillary was connected to copper wire using Wood's metal.<sup>20</sup> Before coating, electrodes were conditioned in aCSF by application of the typical FCV waveform ( $-0.7$  V to  $+1.3$  V vs. Ag/AgCl, scan rate of  $800 \text{ V s}^{-1}$ ) at 100 ms intervals for 20 s. After air drying, electrodes were coated, using methods adapted from previous studies, as noted in Results.<sup>29,32</sup> With the aid of a binocular microscope, a carbon-fiber microelectrode was dipped into a coating solution composed of 22.5 U of AChE and 7.5 U of ChOx, and 1 mg of BSA in 1 mL of a 13.3% (w/v) aqueous solution of poly(vinyl alcohol), *N*-methyl-4(4'-formylstyryl)pyridinium methosulfate acetal (PVA-SbQ). After seven 10 s dips, the electrode was exposed to blue (UV) frequency light (350–450 nm) for one minute, then washed in a 50% ethanol solution and dried for at least 24 h before testing.<sup>29,32</sup> Electrodes were stored dry at 4 °C and could be used within at least four weeks after preparation (longest time tested).

## Electrode calibration

Concentration-response characteristics of the electrodes to  $\text{H}_2\text{O}_2$  and to ACh on uncoated and coated electrodes were determined in aCSF at 32 °C. The relative sensitivity of uncoated and coated carbon-fiber microelectrodes was also assessed for several possible analytes and interferents, including choline, dopamine and adenosine at physiologically relevant concentrations.

## FCV

Evoked ACh release was examined using FCV with coated electrodes in striatal slices. Voltammetric measurements were made with a Millar Voltammeter (available by special request to Dr Julian Miller at St Bartholomew's and the Royal London School of Medicine and Dentistry, University of London). A conventional triangle waveform was used for FCV, with a scan range of  $-0.7$  V to  $+1.3$  V (*versus* Ag/AgCl), scan rate of 400 or 800  $\text{V s}^{-1}$ , and sampling interval of 100 ms. Data were acquired using a DigiData 1200B A/D board controlled by Clampex 7.0 software (Molecular Devices). ACh release was evoked in the caudate-putamen (CPU) of striatal slices using a concentric bipolar electrode for local single-pulse stimulation; stimulus pulse amplitude was 0.4 mA and duration was 100  $\mu\text{s}$ . To quantify evoked  $[\text{ACh}]_o$ , coated electrodes were calibrated with known concentrations of ACh at 32 °C after each experiment in aCSF. All voltammograms were background-subtracted using the voltammogram recorded immediately before a calibration solution reached the electrode, or immediately before single-pulse stimulation in brain slices.

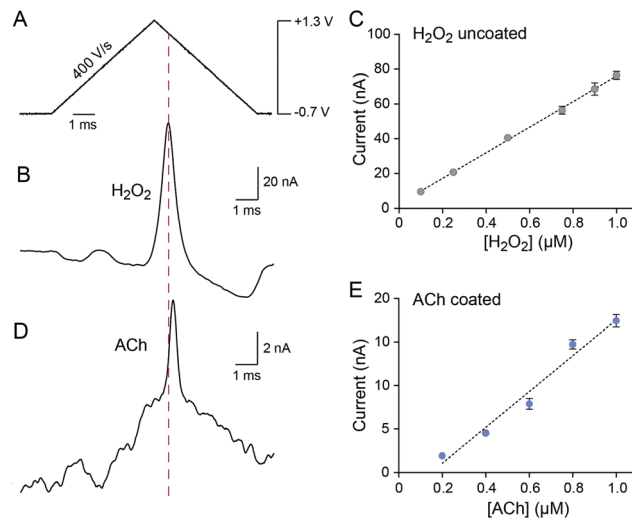
## Statistical analysis

Data are given as means  $\pm$  standard error; linearity of calibration curves was assessed using linear regression with a Runs test. Significance in selectivity of electrodes to ACh was assessed using one-way ANOVA with Dunnett's multiple comparisons test *versus* ACh as control. Comparison between control and ChAT-KO mice was by unpaired Student's *t*-tests. For electrode testing data, *n* is the number of electrodes used; for slice data, *n* is number of slices, with one slice per mouse.

## Results and discussion

### Detection of $\text{H}_2\text{O}_2$ and ACh

In initial FCV studies, we tested the efficacy of the scan rate (400  $\text{V s}^{-1}$ ) (see Fig. 1A) used previously for  $\text{H}_2\text{O}_2$  detection at carbon-fiber microelectrodes<sup>34</sup> in comparison with the faster scan rate we typically use for dopamine detection (800  $\text{V s}^{-1}$ ).<sup>20</sup> In these comparisons, 400  $\text{V s}^{-1}$  provided better resolution of the  $\text{H}_2\text{O}_2$  oxidation peak (data not shown), and was used for all further studies. Subsequently, we worked to optimize methods for electrode fabrication. Most importantly, this involved adjusting several variables in the coating process to improve the sensitivity, mechanical stability, and longevity of the electrodes. First, the amount of each enzyme was altered systematically; the optimal number of units was determined to



**Fig. 1** Electrode sensitivity for  $\text{H}_2\text{O}_2$  and ACh. (A) FCV waveform with scan rate of 400  $\text{V s}^{-1}$  used to detect  $\text{H}_2\text{O}_2$  and ACh; vs. Ag/AgCl. (B) Voltammogram for  $\text{H}_2\text{O}_2$  at an uncoated electrode showing a single oxidation peak at +1.1 V on the falling phase of the FCV waveform. (C) Uncoated electrode calibration for  $\text{H}_2\text{O}_2$  showed a linear response over the range tested ( $R^2 = 0.999$ ,  $n = 7$  electrodes). (D) Voltammogram for ACh at an enzyme-coated electrode showing a single oxidation peak at a similar potential to that of  $\text{H}_2\text{O}_2$ . (E) The response to ACh was linear over the physiologically relevant concentration range tested ( $R^2 = 0.972$ ,  $n = 5$  electrodes). Data points are means  $\pm$  SEM.

be 22.5 U for AChE and 7.5 U for ChOx. Although the ratio of AChE to ChOx was the same as that in previous studies, 15 U AChE to 5 U ChOx,<sup>29,32</sup> higher absolute units of both enzymes increased the sensitivity for ACh at these carbon-fiber based microelectrodes. The duration and frequency of dipping into the polymer solution was also adjusted until the technique used (see Methods) produced a sensitive electrode that maintained the ability to detect ACh when stored before use, and could be lowered into a brain slice without compromising the enzyme coating. Overall, these coated microelectrodes proved to be reliable detectors for ACh in solution and in brain slices.

Because  $\text{H}_2\text{O}_2$  is the product to be detected at the electrode surface, we next assessed the concentration-dependence of peak  $\text{H}_2\text{O}_2$  currents at uncoated carbon-fiber microelectrodes. As reported previously,<sup>35</sup> the oxidation peak for  $\text{H}_2\text{O}_2$  with FCV appeared on the falling phase of the triangle waveform, albeit with the potential still high,  $\sim +1.1$  V vs. Ag/AgCl (Fig. 1B). Peak oxidation currents (in nA) were measured for 0.1  $\mu\text{M}$  to 1  $\mu\text{M}$   $\text{H}_2\text{O}_2$ , and indicated a linear response over this range (Fig. 1C,  $R^2 = 0.999$ ,  $n = 7$  electrodes). Voltammograms recorded on coated microelectrodes in the presence of ACh had a similar oxidation peak potential to that of  $\text{H}_2\text{O}_2$  (Fig. 1D), confirming that the AChE/ChOx couple on the electrode surface generated  $\text{H}_2\text{O}_2$  when exposed to ACh. The concentration dependence of ACh at the coated microelectrode was also linear (Fig. 1E,  $R^2 = 0.972$ ,  $n = 5$  electrodes), although with lower sensitivity than for  $\text{H}_2\text{O}_2$  at an uncoated electrode (slope:  $73.7 \pm 1.1 \text{ nA } \mu\text{M}^{-1} \text{H}_2\text{O}_2$ ;  $20.6 \pm 2.0 \text{ nA } \mu\text{M}^{-1} \text{ACh}$ ).

### Enzyme-coated electrode selectivity for ACh

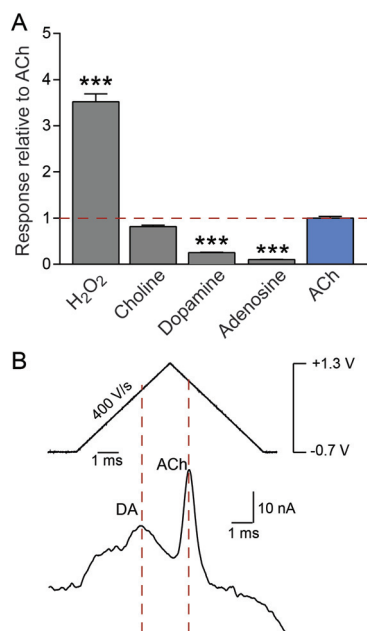
After demonstrating the sensitivity and linearity for ACh detection, the next step was to test selectivity for ACh against other substances – choline, dopamine, adenosine, and  $\text{H}_2\text{O}_2$  – each of which could contribute to Faradaic currents with local electrical stimulation in striatal slices<sup>19,37</sup> (Fig. 2). Notably, possible interferents in the striatum *in vivo*, particularly ascorbate and the dopamine metabolite DOPAC, do not contribute to detected currents with local electrical stimulation in brain slices.<sup>20</sup>

An ideal electrode would be specific for ACh; with the present design, however, sensitivity to choline and  $\text{H}_2\text{O}_2$  is also expected, given the enzyme-mediated reactions involved. Indeed, at coated electrodes, the current response to  $1.0\ \mu\text{M}$   $\text{H}_2\text{O}_2$  was significantly greater than that of  $1.0\ \mu\text{M}$  ACh ( $p < 0.001$ ;  $n = 3$  electrodes for  $\text{H}_2\text{O}_2$ ;  $n = 5$  electrodes for ACh) (Fig. 2A). Sensitivity to choline was similar to that of ACh ( $p > 0.05$ ,  $n = 5$  electrodes). The response to dopamine was significantly lower than to any of these other substances ( $p < 0.001$ ,  $n = 5$  electrodes) (Fig. 2A); moreover, the dopamine peak occurred at  $\sim +0.75\ \text{V}$  vs. Ag/AgCl on the rising phase of the triangle wave (Fig. 2B) in contrast to the  $\text{H}_2\text{O}_2$ /ACh peak on the falling phase (Fig. 1A and C), providing resolution of the two peaks (Fig. 2B). The peak current for adenosine occurred at the same potential as the  $\text{H}_2\text{O}_2$ /ACh peak, but produced negligible current compared to the same concentration of ACh ( $p < 0.001$ ,  $n = 5$  electrodes) (Fig. 2A). Overall, the two most

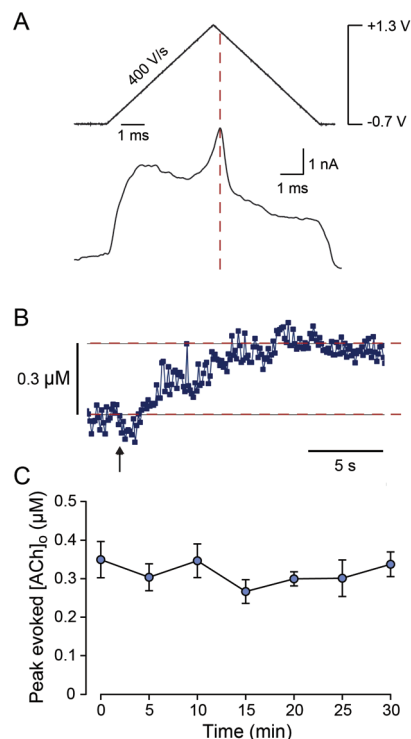
likely interferents, dopamine and adenosine, should not contribute to the ACh signal detected in brain tissue.

### Enzyme-coated electrodes reliably detect stimulated ACh release in brain tissue

The final test for coated, carbon-fiber microelectrodes was whether they could be used to detect ACh release in brain tissue. The electrodes were first tested in striatal slices from control mice, and showed reliable detection ability, with a voltammetric peak that matched that of ACh in calibration (Fig. 1D and 3A). Average  $[\text{ACh}]_0$  evoked by the first stimulus at a given recording site was  $0.41 \pm 0.05\ \mu\text{M}$  (mean  $\pm$  SEM;  $n = 7$  mice). The time course of ACh detection by these electrodes was slower than that for direct detection of changes in  $[\text{DA}]_0$  with FCV: the peak response occurred 8 to 10 s after the stimulus (Fig. 3B), which is comparable to the response seen with other designs (e.g., ref. 30). One caveat arising from this longer response time is that background-subtracted voltammograms for evoked  $[\text{ACh}]_0$  included background drift, in part from stimulus-induced ionic and pH shifts (Fig. 3B). However, this did not interfere with quantification of the ACh-dependent Faradaic current, which was measured from the mean of points immediately before and after the peak. The stability of the electrode in tissue was shown by the ability to detect con-

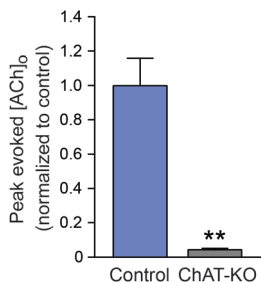


**Fig. 2** Electrode selectivity for ACh, choline, and  $\text{H}_2\text{O}_2$ . (A) Coated electrodes were  $\sim 3.5$ -fold more sensitive to  $\text{H}_2\text{O}_2$  than ACh, equally sensitive to ACh and choline, and less sensitive to dopamine and adenosine;  $1\ \mu\text{M}$  each ( $***p < 0.001$  vs. ACh;  $n = 3$ –5 electrodes); response (nA) for each normalized to that of ACh. (B) Calibration voltammogram showing oxidation peak separation for dopamine (DA) at  $+0.75\ \text{V}$  vs. Ag/AgCl on the rising phase of the FCV sweep and  $\text{H}_2\text{O}_2$  at  $+1.1\ \text{V}$  on the falling phase.



**Fig. 3** Electrodes reliably detect evoked ACh release in brain tissue. (A) Voltammogram of endogenously evoked ACh release in a mouse striatal slice; vs. Ag/AgCl. (B) Representative trace showing the timecourse of ACh release evoked by a single stimulus pulse (arrow) monitored at the peak oxidation potential with time. (C) ACh release could be reliably detected when evoked at five minute intervals over a period of 30 minutes ( $n = 4$ ).





**Fig. 4** ACh release is not detected in ChAT-KO mice. Single-pulse evoked ACh release was undetectable in striatal slices from mice lacking the ACh synthesizing enzyme ChAT in the forebrain (ChAT-KO). Responses are normalized to peak response in littermate control mice ( $n = 3$  mice per group).

sistent responses evoked by repetitive stimulation at 5 min intervals for 30 minutes ( $n = 4$ ) (Fig. 3C).

Coated microelectrodes were also tested in littermate transgenic mice in which ACh production was eliminated in the forebrain by genetic deletion of ChAT.<sup>36</sup> Selective detection of ACh (rather than choline or  $\text{H}_2\text{O}_2$ ) was indicated by the minimal peak current detected on the usual background shift in slices from ChAT-KO mice *versus* controls ( $p < 0.01$ ,  $n = 3$  mice per group) (Fig. 4). The lack of contribution from endogenously generated  $\text{H}_2\text{O}_2$  with local single-pulse stimulation is consistent with the absence of  $\text{H}_2\text{O}_2$ -dependent modulation of evoked  $[\text{DA}]_0$  seen with this stimulus in striatal slices reported previously.<sup>38</sup> Overall, these data support the viability of the designed coated electrodes in brain tissue, and validate their use in *ex vivo* slices for selective detection of ACh. It is possible that the prolonged response in control striatum might include some contribution from choline produced by ACh degradation *via* endogenous AChE. Even if this were the case, however, it would also reflect ACh release, and thus would actually improve our ability to assess factors that regulate ACh release in future work.

## Conclusions

Here we introduce an optimized enzyme-coated carbon-fiber microelectrode to monitor evoked ACh release in brain slices. The electrodes can be stored unused for several weeks after preparation, and are mechanically sturdy for use in tissue. They also show the sensitivity, selectivity, and stability necessary to record evoked ACh release in brain tissue. The introduction of FCV, as opposed to amperometry, gives this tool improved selectivity. The photo-cross-linkable coating methodology can be extended to other enzymes for detection of non-electroactive neurotransmitters.

Overall, this refined electrode allows for a better understanding of neuronal circuitry between several neurotransmitter systems. Future studies will capitalize on the possibility of simultaneous detection of dopamine and ACh in striatal slices. Of course, the value of these electrodes is not

limited to exploration of striatal neurotransmitter interactions, as they could be used to examine ACh release regulation in other brain regions with cholinergic input, like the cortex and hippocampus. Thus, the electrode design holds promise for a variety of neuroscience applications.

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