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# Cortical Second Messengers After NBM Damage: No Change in Responses to Cholinergic Agonists

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SHOHAM, S., M. E. NEWMAN, E. WERTMAN AND R. P. EBSTEIN. *Cortical second messengers after NBM damage: No change in responses to cholinergic agonists.* PHARMACOL BIOCHEM BEHAV **36**(3) 507–513, 1990.—Damage to the nucleus basalis of Meynert (NBM) decreases acetylcholine (ACh) innervation of cortex. We explored transmission of cholinergic messages in cortex 2–3 weeks after such damage. The NBM damage was unilateral and the ipsilateral denervated cortex was compared to the contralateral nondenervated cortex. The response to carbachol, a muscarinic ACh receptor-agonist, was measured by inhibition of forskolin-induced cAMP accumulation in cortical membranes and by formation of inositol phosphate (IP) in cortical slices. No difference was found in the carbachol effects between ipsi- and contralateral cortices. Thus, we find no evidence of either receptor loss or receptor supersensitivity. There was, however, a significant decrease in $K^+$-stimulated IP formation in the cortex ipsilateral to the damage which probably reflected loss of cholinergic terminals. When comparing the cortex contralateral to NBM damage with the cortex contralateral to sham damage in control rats, no difference was found in any of the above parameters. When severe cognitive deficits are observed, 2–3 weeks after NBM damage, loss of presynaptic ACh is the main change in cortical cholinergic transmission.

NBM cAMP Inositol phosphate Acetylcholine Carbachol

MAGNOCELLULAR forebrain nuclei project cholinergic input to cortex and hippocampus (12,17). Cell shrinkage and degeneration in these nuclei in Alzheimer Disease (AD), in particular in the nucleus basalis of Meynert (NBM), attracted attention to the possibility that cortical indices of decreased cholinergic function in AD are due to loss in subcortical cholinergic input from the NBM (22,28). In rats, experimentally induced damage of the NBM causes decreases in cortical cholinergic enzymes: choline acetyltransferase (ChAT) and acetylcholinesterase (AChE) (6, 7, 13, 16). In addition, NBM damage in rats produces a syndrome which includes memory dysfunction that parallels AD (6, 7, 13).

In search of potential sites for pharmacological intervention in AD, it is of interest to explore plasticity in cortical cholinergic function after damage to the NBM. The spared components of the cholinergic system may undergo changes to compensate for the damage; for example, axonal sprouting or changes in numbers of ACh receptors, etc. Perhaps some plasticity phenomena can be amplified or suppressed pharmacologically.

The subcortical cholinergic projection from NBM to cortex originates in each cerebral hemisphere and innervates the respective ipsilateral cortex only (12,17). Therefore, a common model has been the unilateral damage to NBM and subsequent comparison of consequences in ipsilateral vs. contralateral cortex (13,16). The NBM is a diffusely organized nucleus. Thus, the surgical damage induced by a stereotaxically inserted vertical cannula or electrode is partial although sufficiently large to mimic the cholinergic deficits in cortex observed in AD patients.

Plasticity in cholinergic receptor function was explored in several studies in terms of binding of cholinergic receptors to radiolabeled pharmacological agents of known affinity to cholinergic receptors. When pharmacological agents not distinguishing receptor subtypes were used, some authors reported transient changes (5) while others found no change (1,20). The findings were contradictory also when agents distinguishing receptor subtypes were used: McKinney and Coyle (14) found acute reduction in M2 (low affinity muscarinic receptors) and a later rise in number of M1 (high affinity muscarinic receptors). Mash *et al.* (16) confirmed the acute reduction in M2 numbers and showed that it parallels a reduction in M2 in AD patients. However, de Belleroche *et al.* (5) and Watson *et al.* (26) found reduction in both M1 and M2 in the cortex ipsilateral to the damage. Atack *et al.* (1) found early increase in M2 binding and late increase in M1 binding. This discrepancy of findings may be explained in part by variation in the extent and placement of the lesion. An additional variable might be changes in cholinergic receptor-mediated function through intracellular second messengers. Perhaps in some functional pathways an intracellular change makes receptor changes unnecessary.

We explored second messenger responses to cholinergic agonists. In rat brain, muscarinic cholinergic receptors have been shown to be coupled to both major second messenger systems: adenylate cyclase and polyphosphoinositol (PPI) hydrolysis, leading to the formation of diacylglycerol and inositol phosphate (IP) (2–4, 18).

We damaged the NBM unilaterally according to the procedure of LoConte *et al.* (13). This model was chosen because in a series

of studies, LoConte *et al.* (13) and Pedata *et al.* (19) documented changes at many levels behavioral, physiological, histological and neurochemical. We were particularly interested in the possible correlation between neurochemical and behavioral changes early (2–3 weeks) after the lesions.

Carbachol inhibition of forskolin-induced cyclic AMP was examined in cortical membranes. Stimulation of IP formation was measured both in response to KCl in the presence of the AChE inhibitor physostigmine, which had been shown to be a response to endogenously released ACh (11), and in response to carbachol. There was no difference in carbachol-induced effects in ipsilateral vs. contralateral cortex hence no evidence of ipsilateral change. We did find reduced IP accumulation in response to KCl-depolarization in ipsilateral cortex probably reflecting loss of cholinergic terminals.

## METHOD

### *Surgical Procedure*

Male albino Sabra rats were used, weighing 250–350 grams and maintained at an ambient temperature of 25°C and a 12:12 hr light:dark cycle (light off from 0600 to 1800). Under pentobarbital sodium (50 mg/kg) anesthesia, the rat's head was fixed in a David Kopf stereotaxic instrument with skull level between bregma and lambda. Using bregma as reference, the coordinates were anterior 0.5 mm, lateral 2.8 mm, and ventral 7.6 mm (ventral from skull surface). A stainless steel electrode, 250 μm in diameter and insulated except for its cross sectioned tip, was lowered into the brain according to the above coordinates and 1.1 mA anodal direct current passed for 30 seconds; a steel burr inserted into the rectum served as a cathode. Sham operations were identical except that the electrode was lowered only 5.6 mm to avoid insertion damage to the NBM; no current was passed. After a 10–14-day recovery period, testing of memory was begun using the passive avoidance task as described below.

At 2–3 weeks after damage surgery and after behavioral tests had been completed, 20 rats were assigned for cAMP assays (10 experimental and 10 sham controls). Twenty other rats were assigned for IP assays (10 experimental and 10 sham controls). Rats were sacrificed by cervical dislocation and the brain quickly removed. Frontal-parietal cortex, extending from frontal pole anteriorly to approximately the level of stereotaxic A2500 (10) and laterally to the rhinal sulcus, was taken for biochemical assays.

### *Histology and Histochemistry*

In an initial stage of this experiment, a series of 10 rats were sacrificed to verify the location and extent of lesion. They were anesthetized and perfused through the heart with 10% formalin. Brain slices 35 μm thick were stained with the method of hematoxylin and eosin.

A second series of 7 rats was used for parallel histochemical verification of the cholinergic aspect of the lesion and biochemical assay of cortical inositol phosphate hydrolysis. These rats were sacrificed by cervical dislocation. The brain was quickly removed. Cortical tissue taken for biochemical tests included the frontal parietal cortex anterior to the rhinal sulcus. The subcortical tissue block was immersed in 4% paraformaldehyde in phosphate buffered saline (PBS) for 3–4 days and then in 30% sucrose solution in PBS for 3–7 days. Brain sections 30–40 μm thick were stained for acetylcholinesterase by a modified method of Hedreen *et al.* (9). The exposure of tissue to the reaction medium was in the presence of 0.1–0.2 mM Ethopropazine to inhibit butyrylcholinesterase. Exposure time was adjusted to allow visualization of individual NBM cells.

Two additional NBM-damaged rats were used to demonstrate decreased cholinergic innervation of cortex. They were sacrificed by pentobarbital 60 mg/kg and perfused through the heart with saline and then with 4% phosphate buffered paraformaldehyde. The acetylcholinesterase histochemistry methods were same as above.

### *Biochemical Procedures: $^3$H-Inositol Phosphate Formation*

Incorporation of $^3$H-inositol, and $^3$H-inositol-1-phosphate formation and isolation were performed by a modification of the prelabelling procedure described by Berridge *et al.* (2). Brain slices were prepared by cross-chopping using a McIlwain tissue chopper set at 0.35 mm. Slices were preincubated in 100 ml Krebs-Ringer bicarbonate buffer containing 1.29 mM $CaCl_2$ and 10 mM glucose with constant shaking and gassing with 95% $O_2$/5% $CO_2$ for 60 min at 37°C. The medium was then aspirated and the collected slices transferred to incubation vials containing 5 ml Krebs-Ringer's to which $^3$H-inositol had been added to give a final concentration of 0.3 μM. The slices were incubated in this medium with constant gassing for 60 min and then washed three times with Krebs-Ringer's at room temperature to remove excess unincorporated inositol. Fifteen μl aliquots of packed cortical slices were then transferred to glass tubes containing 0.5 ml Krebs-Ringer's to which agonists and 10 mM LiCl had been added. The tubes were gently agitated, briefly gassed, capped and incubated for a further 60 min at 37°C. At the end of this period they were centrifuged, the medium decanted, 1 ml of chloroform: methanol (1:1) added, and left for 10 min at room temperature. A further 0.3 ml of chloroform and 0.3 ml of water were then added, the tubes centrifuged to separate the phases, and 0.8 ml aliquots of the upper aqueous phased applied to columns containing 1 ml Dowex-1-X8 resin (formate forms). The columns were washed four times with 4 ml of 5 mM inositol, and inositol-1-phosphate eluted with 4 ml 0.2 M ammonium formate/0.1 M formic acid directly into scintillation vials for counting.

### *Preparation of Membranes and Adenylate Cyclase Assay*

Tissues were homogenized in 50 mM tris-HCl, pH 7.4 containing 1 mM dithiothreitol and 2 mM EGTA. Nuclei and cell debris were removed by centrifugation at 1000 × g for 10 min, and the resulting supernatants centrifuged at 10,000 × g for 20 min. The pelleted membranes were frozen in 1 ml aliquots in liquid $N_2$. Adenylate cyclase was determined in an assay volume of 0.2 ml containing 25 mM tris-HCl, pH 7.4, 2 mM $MgCl_2$, 0.5 mM ATP, 10 μM GTP, 100 mM NaCl, 1 μCi [alpha-$^{32}$P]ATP, 1.5 mg/ml creatine phosphate, 0.2 mg/ml creatine phosphokinase, and 1 mM 3-isobutyl-1-methylxanthine. $^{32}$P-Cyclic AMP was separated from ATP by the method of Salomon *et al.* (18). Incubations were started by addition of 50 μg protein, and terminated after 10 min at 30°C by addition of 0.1 ml of a solution containing 0.5 mM cyclic AMP and 4 mM ATP, and transferring the tubes to a boiling water bath.

Dose-response curves for carbachol inhibition of forskolin-stimulated adenylate cyclase were transformed by the Eadie-Hofstee method, and values for inhibition constants and maximal inhibition derived by linear regression using the Statpak programme on an IBM-PC.

### *Passive Avoidance Testing*

Rats were tested in a dimly lit room. They were allowed 30–60 min habituation in a holding cage. The passive avoidance box consisted of one compartment lit by two 60-W light bulbs fixed at

the top, and a second dark compartment. For training, the rat was placed in the light compartment and while the rat was facing away from the partition between the compartments, the guillotine door was lifted. Latency was defined as time taken for the rat to enter the dark compartment with all 4 feet. The guillotine door was then lowered to block escape and a 1 mA current passed through the grid floor for 2–3 sec. Ten sec later the rat was taken out of the dark compartment and returned to its holding cage. For testing retention, 24 hr later, latency to cross from the light into the dark compartment was measured. If the rats did not cross into the dark compartment, they were taken out after 600 seconds (criterion).

## RESULTS

### *Histological and Behavioral Characterization of the NBM Syndrome*

Histological examination revealed that at its largest extent, the damage included the ventromedial globus pallidus, a lateral part of the medial forebrain bundle, and ansa lenticularis. The anterior-posterior extend spanned between frontal stereotaxic coordinates A7190–A4890 (10). Acetylcholinesterase histochemistry verified that the lesion decreased cholinergic innervation of cortex ipsilateral to the damage (Fig. 1A). AChE histochemistry of the subcortical brain block (Fig. 1B,C) verified the NBM lesion in 7 rats from which the cortices had been removed for biochemical assays.

Twenty-four hours after one trial passive avoidance training nonoperated ("naive") and sham-operated rats displayed intact memory function: Seven of eight naive and nine of eleven sham-operated rats remained in the light compartment for more than 600 seconds (criterion). In contrast, only two of eleven NBM-damaged rats remained to criterion in the light compartment when tested 24 hr after training. The NBM-damaged group mean latency to enter the dark compartment was $162 \pm 62.8$ seconds which was significantly below mean latency of the sham-damaged controls [Mann-Whitney, $U(18) = 21$, $p < 0.031$].

Another group of NBM-damaged rats was tested 1 hr instead of 24 hr after one trial training. Five of these 11 NBM-damaged rats reached criterion. Their group mean latency ($304.0 \pm 86.22$ seconds) was below that of sham-operated rats ($490.5 \pm 70.18$ seconds) tested 24 hr after training and higher than that of NBM-damaged rats ($162.0 \pm 62.8$ seconds) but not significantly different from either [Mann-Whitney, $U(18) = 36$, compared to shams and $U(20) = 45$, compared to NBM damaged, tested 24 hr after training].

### *Biochemical*

*PPI turnover.* The addition of 20 mM KCl to the cortical slices increased inositol-1-phosphate (IP) response by 50% (data not shown). Physostigmine at 0.1 mM had no effect on the basal response but potentiated the response to elevated KCl so that this was now approximately 2-fold. Atropine at 50 nM had no effect on the basal response or on the response to KCl alone, but significantly reduced the response to a combination of KCl and physostigmine. This response was therefore interpreted as being due to presynaptic release of ACh associated with $K^+$-induced depolarization of cortical cells (11). There were no differences in KCl or carbachol-induced IP accumulation between ipsilateral and contralateral cortices, in both lesioned and sham-operated rats (Fig. 2). However, the response to 20 mM KCl in the presence of 1 mM physostigmine was significantly reduced [$p < 0.0067$, paired $t$-tests, $t(9) = 3.05$] in the ipsilateral cortices from NBM-lesioned rats compared to the contralateral cortices from the same lesioned rats. No difference in (KCl + physostigmine) stimulated PI turnover was observed between ipsilateral and contralateral cortices of sham-operated rats, $t(8) = 0.65$.

The response to a combination of KCl and carbachol was unaltered in ipsilateral cortex from lesioned rats compared to the contralateral cortex. In sham-lesioned rats, this parameter showed a significant decrease in ipsilateral compared to contralateral cortex [paired $t(9) = 1.93$, $p < 0.04$] when results were expressed as increments over basal activity. But when the same results were expressed as ratios of stimulated to basal activities, the effect was not significant [KCl + carbachol/basal $= 4.27 \pm 0.55$ in contralateral cortex of sham-lesioned rats, $3.42 \pm 0.53$ in ipsilateral cortex of sham-lesioned rats]. The reduction in stimulation by KCl + physostigmine in ipsilateral cortex of lesioned rats nevertheless remained significant when the results were calculated in this manner: [KCl + physostigmine/basal $= 1.94 \pm 0.07$ in contralateral cortex of lesioned rats, $1.45 \pm 0.12$ in ipsilateral cortex, paired $t(9) = 3.95$, $p < 0.00225$].

In the group of 7 NBM-damaged rats used for parallel acetylcholinesterase histochemistry of subcortical brain and PI hydrolysis assays of the cortex, similar findings were obtained to those described above. However, the difference between ipsilateral and contralateral cortex in response to KCl + physostigmine did not reach significance. Increase over basal activity: $107 \pm 67.9$ ipsilateral vs. $187 \pm 57.6$ contralateral. Stimulation ratio: $1.31 \pm 0.17$ ipsilateral vs. $1.547 \pm 0.18$ contralateral. A typical example of damage is demonstrated in Fig. 1B. No pattern emerged in terms of correlation between location and size of damage and biochemical results.

*Adenylate cyclase.* No difference was observed in carbachol inhibition of forskolin-stimulated adenylate cyclase activity in cortical membranes between the ipsilateral and contralateral sides in either lesioned or sham-operated rats (Table 1).

In the lesioned animals, forskolin-induced cAMP was significantly reduced in the contralateral cortex compared to ipsilateral cortex [paired $t(8) = 2.748$, $p < 0.012$; see Table 1]. In spite of this difference, maximal inhibition by carbachol was not different between the two hemispheres and was comparable to the degree of maximal inhibition by carbachol reported in the literature (15).

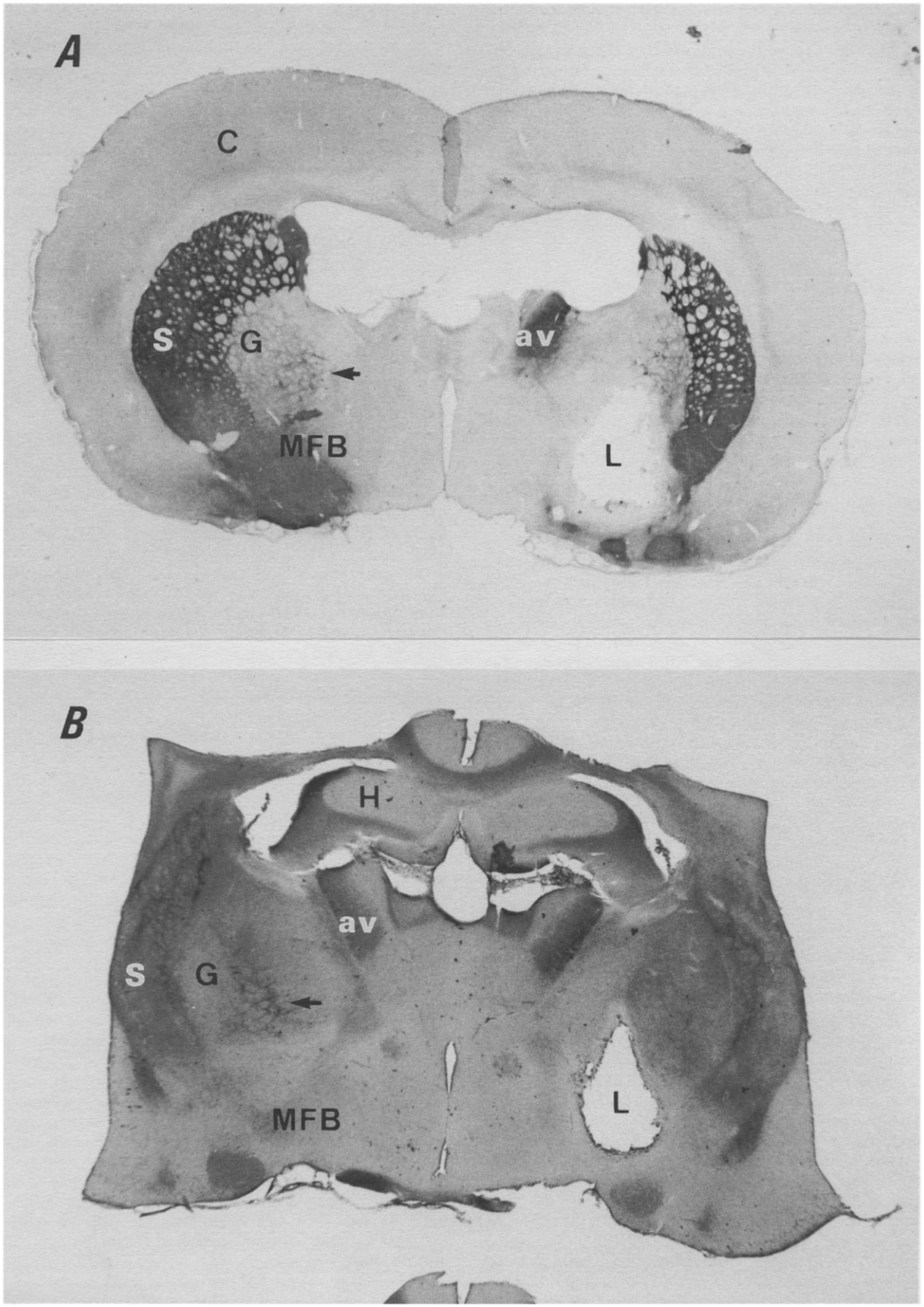
## DISCUSSION

Histological and histochemical examination confirmed unilateral damage to the NBM. At 2–3 weeks post NBM damage, rats used in this investigation displayed a memory deficit expressed in failure of retention of passive avoidance after one trial training. We tested whether at this stage of recovery there was any change in the response to the cyclic AMP and inositol phosphate second messenger systems to cholinergic agonists.

There was no evidence of change in response to carbachol in either second messenger system. This confirms a preliminary report by Scarth *et al.* (24) who found no effect of quinolic acid lesions of the the nucleus basalis on carbachol-induced IP formation in cortical slices and a report by Raulli *et al.* (20) performing the same experiment in ibotenate NBM-damaged rats.

A significant reduction in KCl-stimulated IP accumulation in the presence of physostigmine was observed in cortex ipsilateral to the damage, probably reflecting loss of cholinergic terminals capable of releasing ACh. This parallels a study by Gardiner *et al.* (8) in which unilateral kainate NBM damage reduced $K^+$-induced release of [$^3$H]acetylcholine from slices of cortex ipsilateral to the NBM damage. This also agrees with the LoConte *et al.* (13) finding of reduced scopolamine-induced release of ACh ipsilateral to the damage.

We used frontal-parietal cortical tissue for our assays. This includes a wide field of distribution of cholinergic terminals.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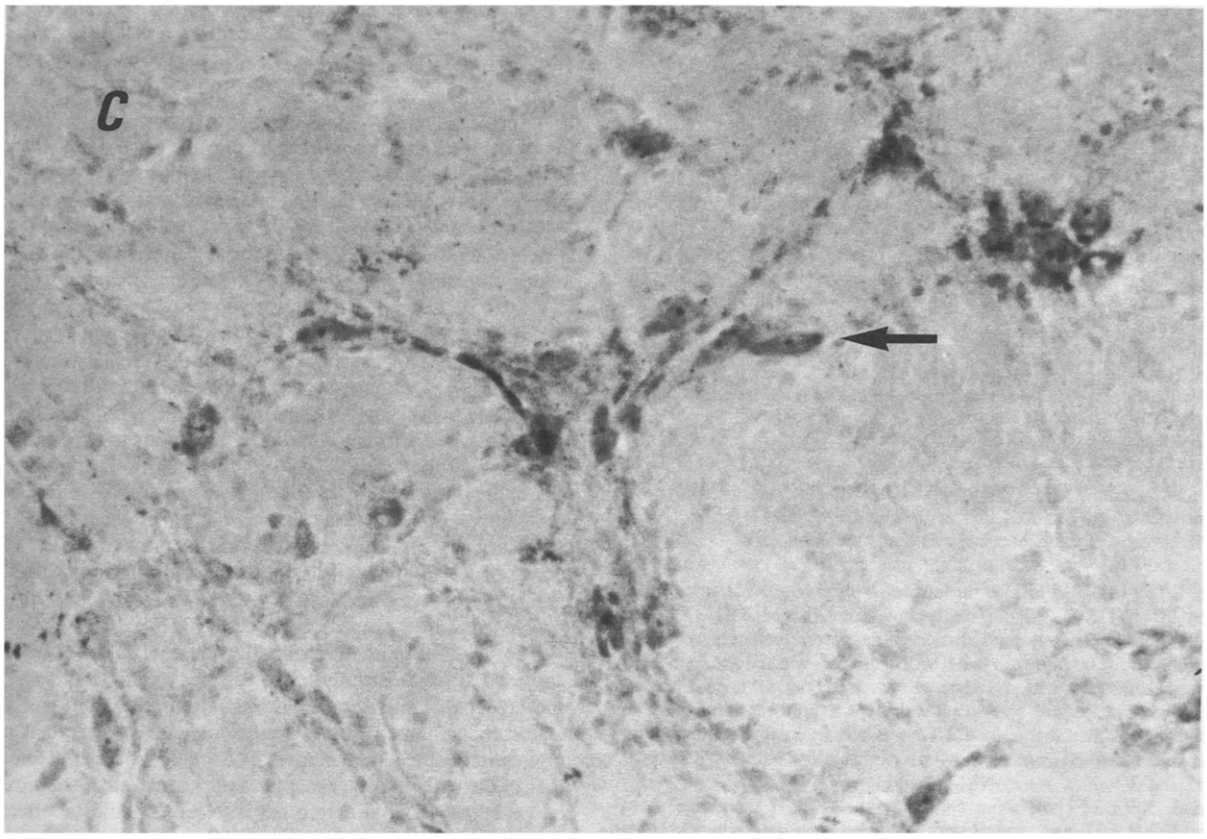


ABOVE AND FACING PAGE

FIG. 1. Acetylcholinesterase histochemistry of unilateral NBM damage. (A) Note decrease in cholinergic innervation in cortex ipsilateral to lesion, as evident from lighter shade compared to contralateral cortex (missing due to tissue handling is the fornix and due to asymmetry in sectioning is the anterior ventral thalamic nucleus). (B) Brain section from which the cortex had been removed for IP assays. The NBM appears as clusters of intense AChE positive neurons (arrow). (C) NBM cell cluster (arrow); magnification 180×. av = anterior ventral thalamic nucleus, C = cortex, G = globus pallidus, H = hippocampus, L = lesion, MFB = medial forebrain bundle, S = striatum.

TABLE 1

ADENYLATE CYCLASE ACTIVITY IN CORTICAL MEMBRANES OF SHAM-TREATED AND LESIONED RATS

| | Lesioned | | Sham | |
|---|---|---|---|---|
| | Ipsi. | Cont. | Ipsi. | Cont. |
| Forskolin-stimulated activity, pmol/min/mg protein | 808.8 ± 66.2 | 625.3 ± 91.3 | 859.0 ± 94.9 | 769.7 ± 99.2 |
| Maximal inhibition by Carbachol, % | 19.9 ± 3.3 | 20.3 ± 3.5 | 23.9 ± 3.3 | 26.1 ± 5.3 |
| $IC_{50}$ for Carbachol, μM | 0.66 ± 0.05 | 0.71 ± 0.06 | 0.72 ± 0.04 | 0.78 ± 0.07 |

Results are mean ± S.E.M. of observations derived from 6 animals in each case.

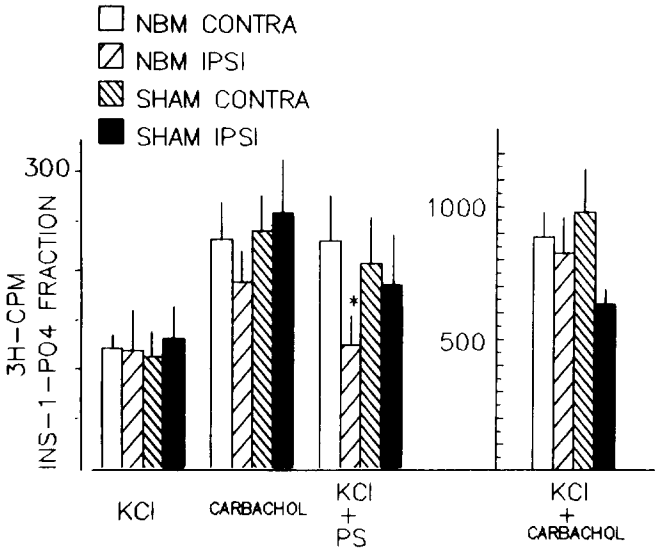


FIG. 2. Effect of NBM lesions on inositol-1-phosphate formation stimulated by 20 mM KCl, 20 mM carbachol, and 20 mM KCl + 1 mM physostigmine, in cerebral cortical slices. Slices were preincubated for 60 min, incubated for 60 min with 0.3 μM $^3$H-inositol, washed and incubated for a further 60 min with agonists as indicated and 10 mM LiCl, as described in the Method section. Results are expressed as cpm/15 μl packed slices obtained in the inositol-1-phosphate fraction after subtraction of basal values derived from corresponding incubations without addition of agonist. Basal values were: NBM contralateral, 312 ± 33 cpm, NBM ipsilateral, 339 ± 32 cpm, sham contralateral, 294 ± 37 cpm, and sham ipsilateral, 294 ± 27 cpm, and did not differ between the various groups. The values given represent mean ± S.E.M. of observations derived from 10 animals in each case, tissue from each animal being assayed in duplicate. *Significantly different ($p<0.05$) from values for NBM contralateral cortex by paired *t*-test.

Therefore, we cannot exclude the possibility of plasticity within restricted regions of cortex which passed undetected in our assays.

We measured biochemical responses 2–3 weeks after damage. Reed and de Belleroche (21) reported a transient supersensitivity, 4 days after damage, expressed in increase in carbachol-stimulated accumulation of IP after unilateral kainic acid lesions of the NBM. Their data for later time points after damage are in agreement with those reported here.

A possible reason why supersensitivity might not develop or was only transient is that due to the diffuse organization of the NBM (12,17) the damage was partial; spared NBM-cortex input may have precluded development of denervation supersensitivity.

Neurochemical plasticity might develop in noncholinergic systems. We made electrolytic lesions which have been shown to reduce dopamine and norepinephrine in ipsilateral cortex (6). Cytotoxic damage was also shown to result in long-term increase in 5-HT$_2$ receptors (27), i.e., a noncholinergic system.

Our results suggest that changes in muscarinic receptor numbers after NBM damage do not affect cortical intrinsic processing of cholinergic messages. As pointed out by McKinney and Coyle (14), the spared intrinsic cortical ACh neurons may be sufficient to compensate for lost NBM input. We found no changes in second messenger systems in the contralateral cortex of NBM-damaged rats compared to the contralateral cortex in sham-operated rats. Our findings suggest that the data of Pedata *et al.* (19), showing increased choline acetyltransferase and high-affinity choline uptake in the cortex contralateral to the damage, do not involve the second messenger amplification systems.

The loss of cognitive function after NBM lesion can be attributed at least partially to loss of presynaptic cholinergic input since receptor and postreceptor signal amplification mechanisms appear to be intact. These results may have important clinical implications since in disorders such as Alzheimer's disease, at least in the early stages, receptor and postreceptor modification of the postsynaptic cholinergic cortical cells should be effective in compensating for loss of cholinergic input. Both the PI and cyclic AMP-linked second messenger cholinergic systems located in cortical postsynaptic cells are apparently undamaged in NBM-lesioned rats. Therapeutic interventions, therefore, might profitably be directed at these receptor and postreceptor sites.

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