We examined the release of acetylcholine (ACh) and dopamine (DA) using a novel probe through which striatal neurons could be both superfused and stimulated electrically in both anesthetized and freely moving awake animals. Optimal stimulation parameters for eliciting ACh release from cholinergic neurons differed from those required for eliciting DA release from dopaminergic terminals: at 0.6 ms pulse duration, 20 Hz and 200 μA, ACh release increased to 357 ± 30% (P < 0.01) of baseline and was blocked by the addition of tetrodotoxin (TTX). Pulse durations of 2.0 ms or greater were required to increase DA release. Unlike ACh release, DA release showed no frequency dependence above 5 Hz. The maximal evoked releases of ACh and DA were 556 ± 94% (P < 0.01) and 254 ± 38% (P < 0.05) of baseline, respectively. Peripheral administration of choline (Ch) chloride (30–120 mg/kg) to anesthetized animals caused dose-related increases in ACh release: basal release rose from 117 ± 7% to 141 ± 5% of initial baseline levels (P < 0.05) and electrically evoked ACh release rose from 386 ± 38% to 600 ± 34% (P < 0.01) in rats given 120 mg/kg. However, Ch failed to affect basal or evoked DA release although neostigmine (10 μM) significantly elevated basal DA release (from 36.7 fmol/10 min to 71.5 fmol/10 min; P < 0.05). In awake animals, Ch (120 mg/kg) also elevated both basal (from 106 ± 7% to 154 ± 17%; P < 0.05) and electrically evoked (from 146 ± 13 to 262 ± 19%; P < 0.01) ACh release. These experiments demonstrate that ACh and DA release can both be reliably evoked from rat striatum and that an intraperitoneal (i.p.) injection of Ch can increase both basal and evoked striatal ACh release.

INTRODUCTION

Numerous studies have applied the technique of in vivo microdialysis to investigate the regulation of DA and ACh release from rat striatum. Such studies have sometimes used high potassium concentrations within the perfusate to evoke neurotransmitter release by depolarizing neurons in the vicinity of the probe. While this technique is useful for releasing large amounts of neurotransmitter, it is less physiologic than providing short-duration electrical pulses. Moreover, high potassium concentrations can produce permanent alterations in synaptic transmission and can, with subsequent challenges, release diminishing quantities of ACh.

The present study used a novel custom-made microdialysis probe, containing a tungsten stimulating electrode, to depolarize neurons electrically in the direct vicinity of the microdialysis membrane. This treatment was found to produce more than a fourfold increase in ACh release and similar responses could be elicited by subsequent stimulations. The use of the hybrid probe enabled us to characterize the electrical parameters that affect both ACh release from striatal interneurons and DA release from nigrostriatal terminals.

After establishing that ACh release could be reliably evoked and entirely suppressed by the infusion of TTX, we explored the effect of exogenous Ch on basal and electrically evoked ACh release. Previous studies had established that dietary Ch could enhance tissue ACh and Ch levels in various regions of rat brain and that addition of Ch to superfused neurons could produce dose-related increases in basal and evoked ACh release from striatal slices. However, studies of the effects of Ch on basal ACh from rat striatum, as assessed by in vivo microdialysis, have provided contra-
dictory data. In the present study, we utilized the hybrid probe to examine the effect of Ch on both basal and electrically stimulated ACh and DA release from rat striata.

MATERIALS AND METHODS

Stimulating electrode and microdialysis probe

Stimulating electrodes were prepared from a 10 cm piece of teflon-coated tungsten wire (A-M Systems) with an uncoated diameter of 51 μm. The teflon was stripped from the tip of the wire, using a dissecting microscope, to expose approximately 0.3 mm of the tungsten. Only electrodes that had an impedance of 50–200 kΩ were used. Each electrode was glued (with nail polish) to a custom-made microdialysis probe (5 mm membrane length) such that its tip extended to half the length of the microdialysis membrane (Fig. 1).

Animals

Experiments were performed on both anesthetized and awake Male Sprague-Dawley rats (Charles River, Cambridge. MA; weighing 280–360 g. The animals were exposed to a 12 h/12 h light/dark cycle and given access to water and food (Charles River Rat. Mouse and Hamster Original Formula) ad libitum.

Anesthetized animal studies

Animals were anesthetized, placed in a stereotaxic frame and kept on a heating pad maintained at 37°C. The anesthetic was prepared by mixing 1.25 g of urethane and 125 mg of chloralose in 10 ml of water. Throughout the experiment the solution was stirred and warmed in a sealed vial. An i.p. injection (0.8 ml/kg body wt.) of chloralose. 50 mg/kg and urethane. 500 mg/kg. was used to anesthetize the animals. The hybrid probe was placed stereotaxically in the right corpus striatum at coordinates 0.4 mm anterior. −2.7 mm lateral from bregma. −6.5 mm ventral from dura and then perfused (4 μl/min) using a microperfusion pump (model 100. CMA) with an oxygenated modified Ringer’s solution (121 mM NaCl. 3.5 mM KCl. 1.2 mM CaCl₂. 1.2 mM MgCl₂. 1.0 mM NaH₂PO₄ and 25 mM NaHCO₃) containing 10 μM neostigmine. Collection vials contained 5 μl of 0.05 M perchloric acid to prevent DA breakdown. The positive lead of an electrical stimulator (model 2100. A-M systems) was connected to the tungsten wire and the negative lead was attached to the metal probe holder (kept electrically isolated from the stereotaxic frame). After 3 h of equilibration, an additional injection of 0.2–0.4 ml of anesthetic was given prior to the start of the experiment. At the end of the experiment some brains were removed, fixed and sectioned to verify probe placement. In all cases examined (n = 8), the probe was located in center of the striatum.

Awake freely moving animal studies

Animals were anesthetized with equithesin (3 ml/kg) and placed in a stereotaxic frame and probes were placed as described above. A ground stud affixed to a gold-plated post was placed in the cortex (2.0 mm anterior and −2.0 mm lateral from bregma). The tungsten electrode was also attached to a gold-plated post. The probe and both posts were affixed to the skull with permabond 910 (Permabond International) and dental acrylic repair material (Healthco). The inlet and outlet tubes of the hybrid probe were cut 4 cm from the skull and sealed with epoxy. Animals were removed from the frame and allowed to recover. After 24 h, the epoxy was cut away and the probe was connected to longer inlet and outlet tubes. Prior to the start of the experiment, animals were perfused for 1 h with Ringer’s solution as described above.

Acetylcholine and dopamine collection and determination

Experiments were started by collecting 3–4 consecutive 10 min dialysate samples for determination of baseline levels of ACh and/or DA. To characterize the excitability of striatal neurons, we stimulated with impulses of defined (and constant) current, duration and frequency for a period of 10 min. The TTX sensitivity of ACh release was determined by measuring release in both basal and stimulated (200 μA. 20 Hz and 0.6 ms) samples prior to and 1 h following. TTX (10 μM) administration through the dialysis probe. To determine the effect of Ch on basal and evoked neurotransmitter release, we administered isotonic saline i.p. to 10 rats and Ch chloride i.p. to 27 rats in the following doses: 30 mg/kg (n = 6). 60 mg/kg (n = 10). 120 mg/kg (n = 11). Three additional samples (10 min each) were collected, then all animals were electrically stimulated for 10 min (200 μA. 20 Hz and 0.6 ms). Rats receiving 120 mg/kg Ch and respective control rats were stimulated for a second period, 30 min after the first stimulation.

Levels of ACh, Ch and DA were assayed by splitting the dialysate sample. Probe recoveries for ACh and Ch (tested in a 10 μM solution) were 9.86 ± 0.37% (mean ± S.E.M. n = 28) and 10.81 ± 0.62% (n = 21), respectively, ranging between 6.00 and 12.75% for ACh and between 6.36 and 16.00% for Ch. Recovery for DA (tested in a 1 μM solution) was 8.60 ± 0.77% (n = 13) and ranged between 5.15 and 13.04%. Data values were not corrected for probe recoveries. ACh and Ch were determined by HPLC on a polymeric re-
versed-phase column (BAS) with a mobile phase of 50 mM phosphate (pH 8.5) containing 0.005% Kathon CG as a bactericide. Once separated, ACh and Ch were converted to betaine and hydrogen peroxide in a postcolumn enzymatic reactor containing Ch oxidase and ACh esterase (BAS). The hydrogen peroxide was detected electrochemically using a platinum electrode set at 500 mV (vs Ag/AgCl) (model 200a, BAS). The DA samples were assayed by HPLC on a C-18 (3.2 mm bore with 3.0 μm packing) column (ESA, HR-80). The mobile phase consisted of 70 mM Na₂HPO₄, 1 mM heptane sulphonic acid, 0.22 mM Na₂-EDTA and 4% methanol v/v at pH 5.5. DA was detected electrochemically by oxidation at 360 mV (Coulochem II, ESA). Release data were expressed both as picomoles released per 10 min and as percents of the three initial baseline values.

**Strength–duration curve**

Data relating the release of ACh to stimulation current (Fig. 2) and pulse duration (Fig. 3a) were used to calculate a strength–duration curve (Fig. 4). First, the equation describing the relation between current and percent ACh release was derived from data in Fig. 2 (y = 0.00730x + 0.00391, r = 0.998). This equation was then used to calculate the respective currents that would be required, at various pulse durations, to increase ACh release to 250% of basal. For example, the release observed at 1 ms (494 ± 39%) was approximately twice that observed at 0.2 ms (250%). By extrapolating from the equation relating release to current, it was determined that 109 mA would be needed to increase ACh release to 250% of control at this pulse duration (1 ms). This calculation was repeated for each point in Fig. 3a. The resulting curve (Fig. 4) fits the predicted equation \( I = I_o (1 + C/T) \) (\( I \) is the current, \( I_o \) the rheobase current, i.e., the current required at very long pulse durations, \( T \) is the pulse duration and \( C \) the chronaxie: the time at twice the rheobase current).

**Statistics**

Groups were compared using one way analysis of variance (ANOVA) with a post-hoc Newman–Keuls test. Significance was assumed for \( P < 0.05 \). Data are expressed as mean ± S.E.M. from 4–12 experiments on individual animals. Data examining the effect of multiple stimulations were analyzed using an one way ANOVA and a multiple regression. The dose–response relationship was analyzed using a linear regression.

**RESULTS**

**Effect of electrical stimulation on striatal acetylcholine, choline and dopamine release**

Basal ACh release (i.e., prior to the first stimulation period) was \( 2.76 ± 0.18 \) pmol/10 min (\( n = 10 \)); this remained stable throughout the experiment. Basal Ch release tended to decrease over time from a starting level of \( 14.40 ± 1.32 \) to 9.92 ± 0.90 pmol/10 min after 3 h (\( n = 10 \)). When the striatum was stimulated using increasing currents with stimulation frequency fixed at 20 Hz and pulse duration at 0.2 ms (Fig. 2). ACh release exhibited a linear relationship to stimulation current between 50 and 300 μA (\( r = 0.955 \)). Using 200 μA, 20 Hz and 4.0 ms pulse duration, electrical stimu-
ulation increased ACh release to 546 ± 32% (n = 4) of baseline (Fig. 3a). The half-maximal response was obtained with pulse durations of 0.2 ms and only a minor additional increase in ACh release was observed at durations greater than 1 ms. The effect on ACh release of increasing the stimulation frequency was studied by keeping the pulse duration and current constant at 200 μA and 0.2 ms, respectively (Fig. 3b). ACh release reached a plateau at 20 Hz with a mean increase of 250 ± 30% (n = 7) of baseline.

The response of dopaminergic terminals to electrical stimulation was markedly different from that of cholinergic striatal interneurons. Basal DA release (0.0854 ± 0.0114 pmol/10 min) was affected in an all-or-none fashion by stimulation at the parameters tested (i.e., evoked release was not influenced by increasing frequency or pulse duration after reaching a threshold level). DA release was significantly increased at pulse durations of 2.0 ms or greater (Fig. 3a) or at frequencies of 20 Hz or greater (Fig. 3b); however, further increases in these parameters did not further augment DA release.

**Electrophysiology of acetylcholine release as determined by strength-duration relation**

A strength-duration curve was calculated for the relationship between current and pulse duration, as described in the methods section. This calculation was possible because ACh release, as expressed as percent increase over basal, was found to be linearly related to current \( y = 0.00730x + 0.00391, \ r = 0.998 \) in the range examined. The obtained curve fit the predicted equation \( I = I_r (1 + C / t), \) where \( I \) is the current, \( I_r \) the rheobase current, i.e., the current required for very long duration pulses, \( t \) is the pulse duration and \( C \) the chronaxie, i.e., the time on the curve for twice \( I_r \). \( C = 0.236 \) ms.

**The effect of repeated stimulation on acetylcholine release**

Two experiments were performed to determine whether the release of ACh by the first stimulation period differed from that evoked by one or two subsequent stimulation periods, provided at intervals of 30 min. Eleven animals were stimulated using identical electrical parameters for two periods; three were stimulated for three periods (Fig. 5). ACh release during the second and third periods did not differ significantly from release during the first period.

In a second experiment, a multiple regression analysis was performed using all of the stimulation data (143 stimulation periods) to assess the effect of stimulation order on ACh release. In the regression analysis, ACh release was designated as the independent variable while current, duration, frequency and order were designated as dependent variables. In the resulting regression, duration, frequency and current all had \( P \)-values of less than 0.001, whereas order had a \( P \)-value of 0.23. The overall \( r^2 \)-value (the percent of variance explained by the regression) was unaffected by the presence or absence of the stimulation order variable (from 0.8361 to 0.8344), further demonstrating that the level of ACh evoked by the second or third stimulation did not differ from that evoked by the first.

**Tetrodotoxin sensitivity of acetylcholine release**

Both basal and evoked ACh release were entirely TTX-dependent. Basal ACh release (3.34 ± 0.33 pmol, \( n = 3 \)) and electrically stimulated ACh release (9.86 ± 1.68 pmol, \( n = 3 \)) were reduced by the addition of TTX.
(10 μM) to levels below the limit of detection (0.2 pmol).

The effect of choline on basal and evoked acetylcholine and dopamine

Ch administration to anesthetized rats after collection of basal dialysate samples increased both basal and evoked ACh release. A typical experiment is illustrated in Fig. 6. Basal ACh release 30 min after injection of 120 mg/kg Ch chloride was significantly increased from a mean of 117 ± 7 to 141 ± 5% of basal DA release 35.6 ± 5.1 to 71 ± 15.0 fmol/l0 min (P < 0.05); lower doses were ineffective (Table I). Ch enhanced evoked ACh release in a dose-dependent manner (r = 0.994, P < 0.01); release after 120 mg/kg Ch chloride (600 ± 34%, P < 0.01) and after 60 mg/kg (502 ± 49%, P < 0.05) differed significantly from release after saline (386 ± 38%). A potentiation of release was also observed in the second stimulation period, from 401 ± 33% to 578 ± 52% of basal levels in the 120 mg/kg group (P < 0.05). A statistically significant increase (P < 0.01) in brain dialysate Ch was observed immediately after the i.p. injection of Ch at all doses (Table I). Under these conditions, we saw no effect of Ch on DA release. However, the addition of neostigmine to the perfusion medium elevated DA release from 35.6 ± 5.1 to 71 ± 15.0 fmol/10 min (P < 0.01, n = 15).

Effect of choline on acetylcholine release from awake animals

In awake animals, ACh release from the corpus striatum was approximately twice that observed in anesthetized animals (from 2.52 ± 0.20 to 4.10 ± .31 pmol/10 min, P < 0.01). Ch (120 mg/kg) significantly

![Graph](image)

Fig. 6. Effect of choline administration on basal and evoked acetylcholine release from anesthetized rat corpus striatum. After three baseline samples, animals received isotonic saline i.p. (open bars) or choline chloride (120 mg/kg) (crossed hatched bars). Three additional samples were collected and then striata were electrically stimulated for 10 min (current 200 μA; pulse duration, 0.6 ms; frequency, 20 Hz). After 30 additional minutes, the stimulation was repeated. Fig. 6 shows the results of a typical experiment.

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<th>Evoked</th>
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<td>(%)</td>
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<td>141 ± 15**</td>
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<td>0.089 ± 0.011</td>
<td>107 ± 6</td>
<td>0.152 ± 0.028</td>
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TABLE I

Effect of choline administration on release of choline, acetylcholine and dopamine from anesthetized rat striatum

Baseline levels of ACh, Ch and DA were determined during the first three 10-min collection periods after which rats received either isotonic saline (n = 10) or choline chloride (30 mg/kg (n = 6), 60 mg/kg (n = 10) or 120 mg/kg (n = 11) mg/kg) i.p. Three additional 10-min samples were collected and then the microdialysis electrodes were stimulated for 10 min (current 200 μA; pulse duration, 0.6 ms; frequency, 20 Hz) and samples were collected for an additional 30 min. Resting levels represent the release immediately prior to stimulation. Data (means ± S.E.M.) are expressed both as pmol/10 min and percent of basal levels (i.e., levels during the initial three pretreatment collection periods). The normalized data were analyzed using an ANOVA with a post-hoc Newman–Keuls test (* P < 0.05; ** P < 0.01).

DISCUSSION

We incorporated a stimulating electrode into a standard microdialysis probe (Fig. 1) in order to characterize the release of transmitters from depolarized DA terminals and ACh interneurons within the rat striatum. Once the electrophysiological properties of such release were determined, we applied this apparatus to examining the effect of Ch administration on striatal ACh and DA release. We observed that Ch in the doses tested potentiated both basal and evoked ACh release, but had no apparent effect on DA release.

Our hybrid probe is similar to one described by Sandberg et al., except that in our study the electrode was used for stimulating and not recording from,
striatal neurons can be stimulated for as much as three ten-minute periods without exhibiting attenuation of evoked ACh release (Fig. 5). In contrast, such release is rapidly attenuated when striata are perfused locally with high potassium solutions. The long periods (10–20 min) of constant depolarization associated with exposure to high potassium concentrations make this method less physiologic than electrical stimulation.

Initially, we validated the use of the hybrid microdialysis probe by measuring ACh release in response to increasing amounts of current, using a frequency (20 Hz) and pulse duration (0.2 ms) typical of neurophysiologic studies involving brain stimulation. Both basal and evoked ACh release were found to be TTX-dependent, indicating that release was indicative of physiologic processes and not the result of nonspecific trauma. Basal ACh release (2.52 ± 0.29 pmol/10 min, using probes with approximately a 10% recovery) was consistent with levels in previous reports, if the data in such reports are corrected for the lower recoveries (4–5%) in those studies. The release of ACh was found to vary linearly with increasing current (r = 0.955; Fig. 2).

ACh release was profoundly affected by pulse durations in the range of 0.2–1.0 ms (P < 0.01; 20 Hz; 200 μA) (Fig. 3a). A pulse duration of 0.2 ms increased ACh release to 251 ± 30% of baseline while leaving DA release unchanged. DA release was significantly increased only at pulse durations of 2.0 ms or greater. This finding might suggest that the physiologic release of ACh within the striatum fails to affect DA release from nigrostriatal terminals. Alternatively, the presence of neostigmine (10 μM) in our dialysis medium might, by elevating synaptic ACh levels, have rendered the dopaminergic terminals insensitive to additional ACh. This is supported by our observation that basal DA levels are increased twofold by the addition of neostigmine.

Maximal ACh release was achieved at a frequency of 20 Hz (Fig. 3b). This frequency may be physiologic for striatal interneurons, inasmuch as Wilson et al. observed spontaneous firing at rates of 3 to 10 Hz, with bursts of up to 23 Hz, in intracellular recordings from such neurons. Unlike ACh release, DA release was increased in an all-or-nothing fashion by intrastriatal stimulation (Fig. 3a and b). Similar findings have been reported for medial forebrain bundle (MFB) stimulation at frequencies of 6.67, 8.33, 12.5 and 25 Hz; all produced approximately the same (50%) increase in DA release despite the fact that the number of action potentials recorded from the region surrounding the probe was increased in proportion to the stimulation

We initially demonstrated that the release of DA is not affected by electrical stimulation of the MFB, but that DA release is profoundly affected by the addition of neostigmine. We therefore investigated whether the release of DA from the rat striatum is affected by electrical stimulation of the MFB in the presence of elevated calcium concentrations.

We demonstrated a linear relationship between the number of pulses delivered to the MFB and the increase in DA release from the rat striatum as determined by in vivo microdialysis in the presence of elevated calcium levels (3.4 mM and 2.3 mM, respectively). The calcium concentration is likely to play a critical role in regulating the release of DA and therefore comparisons between our data, based on experiments using 1.2 mM and those with elevated calcium concentrations, are tenuous. The finding that DA release exhibited all-or-none behavior in response to a range of stimulation frequencies may have resulted from autoreceptor-related mechanisms.

Neuron size, distance from the electrode, degree of myelination, neuronal region (perikaryon vs axon vs terminal) and various electrophysiological properties may all affect the excitability of a neuron in response to electrical stimulation. The DA in rat striatum is localized predominantly in the nerve terminals – which are typically bulbous varicosities with a thickness of 0.4–2.0 μm. ACh is present in both terminals and cell bodies of aspiny striatal neurons with a calculated diameter of 20–30 μm. The larger cholinergic neurons, because of their greater length constant, would be expected to reach firing threshold at lower stimulation currents than the smaller dopaminergic terminals. In our experiments, ACh release was, in fact, more easily evoked than DA, a finding consistent with anatomical data on the relative sizes of these two classes of neurons.

The responsiveness of neurons to electric current can be characterized by the strength–duration curve (Fig. 4), which represents the amount of current and the duration of pulses that yield the same level of response (i.e., the evoked increase in ACh release). We calculated this curve in a novel fashion, using data relating ACh release to current level at a fixed duration and using data relating ACh release to pulse duration at a fixed current (Figs. 2 and 3a). The calculated curve fit the theoretical strength–duration equation; moreover, the calculated chronaxie (0.236 ms), a measure of the excitability of cholinergic striatal neurons, was typical of CNS gray matter. Thus, we have been able to relate neurotransmitter release in vivo to the electrophysiological properties of the cholinergic neurons.

Ch administration to anesthetized animals rapidly
elevated brain Ch release and increased both basal and evoked ACh release, without affecting that of DA (Table I). This lack of response may have been an artifact of the use of neostigmine, an acetylcholinesterase inhibitor, in the dialysate, resulting in synaptic ACh levels which saturated the responsiveness of DA terminals. This is supported by our findings that neostigmine significantly (P < 0.01) elevated basal DA levels. Ch amplified evoked ACh release in a dose-dependent fashion (r = 0.994, P < 0.01), but increased basal ACh release only at a dose of 120 mg/kg (Table I).

In order to establish that the effect of Ch on ACh release was not an artifact of the anesthetic used in this study which might have inhibited striatal glutamate receptors5,8 thus enhancing Ch responsiveness4, we examined the effect of Ch in awake, freely moving rats. Our results indicate that the anesthetic had significantly (P < 0.01) suppressed basal ACh release by approximately 40%. Despite the higher basal ACh levels of awake animals, Ch was still able to elevate both basal (P < 0.05) and evoked (P < 0.01) ACh release at a dose of 120 mg/kg. In fact, the ratio of the evoked ACh release from rats receiving 120 mg/kg Ch vs that in saline-treated controls, was 1.68 in anesthetized animals and 1.78 in awake animals.

Addition of exogenous Ch to striatal slices elevates tissue ACh and Ch levels and also increases ACh release during stimulation17,27,28,30,31. Prior in vivo studies, although contradictory, provided some evidence that Ch might act to potentiate ACh release in vivo. Studies by Consolo et al.4 failed to find an effect of i.p. Ch chloride (100 mg/kg) on basal ACh release from sham operated rats, but did show an effect in rats that had been decorticated by interruption of the cortico-striatal pathway4. Westerink and De Boer32 also found no effect on ACh release of i.p. Ch (100 mg/kg) despite a 30-fold increase in brain Ch release32. This is consistent with our finding that basal ACh release was increased only at doses of 120 mg/kg. However, we observed a twofold increase in brain Ch release following Ch administration. Given that plasma Ch levels increase only 3–4-fold following an i.p. Ch injection of 100 mg/kg11 and that the macromolecular system which transports Ch across the blood-brain barrier operates by facilitated diffusion across a concentration gradient20, it is difficult to understand the 30-fold increase observed by Westerink and De Boer32. While Koshimura et al.16 failed to find an effect of a low Ch dose (7.5 mg/kg, i.p.) on ACh release, they did demonstrate increases after intracerebroventricular Ch (10 μmol, 20 μmol or 50 μmol)16. Our data not only demonstrate that ACh and DA can be reliably evoked from rat striatum in vivo, but that changes in local Ch concentrations can affect ACh release.

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