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N-Methyl-D-Aspartate Increases Acetylcholine Release from Rat Striatum and Cortex: Its Effect is Augmented by Choline[1]

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ABSTRACT

We examined the effects of N-methyl-D-aspartate (NMDA), a glutamate agonist, and of glutamate itself, on acetylcholine (ACh) release from superfused rat striatal slices. In a Mg^{++}-free medium, NMDA (32–1000 μM) as well as glutamate (1 mM) increased basal ACh release by 35 to 100% (all indicated differences, $P < .05$), without altering tissue ACh or choline contents. This augmentation was blocked by Mg^{++} (1.2 mM) or by MK-801 (10 μM). Electrical stimulation (15 Hz, 75 mA) increased ACh release 9-fold (from 400 to 3660 pmol/mg of protein): this was enhanced (to 4850 pmol/mg of protein) by NMDA (100 μM). ACh levels in stimulated slices fell by 50 or 65% depending on the absence or presence of NMDA. The addition of choline (40 μM) increased ACh release both basally (570 pmol/mg of protein) and with electrical stimulation (6900 pmol/mg of protein). In stimulated slices choline acted synergistically with NMDA, raising ACh release to 10,520 pmol/mg of protein. The presence of choline also blocked the fall in tissue ACh. No treatment affected tissue phospholipid or protein levels. NMDA (32–320 μM) also augmented basal ACh release from cortical but not hippocampal slices. Choline efflux from striatal and cortical (but not hippocampal) slices decreased by 34 to 50% in Mg^{++}-free medium. These data indicate that NMDA-like drugs may be useful, particularly in combination with choline, to enhance striatal and cortical cholinergic activity. ACh release from rat hippocampus apparently is not affected by NMDA receptors.

Cholinergic neurons are unique in that they both acetylate and phosphorylate choline, forming ACh and phosphocholine (and, ultimately, PCho). Although both of the enzymes that catalyze these processes [choline acetyltransferase (Cohen and Wurtman, 1975) and choline kinase (Spanner and Ansell, 1979; Millington and Wurtman, 1982)] are unsaturated with their choline substrate *in vivo*, acetylation of choline is favored over its phosphorylation when cholinergic neurons are depolarized (Ando *et al.*, 1987). An important part of the choline used for ACh synthesis derives from the choline in membrane PCho, and perhaps other phospholipids (Blusztajn and Wurtman, 1983; Ulus *et al.*, 1989; Buyukuysal and Wurtman, 1990), especially when cholinergic neurons are stimulated and provided with insufficient exogenous choline (Ulus *et al.*, 1989).

We reported previously that when striatal slices were superfused with a medium that lacked free choline and contained an acetylcholinesterase inhibitor (which blocks the intrasynaptic formation of choline from ACh), electrically evoked ACh release was sustained even after 2 hr of stimulation, without diminishing tissue contents of ACh or choline (Ulus *et al.*, 1989; Buyukuysal and Wurtman, 1990; Buyukuysal *et al.*, 1991). Under these conditions, however, membrane levels of PCho and other major phospholipids declined in proportion to the duration of stimulation and to the amounts of ACh released into the medium. Addition of physiological concentrations of choline (10–40 μM) to the medium both protected against the decline in content of membrane phospholipids as well as amplified the release of ACh (Ulus *et al.*, 1989; Buyukuysal and Wurtman, 1990; Buyukuysal *et al.*, 1991).

The mechanisms that regulate the production of free choline from membrane phospholipids to support ACh synthesis are presently unknown. That such regulatory mechanisms actually exist is indicated by the ability of rat brain slices to divert more or less choline from membrane lipids to ACh synthesis, depending on the firing frequencies of cholinergic neurons (Ulus *et al.*, 1989; Parducz *et al.*, 1976, 1986) and the availability of free choline from other sources. Thus, this diversion is increased in the presence of drugs that inhibit acetylcholinesterase (Ulus and Wurtman, 1988) or choline uptake (Parducz *et al.*, 1976, 1986), but not by drugs like the aminopyridines, 4-aminopryridine and 3,4-diaminopyridine (Buyukuysal and Wurtman, 1990; Buyukuysal *et al.*, 1991), which augment both

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ABBREVIATIONS: ACh, acetylcholine; PCho, phosphatidylcholine; NMDA, N-methyl-D-aspartate; HPLC-EC/ high-performance liquid chromatography-electrochemical detection.

ACh release and choline uptake. Thus, it might be anticipated that any drug which increases ACh release may or may not modulate the flux of choline into membrane phospholipids, depending on whether the drug also affects free choline levels.

Striatal cholinergic neurons are known to receive an excitatory glutamatergic input from the cerebral cortex (McGeer *et al.*, 1977; Storm-Mathisen, 1977; Wood *et al.*, 1979). Glutamate itself and NMDA, a glutamate agonist, are also known to release [^{3}H]ACh from striatal slices preloaded with [^{3}H]choline, in a Ca^{++}-dependent, tetrodotoxin-sensitive manner (Lehmann and Scatton, 1982; Snell and Johnson, 1986; Alberch *et al.*, 1990). Pharmacological evidence indicates that this release is mediated by a specific NMDA-type receptor (Lehmann and Scatton, 1982; Snell and Johnson, 1986). In most previous studies, the effects of NMDA on striatal ACh release were investigated by measuring [^{3}H]ACh in the medium after preloading of the slices with [^{3}H]choline. No data are available about whether NMDA also alters tissue stores of ACh and choline. Present experiments were designed to determine the effects of NMDA on: 1) the basal release of endogenous ACh and choline from, and their tissue levels in, striatal, hippocampal and cortical slices; 2) release of ACh from striatal slices during electrical stimulation; and 3) the effects of NMDA on the previously demonstrated depletion of membrane phosphatides occurring when striatal slices are subjected to prolonged electrical stimulation without exogenous choline. We have also tested the ability of exogenous choline to modulate these NMDA responses.

Methods

Preparation and perfusion of slices. Male Sprague-Dawley rats (280–360 g) were sacrificed by decapitation and their brains were removed rapidly. Striatal, hippocampal and cortical tissues were dissected and slices 0.3 mm thick were prepared with a McIlwain tissue chopper (Brinkmann Instruments, Westbury, NY). The tissue slices obtained from three rats were combined and washed with ice-cold medium to remove most of the membrane debris. Four to six striatal slices were then transferred into each of eight superfusion chambers (volume, 1.0 ml). When cortical or hippocampal slices were used, 10 to 15 slices were placed in each superfusion chamber. The chambers were kept at 37°C in a water bath, and the slices were perfused for 60 min (for equilibration) with a physiological solution (millimolar: NaCl, 120; KCl, 3.5; $CaCl_2$, 1.3; $MgSO_4$, 1.2; NaH_2PO_4, 1.2; $NaHCO_3$, 25; glucose, 10; and eserine salicylate, 0.02) at a constant flow rate of 0.6 ml/min by means of a Rainin Rabbit eight channel peristaltic pump (Rainin Instrument Co. Inc., Woburn, MA). This solution was bubbled continuously with a mixture of 95% O_2 and 5% CO_2. Choline or MK-801 was sometimes added to the perfusion medium, as indicated in the text.

After a 60-min equilibration period, slices were perfused for another 60 min with either the same medium or a Mg^{++}-free medium, the composition of which was the same as that above save for lacking $MgSO_4$. Where indicated, compounds were added to the superfusion medium for the duration of this perfusion period. Perfusates representing an entire 60-min period were collected in beakers on ice, and later assayed for ACh and choline. The slices were taken from the superfusion chambers at the end of the perfusion period and assayed for ACh, choline, phospholipids, protein and DNA.

Extraction of ACh and choline from perfusates. ACh and choline were extracted from the perfusates by a silica column procedure (Gilberstadt and Russell, 1984). Two milliliters of the perfusate were applied to a silica column (5 × 8 mm bed of Bio-Sil A, 200–400 mesh, Bio-Rad Laboratories, CA). The column was then washed successively with 1 ml of 0.001 N HCl, 0.8 ml of 0.075 N HCl and 0.8 ml of 0.03 N HCl in 10% (v/v) 2-butanone. Subsequently, the last two fractions were collected, dried under a vacuum and assayed for choline and ACh, respectively. Standards of ACh and choline (0–400 pmol) prepared in 2 ml of the same solutions as those used for superfusing the slices were processed in parallel with the samples.

Extraction of ACh and choline from the tissues. Tissue ACh and choline were extracted as described by Torn and Aprison (1966). The slices were removed from the chamber and washed twice with ice-cold physiological solution and then homogenized in 1 ml of cold water containing 0.02 M eserine salicylate. Aliquots (0.2 ml) of the homogenates were mixed with 1 ml of 1 M formic acid in acetone (15:85, v/v); the mixtures were vortexed and allowed to stand for about 2 hr in a cold room and then centrifuged (1500 × *g* for 10 min). Supernatant fluids were transferred to a glass tube and dried under vacuum. Residues were dissolved in 2 ml of the same choline-free medium as that used for perfusion. ACh and choline were then extracted from this solution as described above. Standards for ACh and choline (0–800 pmol) were prepared in 0.2 ml of water and processed in parallel with the samples.

Determination of ACh and choline. The ACh and choline contents of the dried samples obtained from the perfusates were determined by the radioenzymatic method of Goldberg and McCaman (1973). In brief, choline was phosphorylated by choline kinase in the presence of ^{32}P-ATP and the labeled phosphocholine was then separated from excess ATP and quantitated. For the determination of ACh, the endogenous choline was first converted to unlabeled phosphocholine; the ACh was then hydrolyzed by acetylcholinesterase, and the choline resulting from this hydrolysis was assayed as above. The ACh in the dried perfusate samples obtained from experiments in which the exogenous choline had been added was further separated from choline and determined by HPLC-EC. The ACh and choline contents of dried tissue samples were determined by HPLC-EC.

Determination of phospholipids, protein and DNA. For the extraction of phospholipids (Folch *et al.*, 1957), a 0.2-ml aliquot of the tissue homogenates was mixed with 3 ml of chloroform-methanol (2:1, v/v) and 1 ml of water. The mixtures were then centrifuged (1500 × *g*, 10 min) to separate an upper phase (discharged by aspiration) and a lower phase (containing phospholipids) which was dried under vacuum and used for determination of phosphorus (Svanborg and Svennerholm, 1961).

Aliquots of tissue homogenates were assayed for protein (Lowry *et al.*, 1951) and DNA (Labarca and Paigen, 1980).

Statistics. Data are expressed as means ± S.E.M. Statistically significant differences between two means were determined by Student's *t* test for unpaired observations. Significant differences between three or more means were determined by analysis of variance and the Newmann-Keuls test for the multiple comparison of means. P value less than .05 were considered to be significantly different.

Materials. NMDA, *l*-Glutamic acid HCl and (+)-MK-801 maleate were purchased from Research Biochemicals, Inc. (Natick, MA). Eserine salicylate, choline chloride, choline kinase and acetylcholinesterase were purchased from Sigma Chemical Co. (St. Louis, MO).

Results

Effects of NMDA on basal ACh and choline release from striatal slices. In the first series of experiments, the effects of NMDA and *l*-glutamate on ACh and choline release from striatal slices were examined in the presence and absence of Mg^{++} in the perfusion medium. Basal ACh release from striatal slices perfused with a normal Krebs' medium containing 1.2 mM of Mg^{++} was 360 ± 18 pmol/mg of protein per hr (n = 13). Under these conditions, the slices also released a large amount of free choline (3380 ± 230 pmol/mg of protein per hr; n = 13) into the medium (table 1). The addition of *l*-glutamate (1 mM) or NMDA (1 mM) to the medium failed to alter ACh or choline release (table 1).

When striatal slices were perfused with a Mg^{++}-free medium, ACh release increased significantly ($P < .05$), by about 27%

TABLE 1

Effects of NMDA on basal release of ACh and choline from the striatal slices

Striatal slices were perfused with an eserine-containing normal Krebs' medium (contains 1.2 mM $MgSO_4$). After a 60-min equilibration period, slices were perfused with the same or a Mg^{++}-free medium for another 60 min. During this latter period the perfusion medium contained the concentrations of NMDA or glutamate indicated in parentheses. Perfusates were collected in iced plastic tubes and assayed for ACh and choline; values were normalized for the protein contents of each set of slices. Data are expressed as picomoles per milligram of protein per hour, and shown as means ± S.E.M. Numbers of measurements are shown in parentheses.

Medium/Groups	ACh	Choline
	pmol/mg protein/hr	*pmol/mg protein/hr*
Mg^{++}-containing (1.2 mM) medium		
Control-1	380 ± 17 (13)	3380 ± 231 (11)
NMDA (1 mM)	380 ± 26 (5)	3450 ± 445 (5)
Glutamate (1 mM)	380 ± 35 (6)	3450 ± 509 (6)
M^{++}-free medium		
Control-2	450 ± 34 (13)*	2220 ± 265 (13)*
NMDA (10 μM)	490 ± 40 (6)	1580 ± 180 (6)*
NMDA (32 μM)	610 ± 35 (6)†	1650 ± 215 (6)*
NMDA (100 μM)	740 ± 54 (6)†	1850 ± 320 (6)*
NMDA (320 μM)	920 ± 42 (6)†	1680 ± 275 (5)*
NMDA (1000 μM)	580 ± 49 (7)†	2130 ± 244 (7)*
Glutamate (1 mM)	540 ± 39 (6)†	2190 ± 252 (6)*

Significantly differs ($P < .05$) from (*) Control-1 or (†) Control-2.

TABLE 2

Contents of ACh and choline in striatal slices perfused with normal or Mg^{++}-free media containing various concentrations of NMDA

Striatal slices perfused as in table 1 were removed from the chambers after the 2nd 60-min period and assayed for ACh and choline. There were no significant differences.

Medium	ACh	Choline
	pmol/mg protein	*pmol/mg protein*
Mg^{++}-containing medium	1830 ± 122 (12)	970 ± 52 (11)
Mg^{++}-free medium	1790 ± 204 (12)	950 ± 92 (11)
Mg^{++}-free medium		
+10 μM NMDA	1910 ± 182 (6)	930 ± 133 (6)
+100 μM NMDA	1910 ± 117 (13)	920 ± 13 (13)
+320 μM NMDA	2160 ± 117 (6)	1080 ± 178 (5)
+1000 μM NMDA		810 ± 42 (4)

(table 1). Under these conditions, choline efflux was reduced ($P < .05$) by about 34% (table 1). The addition at that point of NMDA (1 mM) or *l*-glutamate (1 mM) to the medium further increased ACh release (table 1). Choline efflux was not influenced by the treatments (table 1).

The concentration-response relationship for NMDA and basal ACh release was investigated in separate experiments. The presence of NMDA in the range of 32 to 320 μM in the Mg++-free medium increased ACh release in a concentration-dependent manner (table 1). The presence of NMDA at lower concentrations was ineffective in altering ACh release (table 1). Choline efflux was not significantly affected by NMDA (table 1).

Effects of NMDA on ACh and choline contents of striatal slices at rest. Tissue ACh and choline levels of the slices after a 60-min perfusion with the Mg^{++}-containing medium were 1830 ± 122 pmol/mg of protein ($n = 12$) and 970 ± 52 pmol/mg of protein ($n = 11$), respectively (table 2). These values remained unaltered when the slices were superfused with a Mg^{++}-free medium both in the absence and presence of NMDA.

Effects of NMDA on ACh release from striatal slices in the presence of MK-801 in the medium. In order to determine whether the NMDA-induced increase in basal ACh release was mediated by a specific NMDA receptor, the effect of 100 μM of NMDA on basal ACh release was tested in the presence of 10 μM MK-801 in the medium. As seen in table 3, the NMDA-induced increase in basal ACh was prevented by MK-801.

Tissue ACh and choline levels of the slices (table 4) remained unaltered when the slices were superfused with a Mg^{++}-free medium, both in the absence and presence of NMDA (100 μM) or MK-801 (10 μM).

Effects of NMDA on ACh release from striatal slices both in the presence and absence of choline in the medium, at rest and during electrical stimulation. In separate experiments, the effects of NMDA on ACh release from striatal slices at rest and during stimulation were compared. We also examined the effects of adding choline to the medium on the stimulating effect of NMDA on ACh release. The rate of ACh release from striatal slices perfused with a choline-free medium was 400 ± 29 pmol/mg of protein per hr ($n = 7$) at rest and 3660 ± 371 pmol/mg of protein per hr ($n = 7$) during electrical stimulation (15 Hz; 75 mA) (table 5). The addition of NMDA (100 μM) to the medium augmented ACh release by 45% at rest and by 32% during stimulation (table 5).

When the striatal slices were perfused with a choline-containing medium (40 μM), ACh release increased to 570 ± 45 pmol/mg of protein per hr ($n = 8$) at rest and to 6900 ± 378 pmol/mg of protein per hr ($n = 7$) during electrical stimulation (table 5). Addition of NMDA (100 μM) to the choline-containing perfusion medium increased ACh release in both situations, at rest and during electrical stimulation (table 5). The increase in ACh release during electrical stimulation in the presence of choline (40 μM) and NMDA (100 μM) was 6860 pmol/mg of protein per hr, considerably greater than the sum of the increments produced by choline (3240 pmol/mg of protein per hr) or NMDA (1190 pmol/mg of protein per hr) alone (table 5).

Effects of NMDA on tissue levels of ACh, choline, phospholipids and protein in striatal slices perfused with

TABLE 3

Effects of NMDA on basal ACh and choline release from striatal slices: presence or absence of MK-801 in the medium

Slices ($n = 8$–10) were perfused for 60 min in the normal Krebs'; during this period, half were also exposed to MK-801 (10 μM). They were then perfused for an additional 60 min in a Mg^{++}-free medium also containing, as indicated above: MK-801 (10 μM), NMDA (100 μM), both compounds or neither compounds.

Groups	ACh	Choline
	pmol/mg protein/hr	*pmol/mg protein/hr*
Control	470 ± 43	2030 ± 143
MK-801 (10 μM)	470 ± 51	1510 ± 124*
NMDA (100 μM)	810 ± 85*†	1570 ± 97*
MK-801 (10 μM)		
+NMDA (100 μM)	490 ± 32	1600 ± 73*

Significantly differs ($P < .05$) from (*) control or (†) from MK-801 + NMDA.

TABLE 4

Contents of ACh and choline in striatal slices perfused with a Mg^{++}-free medium containing MK-801, NMDA or both

Striatal slices ($n = 4$) were perfused as in table 3 and then removed from the chambers after the 2nd 60-min period, assayed for ACh and choline and normalized for the protein contents of the slices. There were no significant differences.

Groups	ACh	Choline
	pmol/mg protein	*pmol/mg protein*
Control	1990 ± 65	1030 ± 54
MK-801 (10 μM)	1820 ± 139	960 ± 54
NMDA (100 μM)	1790 ± 252	970 ± 115
MK-801 (10 μM)		
+NMDA (100 μM)	1980 ± 253	980 ± 94

TABLE 5

Release of ACh from striatal slices perfused with or without choline and NMDA, at rest and during electrical stimulation

Striatal slices (n = 6–8) were perfused for 60 min with or without choline (40 μM). Slices were then perfused both at rest and with electrical stimulation (15 Hz, 75 mA) for an additional 60 min with and without choline (40 μM), NMDA (100 μM) or both. ACh was extracted from the perfusate and assayed by HPLC-EC.

Groups	ACh	
	Rest	Stimulation
	pmol/mg protein/hr	
Control	400 ± 29	3660 ± 371
NMDA (100 μM)	580 ± 45*	4850 ± 254*
Choline (40 μM)	570 ± 45*	6900 ± 378*
Choline (40 μM) +NMDA (100 μM)	650 ± 64*	10520 ± 512†

Significantly differs ($P < .05$) from control values (*) and choline or NMDA alone (†).

TABLE 6

Contents of ACh, choline, phospholipid and protein in striatal slices that had been perfused with or without choline and NMDA, at rest and during stimulation

Striatal slices (n = 7–8) perfused as in table 5 were removed from the chambers after the 60-min period of rest or stimulation and assayed for ACh, choline, total phospholipids, protein and DNA. ACh and choline levels were normalized for the protein contents of slices; phospholipids and protein were normalized for the DNA contents of the slices. ACh levels in all stimulated groups differed significantly ($P < .05$) from those in all resting groups; choline, phospholipid and protein levels did not differ between resting and stimulated groups.

Compounds/Groups	Rest	Stimulation
ACh (pmol/mg protein)		
Control	1920 ± 142	1050 ± 72
NMDA	2170 ± 130	780 ± 40*
Choline	2420 ± 113*	1760 ± 92†
Choline + NMDA	2340 ± 145	1690 ± 87†
Choline (pmol/mg protein)		
Control	1130 ± 97	930 ± 70
NMDA	960 ± 97	840 ± 59
Choline	1470 ± 200	1380 ± 190*
Choline + NMDA	1310 ± 164	1230 ± 169*
Phospholipid (nmol/μg DNA)		
Control	33 ± 1	35 ± 1
NMDA	35 ± 2	35 ± 2
Choline	36 ± 3	36 ± 2
Choline + NMDA	33 ± 1	35 ± 2
Protein (μg/μg DNA)		
Control	54 ± 2	55 ± 2
NMDA	54 ± 2	54 ± 3
Choline	54 ± 2	53 ± 3
Choline + NMDA	53 ± 2	53 ± 4

* $P < .05$, different than control group; † $P < .05$, higher than NMDA and control groups.

and without choline, at rest and during stimulation. When striatal slices at rest were perfused with a choline-containing medium (40 μM), tissue levels of ACh and choline showed a tendency to increase (table 6). In agreement with the results shown in table 2, NMDA did not alter the tissue ACh or choline levels of slices at rest (table 6). When the striatal slices perfused with a choline-free medium were stimulated electrically, tissue levels of ACh were reduced by 44% in the absence, or by 65% in the presence, of NMDA (100 μM). Perfusion of the slices with a choline-containing medium (40 μM) protected the slices from this reduction in tissue ACh levels. Electrical stimulation of slices failed to alter the tissue choline levels (table 6).

None of the treatments (*e.g.*, electrical stimulation, choline or NMDA, alone or in combination, significantly altered tissue phospholipid or protein levels (table 6).

Effects of NMDA on ACh and choline release from cortical or hippocampal slices at rest. Rates of ACh release from cortical slices were 49 ± 3 pmol/mg of protein per hr (n = 6) in the first series of experiments and 52 ± 4 pmol/mg of protein per hr (n = 6) in the second series of experiments (table 7), much lower than in the striatal slices. Rates of choline efflux, however, were similar in both tissues (tables 1 and 7). Perfusing the cortical slices with a Mg^{++}-free medium failed to alter ACh release, but decreased ($P < .05$) choline efflux (table 7). The addition of NMDA (32–320 μM) to the Mg^{++}-free medium greatly increased ACh release (table 7) without altering choline efflux (table 7).

ACh and choline release from the hippocampal slices were the same whether the slices were perfused with a Mg^{++}-containing medium (1.2 mM) or Mg^{++}-free medium (table 7). The addition of NMDA (10–320 μM) to the Mg^{++}-free perfusion medium failed to alter ACh and choline release from the slices (table 7).

Effects of NMDA on ACh and choline contents of cortical or hippocampal slices. The ACh and choline contents of hippocampal and cortical slices were not altered by perfusion with a Mg^{++}-free medium in either the presence or absence of NMDA (table 8).

Discussion

These results show that NMDA can increase the release of ACh from resting striatal slices (table 1) without modifying tissue ACh or choline levels (table 2). NMDA can also increase ACh release from electrically stimulated slices (table 5), concurrently enhancing the reduction in tissue ACh induced by

TABLE 7

Effects of NMDA on basal release of ACh and choline from the cortical and hippocampal slices

In two series of experiments, cortical and hippocampal slices (n = 4–8) were perfused with a normal Krebs' buffer (containing 1.2 mM Mg^{++}) for 60 min. After this equilibration period, slices were perfused with the same or a Mg^{++}-free medium, with or without NMDA, for an additional 60 min. Perfusates were then collected for ACh and choline assays.

Tissue/Medium	ACh	Choline
	pmol/mg protein/hr	*pmol/mg protein/hr*
Cortical slices (1st experiment)		
Normal Krebs'	49 ± 3	3510 ± 250
Mg^{++}-free medium	44 ± 2	2570 ± 170†
Mg^{++}-free medium +NMDA (320 μM)	72 ± 7*	2510 ± 240†
Cortical slices (2nd experiment)		
Normal Krebs'	52 ± 4	2990 ± 120
Mg^{++}-free medium	56 ± 7	2350 ± 220†
Mg^{++}-free medium		
+NMDA (10 μM)	ND	2240 ± 180†
+NMDA (32 μM)	77 ± 9	2110 ± 190†
+NMDA (100 μM)	84 ± 6*	2370 ± 240†
Hippocampal slices (1st experiment)		
Normal Krebs'	67 ± 8	2300 ± 279
Mg^{++}-free medium	62 ± 9	1710 ± 164
Mg^{++}-free medium +NMDA (320 μM)	58 ± 4	1910 ± 261
Hippocampal slices (2nd experiment)		
Normal Krebs'	70 ± 4	2290 ± 150
Mg^{++}-free medium	65 ± 3	1910 ± 190
Mg^{++}-free medium		
+NMDA (10 μM)	68 ± 6	2310 ± 180
+NMDA (32 μM)	80 ± 10	1890 ± 100
+NMDA (100 μM)	73 ± 9	2000 ± 190

* $P < .05$, differs from Mg^{++}-free media lacking NMDA and from control; † $P < .05$, differs from control media.

TABLE 8

Contents of ACh and choline in cortical and hippocampal slices perfused with normal or Mg^{++}-free media containing various concentrations of NMDA

Cortical and hippocampal slices perfused as in table 7 (1st experiment) were removed from the chambers after the 2nd 60-min period and assayed for ACh and choline; values were normalized for the protein contents of each set of slices. There were no significant differences.

Tissue/Medium	ACh	Choline
	pmol/mg protein	*pmol/mg protein*
Cortical slices		
Normal Krebs'	98 ± 5	351 ± 6
Mg^{++}-free medium	113 ± 9	37 ± 13
Mg^{++}-free medium +NMDA (320 μM)	104 ± 7	338 ± 10
Hippocampal slices		
Normal Krebs'	282 ± 20	405 ± 31
Mg^{++}-free medium	276 ± 23	401 ± 34
Mg^{++}-free medium +NMDA (320 μM)	293 ± 22	410 ± 29

this stimulation (table 6). Under these conditions, the addition of choline to the perfusion medium both enhances the effect of NMDA on ACh release and protects the slices from depletion of ACh (table 6). NMDA (320 μM) increases ACh release from the cortical slices, but fails to affect ACh release from hippocampal slices (table 7).

The effect of NMDA on striatal ACh release is concentration-dependent, in the range of 32–320 μM (table 1). At 10 μM, NMDA is ineffective and, at 1000 μM, its effect was decreased compared with that observed at 320 μM NMDA. The NMDA concentrations that we found necessary for evoking striatal ACh release were in good agreement with those in most (Lehmann and Scatton, 1982; Snell and Johnson, 1986) but not all (Alberch *et al.*, 1990) previous reports. Lehmann and Scatton (1982) and others (Snell and Johnson, 1986) reported that 10–500 μM N-methyl-DL-aspartate increased [3H]ACh release from striatal slices. However, Alberch *et al.* (1990) found that, in much lower concentrations (0.001–1 μM), NMDA could evoke the release of endogenous ACh or [^{3}H]ACh. The NMDA concentrations (EC_{50} = 7 nM) needed to release endogenous ACh in this study were several times lower than those we observed (table 1) or the concentrations reported by Lehmann and Scatton (1982). There were numerous methodological differences, however, between our study and that of Alberch *et al.* (1990); they used a different incubation medium with higher pH (8.6) and higher concentrations of $CaCl_2$ (5.7 mM) and KCl (5.6 mM).

Two lines of evidence indicate that NMDA-evoked release of striatal ACh is mediated by NMDA-type excitatory amino acid receptors. First, the NMDA effect was evident only in the absence of Mg^{++}; NMDA (1000 μM) was virtually ineffective in releasing ACh in the presence of 1.2 mM Mg^{++} (table 1). [It is well known that Mg^{++} at physiological concentrations produces a voltage-dependent uncompetitive block of NMDA receptor-mediated processes by binding to a specific site within the ionic channel of the NMDA receptor-ion channel complex (Nowak *et al.*, 1984).] Second, the release of ACh evoked by 100 μM of NMDA was blocked by 10 μM MK-801 (table 3), a highly selective antagonist of NMDA-type receptors (Halliwell *et al.*, 1989; Wong and Kemp, 1991).

Omitting Mg^{++} from the medium significantly increased basal ACh release by 27% (table 1). The mechanism of this increase is unknown, but the phenomenon was also observed by Lehmann and Scatton (1982). It could reflect the stimulation of NMDA receptors by an endogenously released agonist (glutamate, aspartate, etc.); however, the failure of MK-801 to block this increase argues against this possibility. Another possibility is that with low levels of release of glutamate and aspartate, MK-801 may not block NMDA receptor function in the absence of stimulation.

The omission of Mg^{++} also decreased choline efflux by 35% (table 1), probably reflecting changes in the production of choline from membrane phospholipids. This view is supported by the observation that Mg^{++} stimulates choline production from rat brain homogenates incubated at 37°C (Zeisel, 1985). In some experiments, the addition of NMDA or of MK-801 to the medium further decreased choline (table 3); this NMDA effect, was not concentration-related, however, and was inconsistent as well (table 1).

Electrical stimulation of the striatal slices increased ACh release several-fold (table 5), as described previously (Ulus *et al.*, 1989: Buyukuysal and Wurtman, 1990; Buyukuysal *et al.*, 1991). The addition of NMDA (100 μM) to the perfusion medium further enhanced this release (table 5). Addition of choline to the perfusion medium both increased ACh release and markedly potentiated the effect of NMDA on ACh release during electrical stimulation (table 5). It is apparent that insufficient free choline was available in cholinergic neurons to support maximal ACh synthesis and release during electrical stimulation.

Under resting conditions, the presence of NMDA in the perfusion medium failed to alter tissue levels of ACh or choline (tables 2, 4 and 6). Hence, the increase in ACh release with NMDA was not the result of the depletion of ACh stores and must have reflected increased ACh synthesis. When slices were stimulated electrically in the absence of exogenous free choline, they released large amounts of ACh into the perfusion medium but also lost about half of their ACh. The presence of NMDA (100 μM) in the medium enhanced the reduction in tissue ACh contents during electrical stimulation (table 6). In all of these situations, the amounts of ACh released into the media were more than 2 times greater than the amounts depleted from the tissue during the 60-min period of electrical stimulation. This again indicates that under these conditions the rate of ACh synthesis is actually increased. Providing supplemental choline enabled the stimulated slices both to increase the release of ACh and to sustain their ACh levels (table 6). We found previously that, by using membrane phosphatides as a source of choline, striatal slices were able to continue releasing large amounts of ACh during prolonged periods of electrical stimulation without exhibiting reductions in their choline or ACh contents (Ulus *et al.*, 1989; Buyukuysal and Wurtman, 1990; Buyukuysal *et al.*, 1991). Under those conditions, their membrane phospholipids declined (Ulus *et al.*, 1989; Buyukuysal and Wurtman, 1990; Buyukuysal *et al.*, 1991). In the present study, the apparent rate of free choline production from membrane phospholipids decreased by about 35% when the slices were perfused without either Mg^{++} or NMDA (table 1). Consistent with this reduction in choline efflux, membrane phospholipid levels of the stimulated slices remained unaltered (table 6).

These findings are consistent with the view that membrane phospholipids constitute a reservoir of choline to be used for ACh (Blusztajn and Wurtman, 1983), and that the tendency of particular cholinergic neurons to divert significant quantities

of choline from this reservoir is enhanced when the neurons are required to fire for prolonged periods while provided with insufficient choline. Membrane phospholipid levels decrease under these conditions (Ulus *et al.*, 1989; Buyukuysal and Wurtman, 1990). If the liberation of free choline from phospholipids decreases, as occurred in the present study, the production of ACh also declines, causing tissue ACh levels to fall (table 6). Providing free choline can enhance ACh synthesis and release (table 5) and protect against the stimulation-induced fall in membrane phospholipids (Ulus *et al.*, 1989; Buyukuysal and Wurtman, 1990) or a reduction in tissue ACh content (table 6).

NMDA receptors are present in very high density in rat cortex and hippocampus (Collingridge and Lester, 1989). NMDA, at micromolar concentrations, evokes neurotransmitter release in both tissues (Jones *et al.*, 1987; Hoehn *et al.*, 1990). No data are available regarding the effects of NMDA on ACh release from cortical cholinergic neurons other than those in the abstract of Johnston and Lodge (1984). According to their abstract (Johnston and Lodge, 1984), NMDA at high micromolar concentrations increased the release of [^{3}H]ACh from cortical slices preloaded with [^{3}H]choline, whereas ketamine (1–5 μM), a competitive antagonist of NMDA receptors, and Mg^{++} (0.1–0.2 mM) inhibited this effect. We found that NMDA (32–320 μM) increased ACh release from the cortical slices (table 7) without altering tissue contents of ACh and choline (table 8), but failed to affect ACh release from the hippocampus (table 7). Lehmann and Scatton (1982) similarly reported that NMDA had no effect on [^{3}H]ACh release from hippocampal slices. The NMDA-evoked release of ACh in the striatum is tetradotoxin-sensitive (Lehmann and Scatton, 1982), indicating it does not occur at the level of the synapse. Whereas the hippocampus contains only a terminal projection field of cholinergic neurons, both cortical and striatal slices contain cholinergic interneurons (Johnston *et al.*, 1981; Woolf *et al.*, 1983). Conceivably, cholinergic nerve endings in the hippocampus lack NMDA receptors, or perhaps the activation of such receptors is not coupled to ACh release.

The decrease in choline efflux from cortical slices during perfusion with a Mg^{++}-free medium, with or without NMDA (table 7), constituted another similarity between the striatal and cortical slices as contrasted with hippocampal slices. The rates of choline release from these tissues were similar to each other, but much higher than choline release from the hippocampus. Regional brain differences in choline release from membrane lipids have also been reported (Freeman and Jenden, 1976; Kosh *et al.*, 1980; Ulus *et al.*, 1989). The regional differences in the rate of ACh release clearly reflect the density of cholinergic innervation and are in good agreement with a previous report (Weiler *et al.*, 1979). Observed regional differences in choline release, however, apparently were not related to the extent of cholinergic innervation as demonstrated in earlier reports (Freeman and Jenden, 1976; Kosh *et al.*, 1980). Surprisingly, the rate of choline efflux from the hippocampal slices was not altered by perfusion with the Mg^{++}-free medium, with or without NMDA (10–320 μM). The mechanisms of the differences in choline release between the hippocampus and the two other brain areas studied are presently unknown.

In summary, these data show that NMDA increases ACh release from resting striatal or cortical cholinergic neurons without altering their tissue levels of ACh and choline. The addition of choline dramatically increases NMDA-induced ACh release when the striatal slices are stimulated electrically. Choline also protects the tissues from the ACh depletion which otherwise occurs during electrical stimulation.

Central cholinergic neurons (Drachman and Leavitt, 1974; Davies and Maloney 1986) and NMDA receptors (Artola and Singer, 1987; Monaghan *et al.*, 1989; Sutor and Hablitz, 1989; Wroblewski and Danysz, 1989) are both involved in learning and memory process. There is, moreover, considerable evidence from clinical studies that the memory impairments and deficits in cognitive functions observed in patients suffering from senile dementia of the Alzheimer's type may be associated with decreases in both cholinergic (Bartus, *et al.*, 1982; Davies and Maloney, 1986; Perry, 1986) and glutamatergic neurotransmission. There is growing clinical interest in the possibility of treating Alzheimer's Disease patients with drugs which increase cholinergic neurotransmission (*e.g.*, muscarinic agonists, acetylcholinesterase inhibitors and agents that increase brain choline levels) or drugs that augment NMDA-type neurotransmission [*e.g.*, milacemide (Herting, 1991); *d*-cycloserine (Jones *et al.*, 1991)]. NMDA agonists are known to be neurotoxic (Wroblewski and Danysz, 1989; Olney, 1990) and they may cause or increase neuronal loss particularly at high doses. Our results suggest that the combination of NMDA receptor-modulating drugs with choline or choline sources may be particularly useful to increase the clinical effectiveness and allow the use of lower doses of NMDA-receptor modulating drugs.

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