

# Rapid assessment of *in vivo* cholinergic transmission by amperometric detection of changes in extracellular choline levels

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## Abstract

Conventional microdialysis methods for measuring acetylcholine (ACh) efflux do not provide sufficient temporal resolution to relate cholinergic transmission to individual stimuli or behavioral responses, or sufficient spatial resolution to investigate heterogeneities in such regulation within a brain region. In an effort to overcome these constraints, we investigated a ceramic-based microelectrode array designed to measure amperometrically rapid changes in extracellular choline as a marker for cholinergic transmission in the frontoparietal cortex of anesthetized rats. These microelectrodes exhibited detection limits of 300 nM for choline and selectivity (> 100 : 1) of choline over interferents such as ascorbic acid. Intracortical pressure ejections of choline (20 mM, 66–400 nL) and ACh (10 and 100 mM, 200 nL) dose-dependently increased choline-related signals that were cleared to background levels within 10 s. ACh, but not choline-induced signals, were significantly attenuated by co-ejection of the acetylcholinesterase inhibitor neostigmine (Neo; 100 mM). Pressure ejections of drugs known to increase cortical ACh efflux, potassium (KCl; 70 mM, 66, 200 nL) and scopolamine (Scop; 10 mM, 200 nL), also markedly increased extracellular choline signals, which again were inhibited by Neo. Scop-induced choline signals were also found to be tetrodotoxin-sensitive. Collectively, these findings suggest that drug-induced increases in current measured with these microelectrode arrays reflect the oxidation of choline that is neuronally derived from the release and subsequent hydrolysis of ACh. Choline signals assessed using enzyme-selective microelectrode arrays may represent a rapid, sensitive and spatially discrete measure of cholinergic transmission.

## Introduction

There is considerable interest in the dynamics of cholinergic transmission given the role of forebrain acetylcholine (ACh) in mediating cognitive functions (Everitt & Robbins, 1997; Sarter & Bruno, 1997; Bruno *et al.*, 1999). Microdialysis methods reveal that performance in certain cognitive tasks is sufficient to stimulate cortical ACh release (Himmelheber *et al.*, 2000; Passetti *et al.*, 2000; Dalley *et al.*, 2001; Arnold *et al.*, 2002). However, future questions pursuing the relationship between cholinergic transmission and specific stimuli or behavioral responses (i.e. correct vs. incorrect responses, omissions, anticipatory responses), and regional variations for such cholinergic regulation require a method with higher temporal and spatial resolution than conventional microdialysis/high-performance liquid chromatography (HPLC).

Voltammetric methods have been used to study neurotransmitter release and reuptake (Capella *et al.*, 1993; Cass *et al.*, 1993; Gerhardt, 1995; Bunin & Wightman, 1998). Microelectrodes configured for enzyme-based measurements by converting non-electroactive molecules into electroactive substances, allow for detection of glucose, glutamate or choline (Hu *et al.*, 1994; Burmeister *et al.*, 2000, 2002; Burmeister & Gerhardt, 2001; Pomerleau *et al.*, 2003). These

microelectrodes involve the enzymatic conversion, via a surface-bound enzyme (oxidase), of the non-electroactive analyte to hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>), which is then oxidized and the resulting current is measured.

Several groups have developed choline-sensitive microelectrodes in order to estimate changes in ACh release by variations in extracellular choline concentrations. Michaels and co-workers developed a redox polymer layer with horseradish peroxidase and choline oxidase (CO) to measure H<sub>2</sub>O<sub>2</sub> using carbon fibers (Garguilo & Michael, 1996; Cui *et al.*, 2001). Although very selective, these microelectrodes are relatively slow, with response times of 10–30 s. A different approach was recently introduced by Gerhardt and co-workers, using a ceramic-based multisite microelectrode array for the detection of choline (Burmeister *et al.*, 2003). Recording sites on this platinum electrode are coated with a layer of Nafion<sup>TM</sup> and CO, yielding a linear response up to 200 μM choline, a sensitivity of < 0.5 μM and a response time of less than 1 s. An important advantage of these microelectrode arrays is that adjacent sites can be selectively coated and used for self-referencing against electroactive interferents, such as ascorbic acid (AA) or dopamine (DA), to improve signal-to-noise ratios and detection limits.

The present experiments tested the ability of the multisite microelectrodes to measure rapid changes in extracellular choline and the potential use of such changes to assess *in vivo* cholinergic transmission. First, the ability of the microelectrode to detect exogenously applied choline and ACh was determined in the frontoparietal cortex of anesthetized rats. Second, the effects of two compounds that

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stimulate cortical ACh release, potassium and scopolamine (Scop), on cortical choline signaling were determined. Third, the extent to which stimulated choline signals depend upon the hydrolysis of endogenous ACh was determined by co-administration of the acetylcholinesterase inhibitor neostigmine (Neo). In addition, the extent to which choline signals depend upon neuronal depolarization was determined by co-administration of tetrodotoxin (TTX). The results suggest that rapid changes in extracellular choline, as measured with microelectrode arrays, provide a valid method for the measurement of rapid changes in ACh release.

## Materials and methods

### Animals

Three- to 4-month-old male Fisher-344/Brown Norway F1 hybrid rats were obtained from Harlan Laboratories (Indianapolis, IN, USA). Animals were treated in accordance with the guidelines of the Institutional Laboratory Animal Care and Use Committee (ILACUC) at The Ohio State University, in facilities accredited by the American Association of Accreditation of Laboratory Animal Care (AAALAC).

### Drugs and chemicals

Nafion<sup>TM</sup> (5% in a mixture of aliphatic alcohols and water) was obtained from Aldrich (Aldrich Chemical, Milwaukee, WI, USA). CO (EC 1.1.3.17), bovine serum albumin (BSA), glutaraldehyde, AA, DA, choline, ACh, Neo and TTX were obtained from Sigma (Sigma Chemical, St. Louis, MO, USA). KCl was obtained from Fluka (Fluka Biochemica, Buchs, Switzerland). All other chemicals and reagents were from Fisher Scientific. Ultra pure water (RO-HPLC grade, Millipore, Billerica, MA, USA) was used to prepare all solutions. Solutions used for intracranial injections were prepared in 0.05 M phosphate-buffered saline (PBS), adjusted to pH 7.4 and filtered through 0.22  $\mu\text{m}$  sterile non-pyrogenic filters (Costar, Corning, NY, USA) prior to use.

### Preparation of choline-sensitive microelectrode

The ceramic-based microelectrode has four  $15 \times 333 \mu\text{m}$  recording sites that are arranged in side-by-side pairs beginning  $\sim 1000 \mu\text{m}$  from the electrode tip (Fig. 1). The microelectrodes were dipped in the negatively charged polymer Nafion<sup>TM</sup> and then dried at  $170^\circ\text{C}$  for 4–5 min in order to repel anions such as AA, increasing the selectivity for choline. This process was repeated four to five times. A mixture containing 1% CO, 1% BSA and 0.125% glutaraldehyde in water was then applied (100 nL) to the bottom pair of recording sites using a  $1.0\text{-}\mu\text{L}$  syringe (Hamilton, Reno, NV, USA). These recording sites were sensitive to choline (choline recording channels). The other two recording sites were coated only with BSA and glutaraldehyde solution, and served as background sentinel channels (see self-referencing procedure below). All coated microelectrodes were allowed to dry in air and stored at  $4^\circ\text{C}$  for at least 24 h prior to use. Figure 1 depicts a schematic illustration of the multisite microelectrode array, the coatings of the recording sites and the major steps involved in the conversion of choline to  $\text{H}_2\text{O}_2$ .

### In vitro calibration of microelectrode

The microelectrode was calibrated using a FAST-16 electrochemical recording system (Quanteon, LLC, Nicholesville, KY, USA) prior to

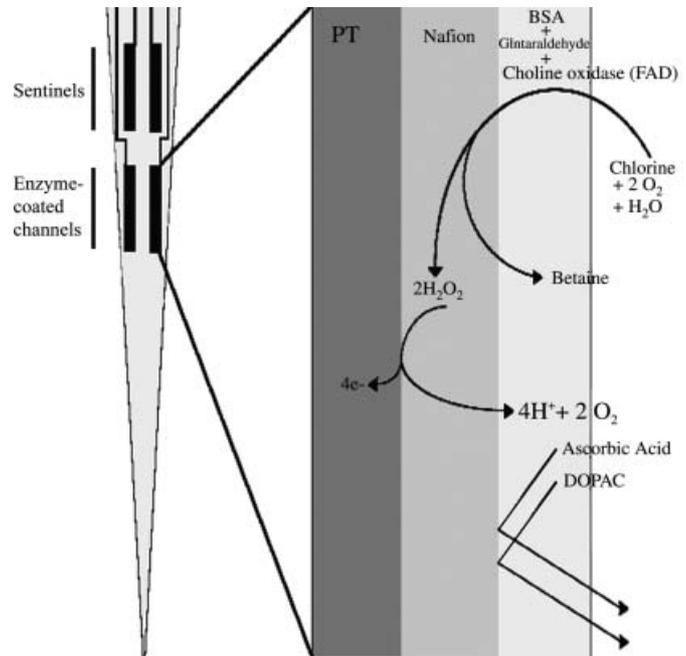


FIG. 1. *In vitro* calibration of the multisite microelectrode used to detect choline signals. A microelectrode, indicating the four platinum (PT) recording sites (or channels) is shown. Each recording site is  $15 \mu\text{m}$  wide and  $333 \mu\text{m}$  long, and the distance between the lower and upper pair of recording electrodes is about  $100 \mu\text{m}$ . As schematically illustrated, recording sites were coated with Nafion<sup>TM</sup>, which serves to reject electroactive species such as ascorbic acid and DOPAC (3,4-dihydroxy phenyl acetic acid) while still allowing passage of  $\text{H}_2\text{O}_2$ . CO was immobilized on the lower two recording sites; the upper control channels were coated with bovine serum albumin (BSA) only (called sentinels). Choline in the presence of oxygen and water is oxidized by CO and generates  $\text{H}_2\text{O}_2$ , which is oxidized at the electrode surface and thus generates current. The activity recorded at sentinels is subtracted from that recorded at CO-coated sites ('self-referencing').

implantation in animals. Constant voltage amperometry was used, with a potential of  $+0.7 \text{ V}$  applied vs. an Ag/AgCl reference electrode. Calibration was performed in a stirred solution of 0.05 M PBS (40 mL) at  $37^\circ\text{C}$ . After 15–20 min, to allow for a stable baseline, aliquots of stock solutions of AA (20 mM), choline (20 mM) and DA (2 mM) were added to the calibration beaker such that the final concentrations were  $250 \mu\text{M}$  AA, 10, 20, 30 and  $40 \mu\text{M}$  choline, and  $2 \mu\text{M}$  DA. The slope, limit of detection (LOD) and linearity ( $R^2$ ) for choline, as well as selectivity ratios for AA and DA, were calculated. Changes in current that reflect the oxidation of choline were assessed using a self-referencing procedure. In this procedure, responses from the individual channels coated with CO were normalized by dividing the current by the microelectrode response obtained following the addition of DA and then by subtracting the background current from sentinel channels. This procedure increases the signal-to-noise ratio and subsequent limit of detection for choline. Calibration data from both the choline and sentinel recording channels were transformed in this fashion before the selectivity, slope, LOD and  $R^2$  were recalculated.

### In vivo amperometric recordings in anesthetized rat

Animals were anesthetized with urethane (1.25–1.5 g/kg, i.p.) and placed in a stereotaxic frame (David Kopf Instruments, Model # 962, Tujunga, CA, USA). Compared with the effects of barbiturates,

urethane-induced anesthesia in general is associated with less pronounced effects on the activity of various neurotransmitter systems, including the cholinergic system (Keita *et al.*, 2000). A heating pad was used throughout the experimental session to maintain rat body temperature at 37 °C. A miniature (200 µm diameter) Ag/AgCl reference electrode was implanted at a site remote to the recording area. Single-barrel glass capillaries (1.0 mm × 0.58 mm, 6 in., A-M systems, Everett, WA, USA) were pulled using a micropipette puller (Model # 720, David Kopf Instruments) and then bumped until the inner tip diameter was ~20 µm. The micropipette was loaded with one of the test solutions (see below) and then attached to the ceramic platform of the microelectrode using sticky wax (Kerr, Romulus, MI, USA). The spacing between the tip of the micropipette and the choline microelectrode was maintained at 50–100 µm. The microelectrode/micropipette arrays were inserted into frontoparietal cortex (AP, +1.0 mm; ML, -2.7 mm; DV, -1.0 mm, measured from bregma). Drug solutions were ejected from the micropipettes using a pressure ejection system (Picospritzer II, Parker Hannifin, Fairfield, NJ, USA), and the ejected volumes were monitored using a stereomicroscope fitted with a reticule (Friedemann & Gerhardt, 1992). The resulting choline or sentinel microelectrode signals were recorded from the four microelectrode recording sites using a FAST-16 recording system. All experiments were initiated after 30–45 min of microelectrode implantation, the time required to achieve stable baseline current.

Several experiments were conducted in order to test the hypothesis that this technique can monitor ACh release by measuring the oxidation of choline generated from the hydrolysis of ACh by extracellular esterases. First, we determined the ability of exogenously applied choline and ACh to generate a choline-induced current when the microelectrode was placed into the frontoparietal cortex. One group of animals received control applications of PBS to determine the effect of pressure ejections *per se* on the background currents measured by the microelectrode channels. A second group of animals received a series of ejections (66, 200 and 400 nL) of choline (20 mM) in order to determine whether there was a concentration-dependent change in the amplitude and/or clearance of the choline-induced signal. In these initial parametric studies (as well as those with KCl), graded changes in choline-induced signals were achieved by varying volumes of a single concentration rather than by ejecting a single volume of different concentrations (as was done in subsequent experiments). This procedure was guided by two considerations: (i) our intent to determine graded changes within the same animal and recording session; and (ii) the fact that we utilized single-barrel ejection cannulae and did not want to potentially disrupt the pipette/microelectrode assembly by continuing to change lines from the picospritzer to the pipette. A third group of animals received several choline ejections (20 mM, 200 nL) followed by ejections containing a combination of choline (20 mM) and Neo (100 mM) in order to determine whether acetylcholinesterase activity was necessary for any measured current change. A fourth and fifth group of animals received ejections (200 nL) of either 10 mM or 100 mM ACh, respectively. These ejections were followed by ejections containing both ACh and Neo (100 mM). Next, we determined the ability of pharmacological challenges, known to increase cortical ACh efflux, to increase choline-related signals, presumably via the release of endogenous ACh. Two groups of animals received ejections (66 or 200 nL) of potassium (KCl 70 mM; also containing 2.5 mM CaCl<sub>2</sub> and 75 mM NaCl to ensure adequate release of ACh, see Moore *et al.*, 1996). These ejections were then followed by a series of ejections of the KCl solution + Neo (100 mM). An additional group of animals received ejections

(200 nL) of Scop (10 mM) followed by similar ejections containing Scop + Neo (100 mM). A final group of animals was used to determine the necessity of voltage-dependent Na<sup>+</sup> channels on Scop-induced increases in choline current by studying the ability of TTX (100 µM) to attenuate Scop-induced signals. In all cases, self-referencing was utilized to remove interferent signals including background noise.

### Histology

At the end of the experiment, the animals were perfused with ice-cold heparinized saline and 10% formalin. Brains were removed and stored in 10% formalin at 4 °C for 24 h and then cryoprotected in 30% sucrose phosphate buffer for at least 72 h. Coronal sections (50 µm) from frontal cortex were stained with Cresyl violet solution to verify microelectrode placement.

### Data analysis and statistics

Five parameters derived from the choline signal were subjected to statistical analysis, including: current (pA), amplitude (µM of equivalent choline), time (s) required for the signal to decline by 80% of the maximal amplitude ( $T_{80}$ ), uptake rate constant ( $s^{-1}$ ) and uptake rate of choline (µM/s). The uptake of choline, which follows first order rate kinetics, is described by the following equation:

$$-\ln[\text{Choline}]_t = Kt - \ln[\text{Choline}]_0$$

The uptake rate constant  $K^{-1}$  depicts the slope of  $\ln[\text{Choline}]$  vs. time ( $t$ ) plot. The uptake rate was calculated by multiplying  $K^{-1}$  by the amplitude (in µM) of the peak. The average of three responses was used for the statistical analysis of clearance data. One-tailed Student's  $t$ -test was used for unpaired and paired data because of unidirectional predictions of responses. Repeated-measures ANOVAs were used to analyse the effects of multiple concentrations or volumes. Significant differences were followed up by a limited number of *post-hoc* comparisons using the least significance difference test ( $\alpha = 0.05$ ). Exact  $P$ -values were given in accordance with the recommendation by Greenwald *et al.* (1996).

## Results

### In vitro calibration

Successive additions of choline yielded linear increases in current recorded from CO-coated sites (Fig. 2A and B). Addition of AA produced negligible currents measured via either CO-coated sites or sentinels. In contrast, the addition of DA resulted in similar increases in current recorded from CO-coated recording sites and sentinels (Fig. 2A). Therefore, self-referencing completely removed currents produced by DA (Fig. 2C).

Based on calibrations of 40 microelectrodes, their average slope, indicating the sensitivity for detecting choline, was  $18.7 \pm 1.7$  pA/µM, with a background current of  $130.3 \pm 13.1$  pA. The LOD was  $333 \pm 30$  nM choline,  $R^2$  was  $0.998 \pm 0.001$  and selectivity for choline : AA was  $>100 : 1$ . These values were identical irrespective of whether data were analysed in the single microelectrode or self-referencing mode. Because of the high linearity of the calibration curve, the description of the results from the *in vivo* experiments, with the exception of Fig. 4D, will be expressed as equivalents of choline concentration (µM).

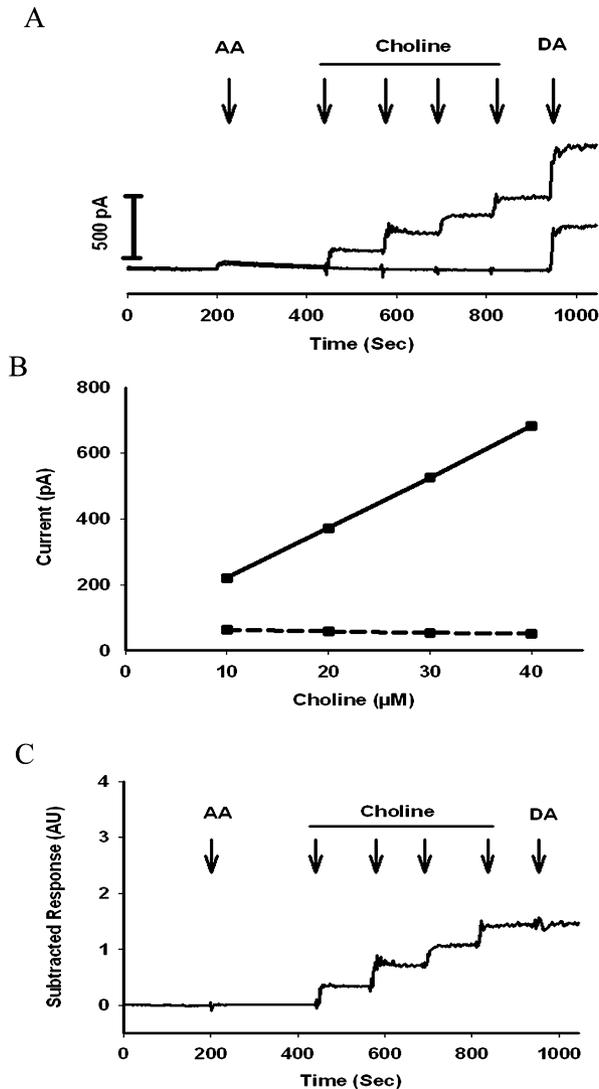


FIG. 2. (A) Recordings of raw current resulting from addition of 250 μM ascorbic acid (AA), 10 μM choline (four cumulative additions) and 2 μM dopamine (DA) via a CO-coated site (upper trace) and a sentinel (lower trace). (B) The linear response of a single recording site to increasing concentrations of choline (solid line, CO-coated channel; dashed line, sentinel). (C) The usefulness of self-referencing by depicting the attenuation of DA-induced currents by self-referencing (see, in comparison, the raw currents in A).

### Intracortical placements

Because of the small size of the microelectrodes, insertion of the microelectrode/micropipette assembly into the cortex produced minimal damage. As shown in Fig. 3A–D, recording sites targeted the middle layers of the frontoparietal cortex.

### Choline signals resulting from pressure ejection of choline and PBS

Basal choline level in frontoparietal cortex was determined by subtracting background current measured via sentinels from the current recorded from CO-coated sites and was  $6.1 \pm 0.6 \mu\text{M}$  ( $n = 5$ ). This value corresponds with the low micromolar range of brain choline concentrations reported in the literature (e.g. Klein *et al.*, 2002; Lockman & Allen, 2002).

Pressure ejections of 66, 200 and 400 nL of choline (20 mM) produced volume-dependent increases in current ( $F_{2,15} = 15.76$ ;  $P = 0.013$ ; Fig. 4D) and signal amplitude ( $F_{2,15} = 19.57$ ;  $P = 0.009$ ; Fig. 4A and E). The measurement of the rise time of signals was limited by the data sampling rate (1/s) and thus was 1 s or less. At sentinels, choline pressure ejections did not produce any appreciable effects (Fig. 4B). Self-referenced signals are shown in Fig. 4C.

Choline pressure ejections into the cortex also resulted in volume-dependent increases in  $T_{80}$  ( $F_{2,15} = 7.05$ ;  $P = 0.049$ ; from  $5.3 \pm 0.3$  s after 66 nL to  $7.1 \pm 0.5$  s after 400 nL) and increases in uptake rate ( $F_{2,15} = 9.27$ ;  $P = 0.031$ ; from  $1.93 \pm 0.20 \mu\text{M/s}$  to  $3.17 \pm 0.29 \mu\text{M/s}$ ). In contrast, the calculation of  $K^{-1}$ , that reflects the clearance rate for the first five data points (or 5 s) after the peak amplitude, did not indicate an effect of increasing choline volumes ( $F_{2,15} = 0.812$ ;  $P = 0.51$ ;  $0.143 \pm 0.004 \text{ s}^{-1}$ ).

To control for potential non-specific effects of pressure ejections, the effects of ejections of PBS (200 nL) were recorded. Relative to background current measurements and compared with the effects of choline pressure ejections (which produced a 145.8% increase in signal amplitude following ejections of 200 nL), PBS ejections produced minor effects (an 8.8% increase in amplitude) which, however, reached significance ( $t_4 = 3.71$ ;  $P = 0.02$ ) when compared with baseline (non-evoked) current changes. Thus, pressure ejections of PBS produced a small signal, possibly reflecting non-specific effects of pressure changes and pipette movement.

### Effects of Neo on ACh or choline-induced signals

Pressure ejections of ACh (10 and 100 mM; 200 nL) generated concentration-dependent increases in choline amplitudes (Fig. 5A and C and Table 1). ACh-induced signals were attenuated by co-ejection of the cholinesterase inhibitor Neo (100 mM; Fig. 5B and D). In contrast, choline-induced signals (Fig. 5E) were not affected by co-ejection of Neo (Fig. 5F; for statistical results see Table 1). Pressure ejections of Neo (100 mM; 200 nL) alone did not produce any signals or changes in baseline current (data not shown).

Compared with the effects of 10 mM ACh, pressure ejections of 100 mM ACh resulted in a significantly increased signal amplitude ( $t_8 = 2.72$ ;  $P = 0.013$ ).  $T_{80}$  remained unchanged ( $t_8 = 1.23$ ;  $P = 0.128$ ), while  $K^{-1}$  ( $t_8 = 2.49$ ;  $P = 0.019$ ) and uptake rate increased ( $t_8 = 2.44$ ;  $P = 0.020$ ; Table 1). Co-ejections of Neo significantly attenuated the effects of 10 mM or 100 mM ACh on all measures (except  $K^{-1}$  at 100 mM ACh; Table 1). The amplitude of the choline signal produced by 10 mM ACh was reduced by over 80% ( $t_4 = 3.57$ ;  $P = 0.011$ ), and the 100 mM ACh-induced amplitude decreased by over 60% ( $t_4 = 3.55$ ;  $P = 0.012$ ) following co-ejection of Neo.

In contrast to the ability of Neo to attenuate choline signals produced by ACh, the effects of choline pressure ejections were not altered by Neo co-ejections (Fig. 5E and F; Table 1; all  $P > 0.05$ ). This finding indicates that choline signals measured in response to ACh pressure ejections reflect choline hydrolysed from ACh, and that the signal-attenuating effects of Neo were not confounded by potential artefacts resulting from ejections of this cholinesterase inhibitor *per se*.

### Effects of Neo on choline signals reflecting endogenous ACh release

Pressure ejections of potassium (KCl 70 mM; 66, 200 nL) or Scop (10 mM; 200 nL) produced choline signals (Fig. 6A and E; Table 2), reflecting the effects of cholinergic terminal depolarization and

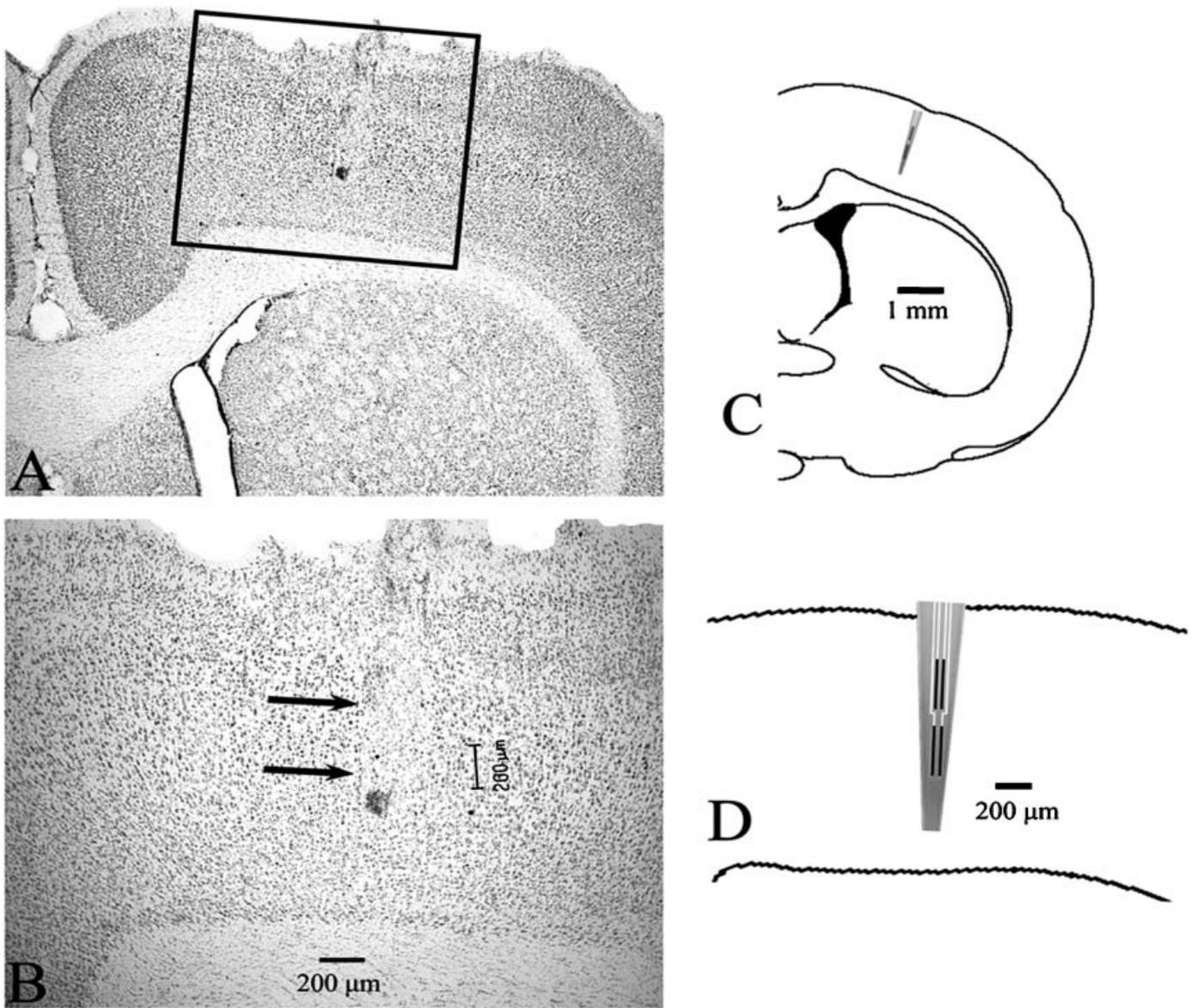


FIG. 3. (A–D) The placement of the microelectrode in the frontoparietal cortex. The insert in A is magnified in B and depicts the minimal damage produced by insertion of the electrode/micropipette array. The arrows in B mark the approximate location of the CO-coated recording sites in layers III and IV (200 μm bar is inserted in B). (C and D) Schematic representations of the location of the microelectrode. The dimensions of the electrode and the recording areas in C and D are drawn approximately to the scales (1 mm in C and 200 μm in D).

presynaptic muscarinic receptor blockade. Co-ejection of Neo potently attenuated these signals (Fig. 6C and F), decreasing amplitudes by 55–71% (see Table 2 for additional data).

Specifically, Neo attenuated the increases in choline amplitude that were produced by 66 nL ( $t_5 = 5.68$ ;  $P = 0.001$ ) or 200 nL ( $t_5 = 4.57$ ;  $P = 0.003$ ) of potassium; see Table 2 for a summary of the effects on  $T_{80}$ ,  $K^{-1}$  and uptake rate). Likewise, choline signals resulting from Scop ejections were attenuated by Neo (amplitude,  $t_4 = 3.85$ ,  $P = 0.009$ ; see Table 2 for additional data). These results indicate again that the recorded signals reflect hydrolysed choline, in this case from endogenously released ACh.

#### Attenuation of Scop-induced choline signals by TTX

The amplitude of choline signals produced by pressure ejections of Scop (10 mM; 200 nL) was attenuated by co-ejection of TTX

(100 μM; see Table 2). TTX did not affect  $T_{80}$  ( $t_5 = 1.30$ ;  $P = 0.125$ ), but reduced the uptake rate constant ( $t_5 = 3.59$ ;  $P = 0.007$ ) and uptake rate ( $t_5 = 3.35$ ;  $P = 0.010$ ). These data indicate that the generation of Scop-induced choline signals depended to a significant degree on the  $Na^+$ -dependent depolarization of terminals.

#### Discussion

The present experiments demonstrated that choline signals, recorded using an amperometric method and ceramic-based microelectrodes equipped with four recording sites, exhibit rapid rise times (1 s) and are rapidly cleared (< 10 s). Furthermore, local inhibition of acetylcholinesterase or interruption of impulse flow by TTX attenuated signals elicited by pressure ejections of ACh, Scop or potassium, but not, as would be expected, following choline ejections. Collectively, these data

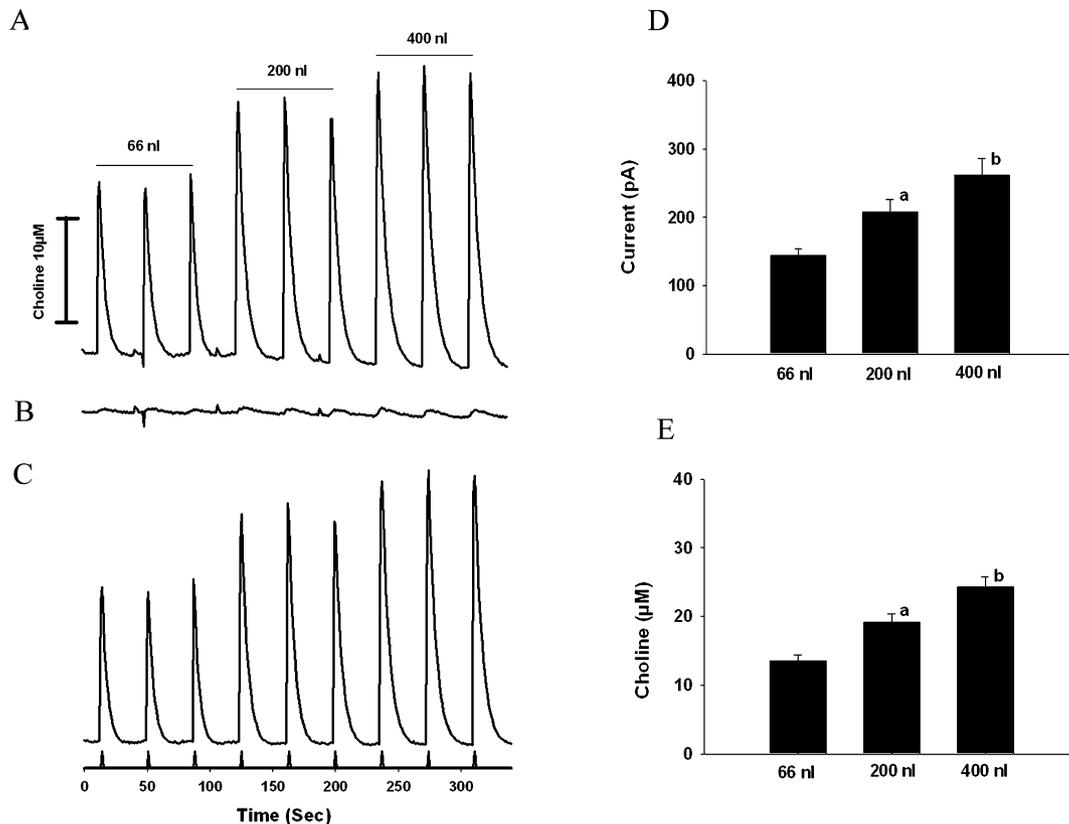


FIG. 4. Choline signals recorded *in vivo* in the frontoparietal cortex in response to choline pressure ejections. Ejection of 66, 200 and 400 nL of choline (20 mM) produced volume-dependent increases in amplitudes (A), while no effects were seen at sentinels (see trace in B). (C) The results of self-referencing, based on the traces shown in A and B. Pressure ejections are marked on the abscissa by tick marks. The effects of choline on current and equivalent choline amplitude are summarized in D and E, respectively. Statistical comparisons were performed using repeated-measure ANOVAs followed by least significant difference test. a,  $P < 0.05$ , 200 nL vs. 66 nL; b,  $P < 0.05$ , 400 nL vs. 66 and 200 nL.

support the usefulness of this method for the sensitive and rapid detection of increases in extracellular choline concentrations. Furthermore, these results support the hypothesis that the increases in choline concentrations measured by this method reflect choline derived from hydrolysis of ACh. Analyses of choline signals potentially provide detailed information concerning the mechanisms mediating the clearance of choline, and thus this method appears suitable for research on the regulation of mechanisms involved in choline metabolism, including the high-affinity choline transporter (Okuda & Haga, 2000, 2003; Okuda *et al.*, 2000, 2002; Lockman & Allen, 2002; Ferguson & Blakely, 2004). Below, major issues concerning the validity and usefulness of this method will be evaluated.

The selectivity of the recorded signals in terms of reflecting choline and, specifically, choline derived from hydrolysed ACh, results from several methodological steps. First, CO-coated recording sites exhibit a highly linear response to increasing choline concentrations and, largely due to Nafion<sup>TM</sup> coating, the selectivity of these responses over interferents (such as AA) is adequate under present testing conditions (see also Burmeister *et al.*, 2003). Second, Burmeister *et al.* (2003) demonstrated that signals produced by various choline concentrations did not differentially depend on oxygen concentrations, thus minimizing and even removing the potential concern that the signals recorded with an oxidase enzyme-based microelectrode are confounded by variations in brain oxygen concentrations (see also Mitchell, 2004). Third, signals generated by interferents (such as DA) were detected on both CO-coated and non-coated (sentinel) recording sites, and thus can be removed using the self-referencing method. This

method in essence subtracts such 'noise' from the signals reflecting choline concentrations and thus enhances the sensitivity and accuracy of choline signal measurements. With respect to the potential interfering signals produced by DA, it should be noted that, *in vivo*, DA-induced responses were not observed in the present study, most likely because the frontoparietal cortex does not receive dopaminergic inputs (Descarries *et al.*, 1987; Berger *et al.*, 1991; but see Valentini *et al.*, 2004). Fourth, the collective data, particularly the minimal effects of PBS pressure ejections, indicate that signals did not result from unspecific consequences of pressure ejections, such as tissue compression or other ejection-induced artefacts.

The available data support the hypothesis that microelectrode-recorded choline signals reflect choline generated by enzymatic degradation of ACh. Pressure ejections of the acetylcholinesterase inhibitor Neo completely attenuated choline signals produced by exogenous (pressure-ejected) ACh, or by potassium- or Scop-induced release of endogenous ACh. Importantly, the effects of Neo were unlikely due to potential confounds associated with pressure ejections of this compound *per se*, because Neo ejections did not affect signals produced by pressure ejections of choline. Moreover, the present data show that the choline signals produced by Scop, which presumably reflect presynaptic muscarinic receptor blockade-induced ACh efflux (e.g. Vannucchi & Pepeu, 1995), are attenuated by TTX and thus are impulse-dependent. Although Neo and TTX significantly attenuated potassium and Scop-induced choline signals, residual choline amplitudes following Neo or TTX co-ejections were observed. Residual signals may have been the result of numerous variables,

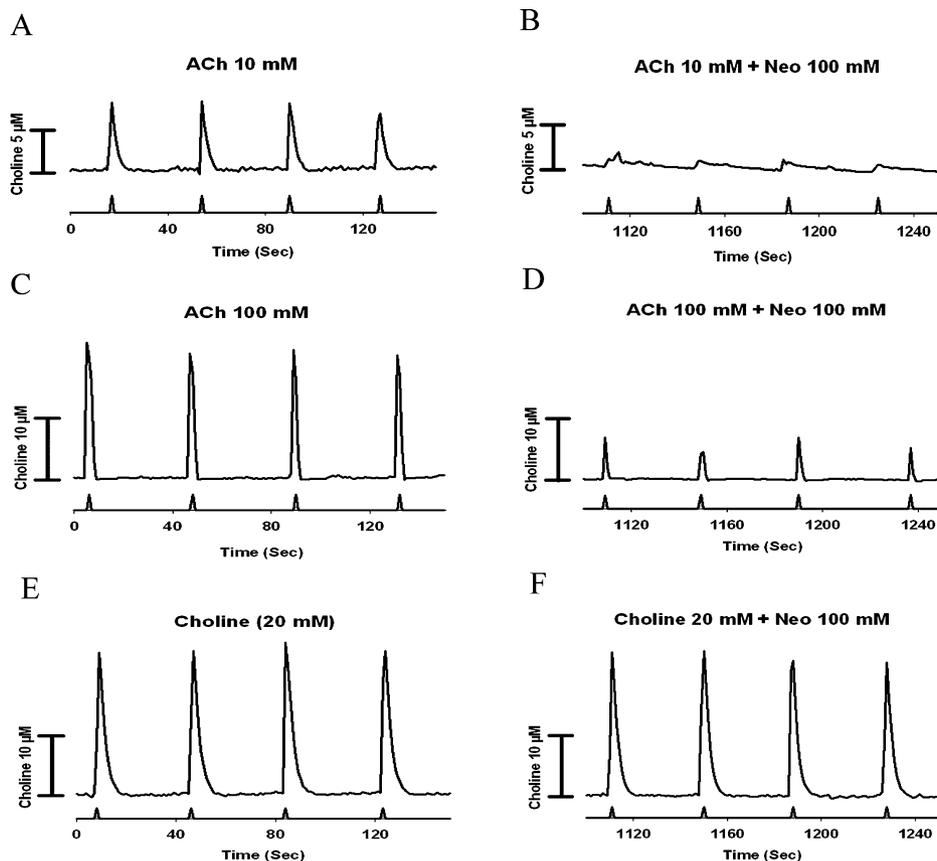


FIG. 5. Attenuation of acetylcholine (ACh)-induced, but not choline-induced, signals by co-ejection of the acetylcholinesterase inhibitor neostigmine (Neo) (self-referenced). Ejections (200 nL) of ACh (A, 10 μM; C, 100 μM) produced concentration-dependent increases in choline amplitude. Co-ejection of Neo (100 mM) markedly attenuated these signals (B and D). In contrast, signals recorded following ejections (200 nL) of choline (20 mM; F) were not affected by Neo (E; see also Table 2).

TABLE 1. Effect of neostigmine (Neo) on choline signals generated by acetylcholine (ACh) or choline ejections

Drugs	Ejection volume (nL)	Signal amplitude (μM)	$T_{80}$ (s)	$K^{-1}$ (s <sup>-1</sup> )	Uptake rate (μM/s)
ACh 10 mM	200	9.5 ± 0.9	4.0 ± 0.2	0.147 ± 0.013	1.56 ± 0.25
ACh 10 mM + Neo 100 mM	200	1.5 ± 0.3 <sup>†</sup>	2.9 ± 0.2 <sup>†</sup>	0.039 ± 0.007 <sup>†</sup>	0.06 ± 0.01 <sup>†</sup>
ACh 100 mM	200	22.7 ± 1.9*	5.4 ± 0.4	0.331 ± 0.031*	7.75 ± 1.11*
ACh 100 mM + Neo 100 mM	200	9.2 ± 0.7 <sup>‡</sup>	4.1 ± 0.3 <sup>‡</sup>	0.263 ± 0.032	2.68 ± 0.49 <sup>‡</sup>
Choline 20 mM	200	25.5 ± 1.7	4.9 ± 0.3	0.178 ± 0.014	4.31 ± 0.46
Choline 20 mM + Neo 100 mM	200	23.1 ± 1.9	4.9 ± 0.3	0.159 ± 0.017	3.34 ± 0.44

Data are expressed as mean ± SEM,  $n = 5-6$  per group. \* $P < 0.05$ , ACh 100 mM vs ACh 10 mM (unpaired  $t$ -test); <sup>†</sup> $P < 0.05$ , ACh 10 mM + Neo 100 mM vs ACh 10 mM (paired  $t$ -test); <sup>‡</sup> $P < 0.05$ , ACh 100 mM + Neo 100 mM vs ACh 100 (paired  $t$ -test).

including the test of suboptimal concentrations of Neo and TTX, unsettled complexities associated with the pressure ejections of multiple compounds such as unequal diffusion of potassium and Scop, and Neo and TTX across the cholinergic terminal space sampled by this method, and the possibility that choline is co-released with ACh (Klein *et al.*, 2002).

Although the present evidence suggests that the choline signals recorded by this method reflect choline derived from ACh hydrolysis and that therefore this method may be employed to monitor, at a relatively high temporal resolution, ACh release, potential complexities need to be examined by future experiments. For example, as already mentioned, the possibility that choline is co-released from

cholinergic terminals during depolarization has been raised (Klein *et al.*, 2002); such a co-release would limit the validity of the present hypothesis that choline signals indicate ACh release. However, the finding that Neo attenuates, to a significant degree, choline signals elicited by ACh, potassium and Scop suggests that choline signals reflect primarily choline hydrolysed from ACh. Furthermore, potential complex interactions between increases in extracellular choline concentrations, even if brief, and the regulation of ACh release via direct or even *trans*-synaptic mechanisms (e.g. Ulus *et al.*, 1989; Alkondon *et al.*, 1997) may constrain the validity of this method. Ongoing efforts aimed at the direct measurement of ACh along with choline will assist in resolving some of these questions.

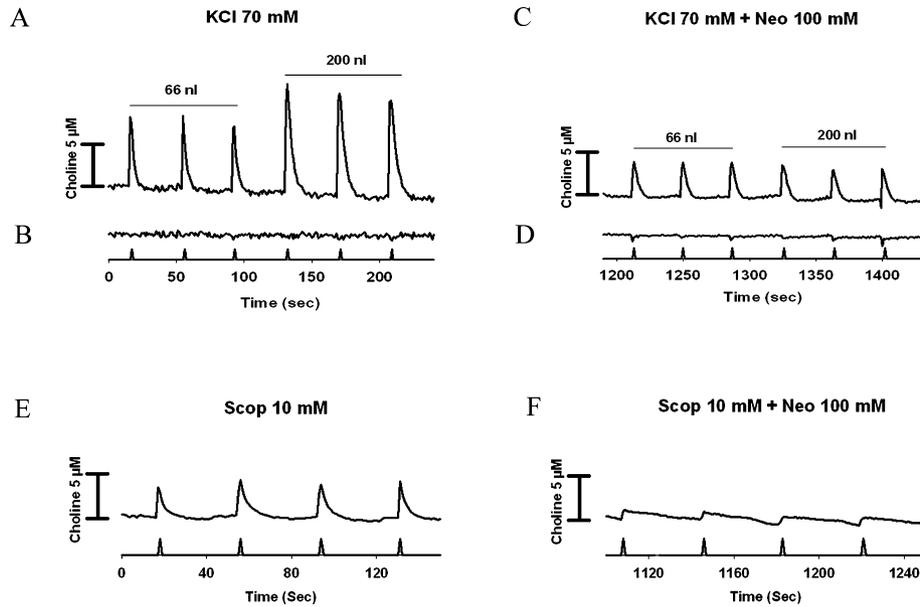


FIG. 6. Attenuation of potassium (KCl)- and scopolamine (Scop)-evoked choline signals by co-ejection of neostigmine (Neo). Raw tracings from pressure ejection of KCl (70 mM; 66, 200 nL) in the absence (A, enzyme-coated site; B, sentinel site) and presence (C, enzyme-coated site; D, sentinel site) of Neo (100 mM) are shown. KCl produced volume-dependent increases of choline amplitudes and Neo markedly reduced KCl-evoked choline signals (A and C). Currents recorded via sentinel sites did not change as a result of pressure ejections of KCl or co-ejections of KCl and Neo (B and D). (E) The effects of Scop (10 mM; 200 nL) ejections (self-referenced tracing). The signals produced by Scop were attenuated by co-ejection of Neo (F, self-referenced). These data indicate that potassium- and Scop-induced signals reflect choline hydrolysed from endogenously released Ach.

TABLE 2. Attenuation of potassium (KCl) and scopolamine (Scop) -induced choline signals by neostigmine (Neo) or tetrodotoxin (TTX)

Drugs	Ejection volume (nL)	Signal amplitude ( $\mu\text{M}$ )	$T_{80}$ (s)	$K^{-1}$ ( $\text{s}^{-1}$ )	Uptake rate ( $\mu\text{M}/\text{s}$ )
KCl 70 mM	66	$5.2 \pm 0.4$	$4.6 \pm 0.2$	$0.143 \pm 0.007$	$0.77 \pm 0.08$
KCl 70 mM + Neo 100 mM	66	$1.9 \pm 0.3^\dagger$	$3.8 \pm 0.2^\dagger$	$0.101 \pm 0.008$	$0.24 \pm 0.05^\dagger$
KCl 70 mM	200	$8.5 \pm 0.7^*$	$6.2 \pm 0.2^*$	$0.151 \pm 0.010$	$1.41 \pm 0.15^*$
KCl 70 mM + Neo 100 mM	200	$2.5 \pm 0.4^\ddagger$	$3.6 \pm 0.2^\ddagger$	$0.077 \pm 0.011^\ddagger$	$0.31 \pm 0.07^\ddagger$
Scop 10 mM	200	$3.5 \pm 0.2$	$4.7 \pm 0.3$	$0.097 \pm 0.012$	$0.36 \pm 0.06$
Scop 10 mM + Neo 100 mM	200	$1.7 \pm 0.2^\S$	$3.3 \pm 0.2^\S$	$0.062 \pm 0.011$	$0.15 \pm 0.02$
Scop 10 mM	200	$3.4 \pm 0.2$	$4.2 \pm 0.2$	$0.116 \pm 0.01$	$0.43 \pm 0.05$
Scop 10 mM + TTX 100 $\mu\text{M}$	200	$1.1 \pm 0.1^\P$	$3.6 \pm 0.2$	$0.061 \pm 0.01^\P$	$0.08 \pm 0.01^\P$

Data are expressed as mean  $\pm$  SEM,  $n = 5-6$  per group. \* $P < 0.05$ , KCl 70 mM (200 nL) vs KCl 70 mM (66 nL) (unpaired  $t$ -test);  $^\dagger P < 0.05$ , KCl 70 mM + Neo 100 mM (66 nL) vs KCl 70 mM (66 nL) (paired  $t$ -test);  $^\ddagger P < <0.05$ , KCl 70 mM + Neo 100 mM (200 nL) vs KCl 70 mM (200 nL) (paired  $t$ -test);  $^\S P < 0.05$ , Scop 10 mM + Neo 100 mM vs Scop 10 mM (paired  $t$ -test);  $^\P P < <0.05$ , Scop 10 mM + TTX 100  $\mu\text{M}$  vs Scop 10 mM (paired  $t$ -test).

The rapid clearance of choline signals represents a major factor contributing to the usefulness of this method as a measure of evoked ACh release. Given conventional assumptions about relatively stable and, as indicated by micromolar plasma choline concentrations required to produce net flux into the brain (Klein *et al.*, 1990), relatively high extracellular choline concentrations in the brain (Klein *et al.*, 1991; Lockman & Allen, 2002), the presence of highly effective clearance mechanisms for transmitter-derived choline may have been unexpected. The present results agree with hypotheses indicating that the activity and capacity of (high-affinity) choline transporters are tightly coupled to ACh release and cholinergic terminal depolarization, and are not in equilibrium with choline pools measured by more conventional methods (Ferguson & Blakely, 2004). The existence of a choline pool that is accessible by the microelectrode and that is more dynamically regulated by cholinergic transmission has also been indicated by the finding that delivery of Neo, via a microdialysis probe located 1 mm away from a microelectrode, resulted in a profound

decrease in the basal choline signal (Mitchell, 2004). These data imply that a significant amount of the basal signal detected by the microelectrode may be linked to cholinergic transmission and does not just indicate a stable extracellular pool in equilibrium with plasma. Furthermore, recent evidence suggests that the majority of hemicholinium-sensitive choline transporters reside in the vesicular membrane, and that they can be delivered to the terminal membrane during ACh release (Ferguson *et al.*, 2003). These observations begin to reveal the complex mechanisms mediating the rapid and effective clearance of choline derived from released ACh and, perhaps, the removal of a proportional concentration of choline from the extracellular pool (see also Ribeiro *et al.*, 2003; Ferguson & Blakely, 2004).

Further studies on the regulation of choline transporters will benefit from the richness of information about clearance that can be extracted from amperometrically recorded choline signals. Clearly, the description of more complex clearance characteristics, particularly combined effects on choline signal amplitudes and transporter

mechanisms, or polynomial clearance functions, require analyses in addition to the present measures of  $T_{80}$ , uptake rate constant and uptake rate. Furthermore, and although blockade of the high-affinity choline transporter by hemicholinium predictably decreased the clearance of evoked signals from the extracellular space (Burmeister *et al.*, 2003), the specific neurobiological significance of the various measures of clearance remains poorly understood. However, the present clearance data already indicate that uptake rate is not saturated at baseline as, for example, increasing ACh concentrations resulted in substantial increases in uptake rates while  $T_{80}$  remained relatively stable (see Table 1). Thus, microelectrode-recorded choline signals appear to represent an informative approach toward the determination of mechanisms regulating the metabolism of choline derived from ACh, including by choline transporters. Similar to the more advanced analyses of monoamine clearance mechanisms by using electrochemical methods (e.g. Cass & Gerhardt, 1995; Zahniser *et al.*, 1999), future studies using the choline microelectrode may reveal further unexpected, and potentially regionally specific, choline clearance capacities and mechanisms.

An evaluation of the potential usefulness of the relatively high temporal resolution of choline signals is inherently linked to the nature of the signals that can be recorded by this method. As indicated by the present data, choline signals elicited by local administration of pharmacological stimuli are characterized by relatively rapid rise times and clearance. Future research will determine the nature of choline signals evoked by systemic treatments, or elicited by behavior in freely moving or even task-performing animals. The present data suggest that the current methodology used to record choline signals in anesthetized animals may be useful in characterizing local neuropharmacological regulation of ACh release and, given the spatial resolution of the microelectrode, regional variations in such release. They may also reveal changes in choline transporter function that result from mutations of enzymes associated with cholinergic transmission or of cholinergic signaling proteins (e.g. Erb *et al.*, 2001; Klingner *et al.*, 2003; Volpicelli-Daley *et al.*, 2003), or from developmental manipulations of cholinergic transmission (e.g. Sarter & Bruno, 2004). Likewise, the present method may be appropriate to examine the cholinergic mediation of the organizational effects of developmental choline supplementation (e.g. Ricceri & Berger-Sweeney, 1998; Albright *et al.*, 1999; Meck & Williams, 2003). In general, plastic changes in cholinergic transmission are likely to involve, in addition to changes in the regulation of ACh release, persistent alterations in choline transporter function (e.g. Wenk *et al.*, 1984; Holley *et al.*, 1993), and the properties of choline signals recorded by the present method suggest that it may be useful in determining such changes *in vivo*.

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## Abbreviations

AA, ascorbic acid; ACh, acetylcholine; BSA, bovine serum albumin; CO, choline oxidase; DA, dopamine; HPLC, high-performance liquid chromatography; LOD, limit of detection; Neo, neostigmine; PBS, phosphate-buffered saline; Scop, scopolamine; TTX, tetrodotoxin.

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