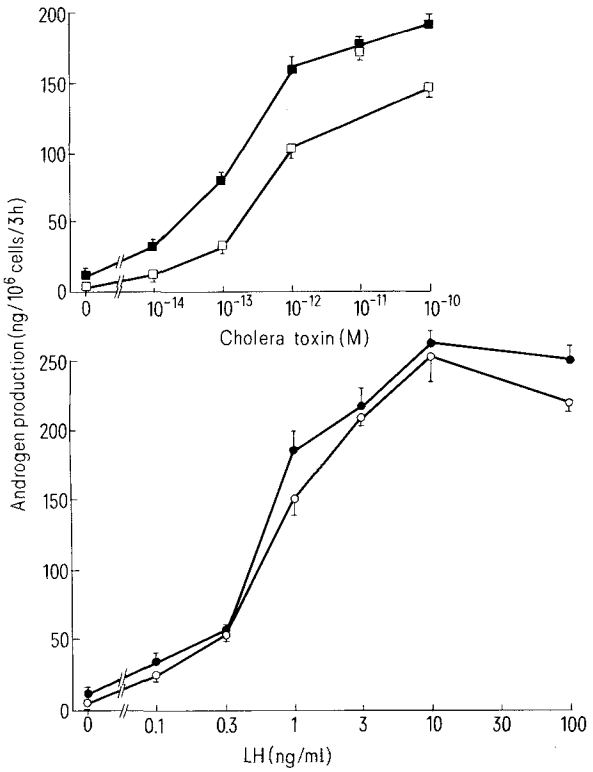
Results and discussion. Androgen production was increased 7.7 and 9.2 times basal unstimulated production by treatment with submaximum concentrations of cholera toxin and LH, respectively, in the absence of inhibitors (table). 3-Aminobenzamide reduced by 60% cholera toxin's ability to stimulate androgen production but did not affect basal production or that stimulated by LH. The non-inhibitory analog 3-aminobenzoic acid had no effect on androgen production.



LH (lower panel) or cholera toxin (upper panel) stimulated androgen production in the presence (open symbols) or absence (closed symbols) of 5 mM 3-aminobenzamide.

The failure of 3-aminobenzamide to inhibit LH-stimulated-androgen production was not due to the LH concentration employed. With the exception of a small (12%), but significant, inhibition at 100 ng LH/ml, 3-aminobenzamide was without effect over the entire range of LH concentrations capable of augmenting androgen production (fig.).

3-Aminobenzamide at concentrations of 1–10 mM has been used by others[11–13] to investigate the role of poly (ADP-ribosyl) transferase in the control of cellular differentiation. In this present study 3-aminobenzamide (5 mM) inhibited cholera toxin's ability to stimulate androgen production, presumably by inhibiting the mono (ADP-ribosyl) transferase activity of the toxin's A subunit. The failure of 3-aminobenzamide to inhibit LH-stimulated steroidogenesis is therefore reasonable evidence that ADP-ribosylation is not part of (or at least not an obligatory part of) the mechanism by which LH stimulates steroidogenesis.

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Cholinomimetics produce seizures and brain damage in rats

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Summary. Microinjections of the cholinergic agonists, carbachol and bethanechol, either into the amygdala or into the dorsal hippocampus produced sustained limbic seizures and brain damage in rats. Systemic administration of pilocarpine in rats resulted in a sequence of convulsive disorders and widespread brain damage as well. Scopolamine prevented the development of convulsive activity and brain damage produced by cholinomimetics. These results suggest that the excessive stimulation of cholinergic muscarinic receptors can lead to limbic seizures and brain damage. It is postulated that muscarinic cholinergic mechanisms are linked to the etiology of temporal lobe epilepsy and epileptic brain damage.

The hippocampus receives via the fornix/fimbria major input from the medial septum and vertical limb of the diagonal band[3]; acetylcholine (ACh) is considered to be the neurotransmitter of this pathway[3,4]. The axons of the cell bodies of cholinergic neurons located in the medial septum and nucleus of the diagonal band enter the hippocampal formation at the level of the subfield CA 3[5], where they divide and innervate the subfields CA 1, CA 2 and CA 4[6]. A massive cholinergic innervation of the amygdala originates in the substantia innominata and the horizontal limb of the diagonal band[3]. A number of electrophysiological studies have provided evidence that the bursts elicited by ACh in

hippocampal pyramidal cells[7] were similar to those seen during hippocampal epileptogenesis[8]. Furthermore, ACh may tonically enhance the excitability of hippocampal[7] and cerebral cortical neurons[9], which might account for an increase in the effectiveness of other excitatory inputs and facilitate the development of epileptogenesis[10]. Thus, there is good anatomical and electrophysiological evidence that the excessive stimulation of muscarinic cholinergic receptors located either in the amygdala or in the hippocampus, and consequent activation of excitatory limbic circuits, can easily lead to status epilepticus, similar to that previously described after intraamygdaloid[11] and intrahippocampal[12] kainic acid. Accordingly, it is reasonable to assume that stimulation of muscarinic cholinergic receptors either in the amygdala or in the hippocampus may lead to seizure-related brain damage. In order to verify this assumption, cholinergic agonists, carbachol and bethanechol, have been injected into the amygdala and into the dorsal hippocampus of adult rats.

The phenomenon of cholinergically-induced cytotoxicity has been previously demonstrated in mammalian muscles[13], where inactivation of acetylcholinesterase with bath application of diisopropylfluorophosphate, or bath application of carbachol to isolated nerve-muscle preparations, produced extensive degeneration of the muscle cells. The observation on the cytotoxicity of ACh analogs in mammalian skeletal muscles reinforces the inference that ACh is involved in certain neurodegenerative processes in the mammalian brain[14]. Therefore, we have tested whether pilocarpine, a potent cholinergic agonist, is capable of inducing seizures and brain damage after systemic administration in adult rats.

Methods. For injections into the amygdala or into the hippocampus, adult male Wistar rats weighing 220–240 g were stereotaxically implanted with permanent guide cannulae under pentobarbital anesthesia (50 mg/kg i.p.). Microinjections were performed 4–5 days after surgery. Bethanechol chloride and carbachol chloride (Sigma, St. Louis, MO, USA) were dissolved in physiological saline immediately before use, and the pH adjusted to 7.35 by adding 0.01 N NaOH. They were administered in a volume of 0.5–2.0 µl into the amygdala (AP + 4.6, L + 4.0, V − 3.6)[15] or into the hippocampus (AP + 4.0, L + 2.6, V + 1.7)[15] in unanesthetized rats. Bethanechol was administered into the amygdala in doses of 50 and 100 µg, but into the hippocampus in doses of 50, 100 and 200 µg. Carbachol was injected into the amygdala in doses of 10 and 20 µg, but into the hippocampus in doses of 25 and 50 µg. Intracerebral injections were made over 5 min. Pilocarpine hydrochloride (Sigma, St. Louis, MO, USA) was freshly dissolved in saline and administered i.p. in doses of 300 and 400 mg/kg. Methyl scopolamine nitrate (Sigma, St. Louis, MO, USA) was injected s.c. in the dose of 1 mg/kg 30 min prior to all dosages of pilocarpine in order to minimize the peripheral cholinergic effects[16]. Scopolamine hydrochloride (Sigma, St. Louis, MO, USA) was dissolved in saline and administered s.c. in the dose of 20 mg/kg 30 min prior to 400 mg/kg pilocarpine or co-administered in the dose of 10 µg with either carbachol or bethanechol into the amygdala or into the hippocampus.

For determination of behavioral responses each rat was individually placed in a Plexiglas compartment (40 × 25 × 17 cm). Behavioral alterations were noted for periods ranging from 2 to 6 h. For histological examination by light microscopy brains were processed 24–27 h after intracerebral microinjections of cholinomimetics or systemic administration of pilocarpine. By this time, neurotoxic manifestations are normally evident in histological sections[17]. The rats were deeply anesthetized and intracardially perfused with saline followed by a 10% formalin solution. The brains were removed, stored in formalin, embedded in paraffin and sectioned coronally at 10 µm. Every 10th section was preserved and stained with cresyl violet.

Results. Behavioral alterations produced by cholinomimetics applied intracerebrally were dose- and time dependent. At 50 µg (N = 7) and 100 µg (N = 4) of bethanechol, and 25 µg of carbachol (N = 4), immediately after injection into the hippocampus, animals were hyperactive and showed prominent wet dog shakes (WDS). This period preceded the second phase in which olfactory and gustatory automatisms predominated. This activity persisted for up to 60–90 min after injection into the hippocampus. The sequence of behavioral alterations after 200 µg bethanechol (N = 5) and 50 µg carbachol (N = 7), when administered into the hippocampus was qualitatively similar, although at these doses WDS and limbic automatisms were less developed and seizure activity more severe. Convulsive behavior included 'motor limbic seizures' with intense salivation, rearing, upper extremity clonus and falling, which commenced after 30–60 min and lasted for 90–180 min. Co-administration of 10 µg scopolamine with either 200 µg bethanechol (N = 3) or 50 µg carbachol (N = 3) prevented the development of limbic seizure activity.

Immediately after injections of bethanechol in doses of 50 µg (N = 5) and 100 µg (N = 6) and carbachol in doses of 10 µg (N = 5) and 20 µg (N = 6) into the amygdala animals were motionless, and crouched on all four limbs. In the second phase, prominent WDS and gustatory automatisms predominated. This activity persisted up to 15–30 min after injection and evolved into the motor limbic seizures and limbic status epilepticus. Convulsive behavior observed after injections of either 100 µg bethanechol or 20 µg carbachol into the amygdala was prevented by co-administration of scopolamine in the dose of 10 µg (N = 6). At doses of 300 mg/kg (N = 8) and 400 mg/kg (N = 8) pilocarpine produced a sequence of behavioral alterations including staring spells, olfactory and gustatory automatisms, and motor limbic seizures that developed over 30–90 min and progressively evolved into the limbic status epilepticus. Pretreatment of animals with scopolamine in the dose of 20 mg/kg s.c. (N = 5) prevented the development of convulsive activity caused by 400 mg/kg pilocarpine.

Histological examination of frontal forebrain sections revealed widespread brain damage after both intracerebral and systemic administrations of cholinomimetics. Neuropathological alterations were found within the olfactory cortex, amygdaloid complex, thalamus, hippocampal formation and neocortex. Injections of bethanechol and carbachol, in all dosages tested, resulted in moderate local destruction in both the hippocampus and the amygdala. The tissue reaction at the injection site ranged from mild to non-existent and was apparently present in the region immediately surrounding the injection cannula tip (fig. 1, C). The intensity and extent of neuropathological alterations was less severe in rats treated intrahippocampally with both bethanechol and carbachol. However, regardless of variance in the location and intensity of distant damage abnormal neurons were seen in the pyriform cortex (fig. 1, B), entorhinal cortex, anterior olfactory nucleus, amygdaloid cortical nucleus (fig. 1, D) and neocortex (fig. 1, E). Degenerating neurons were found scattered throughout the affected areas, some of these neurons appeared dark and shrunken, while others appeared swollen and disrupted. In rats injected intrahippocampally with even the highest doses of cholinomimetics the neuropil of affected areas appeared relatively unaffected.

After injection of cholinomimetics into the amygdaloid complex neuropathological alterations were observed in pyriform cortex, entorhinal cortex, CA 1 and CA 3 subfields of the dorsal hippocampus, dorsomedial thalamic

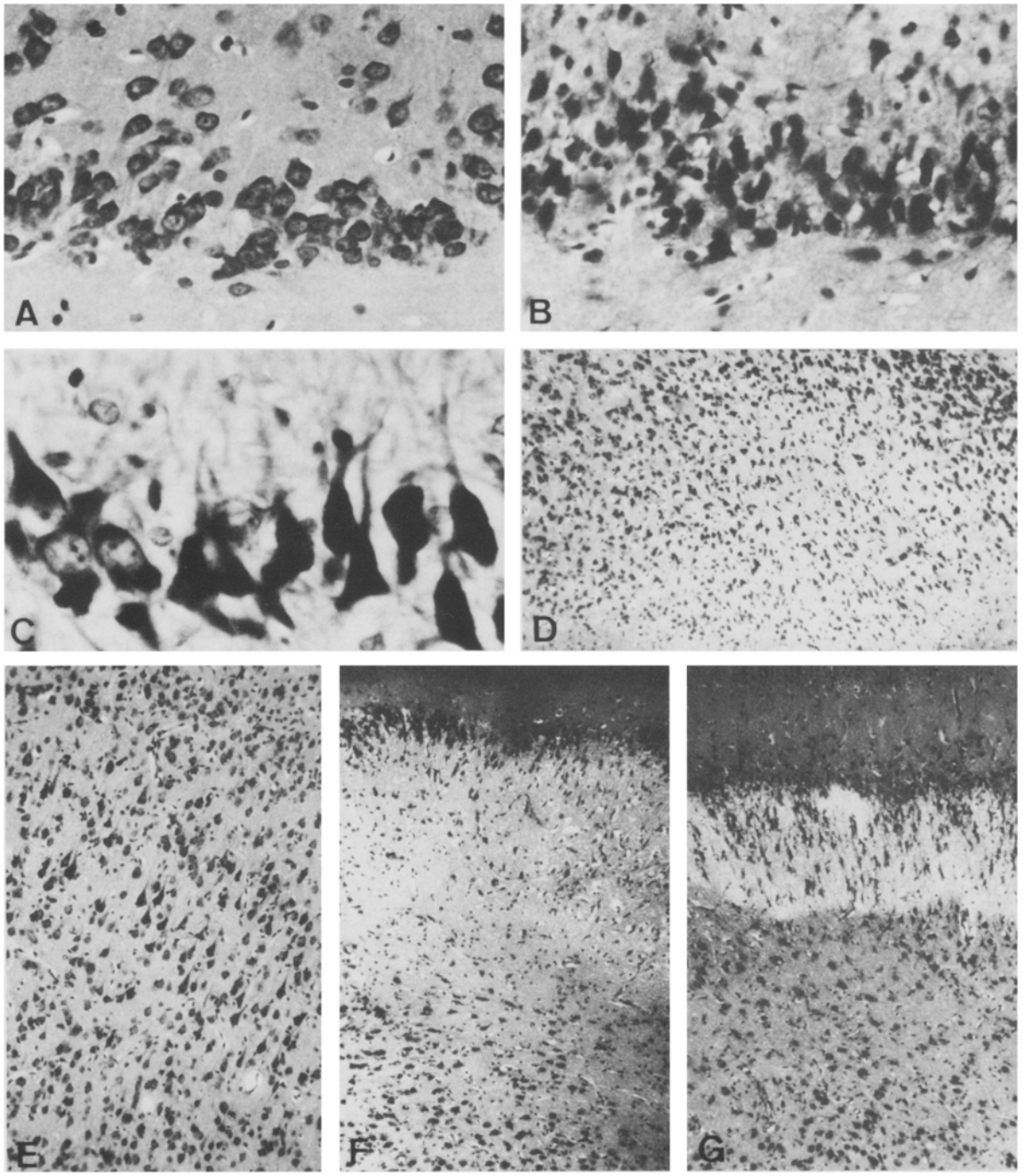

Figure 1. Neuropathological alterations in the rat brain produced by cholinomimetics. Cresyl violet stain. Survival time: 24 h. *A* Photomicrograph of a portion of the posterior pyriform cortex of the rat injected into the ipsilateral hippocampus with 0.5 µl saline. × 196. *B* Representative section illustrating the destruction of the posterior pyriform cortex of the rat injected into the hippocampus with 50 µg carbachol. Shrunken and darkened neuronal somata appear throughout all layers of the pyriform cortex. × 196. *C* Photomicrograph of CA 3a subfield demonstrating the type of tissue reaction observed within the hippocampus of the rat injected with 50 µg bethanechol. Pyramidal cells stain darkly and appear shrunken compared to the normal-appearing cells nearby. × 272. *D* Cortical amygdaloid nucleus of the rat injected into the hippocampus with 50 µg carbachol. Note the nearly total disruption of the cytoarchitecture of the cortical amygdaloid nucleus. Neurons stain darkly and appear shrunken. × 40. *E* Parietal cortex in the rat injected into the amygdala with 100 µg bethanechol. Shrunken and darkened pyramidal neurons are present in the layer V. Darkened and shrunken neurons are widely interspersed among normal-appearing cells. × 25. *F* Photomicrograph illustrating the destruction of the posterior pyriform cortex of the rat injected into the amygdala with 20 µg carbachol. Shrunken and darkened neuronal somata appear throughout all layers of the pyriform cortex. Note extensive edema and disruption of the neuropil in the layers II and III. × 40. *G* Posterior pyriform cortex in the rat injected systemically with 400 mg/kg pilocarpine. Almost complete or nearly complete destruction of the neuronal population within the pyriform cortex is apparent. The cells become dark and severely shrunken. Extensive edema and disruption of the neuropil in the layers II and III are prominent. × 40.

nucleus and neocortex. The intensity of convulsive disorders and the brain damage observed after intraamygdaloid bethanechol and carbachol were more pronounced than those produced by comparable doses of intrahippocampal cholinomimetics. At 20 µg carbachol and 100 µg bethanechol injected into the amygdala dropout of neurons, edema with disruption of the surrounding neuropil was encountered in the pyriform cortex (fig. 1,F). The technique used for the microinjections themselves or microinjections of saline into the hippocampus or amygdala caused no local or distant brain damage (fig. 1,A) except for mild trauma along the cannula track.

Extensive histological changes were produced by i.p. injections of pilocarpine as well. Both doses induced widespread degeneration of neurons and disruption of the surrounding neuropil (fig. 2). Neuropathological alterations were found in the pyriform cortex (fig. 1,G), entorhinal cortex, claustrum, amygdala, mediodorsal, dorsal lateral and ventromedial thalamic nuclei, anterior olfactory nuclei, hippocampal CA 1, CA 3 and CA 4 subfields, substantia nigra and neocortex. Temporal cortex dorsal to the rhinal sulcus and cingulate cortex was particularly sensitive to pilocarpine. Somatomotor, retrosplenial and visual cortices were likewise frequently affected. Scopolamine co-administered intrahippocampally or into the amygdaloid complex prevented the development of brain damage produced by both carbachol and bethanechol. Pre-treatment with scopolamine prevented the evolution of widespread brain damage caused by pilocarpine as well.

Discussion. Microinjections of cholinomimetics into the rat amygdala and into the hippocampus, and systemic administration of pilocarpine in rats produce a sequence of convulsive disorders accompanied by widespread brain damage. These findings are consistent with evidence indicating that repeated application of carbachol into the rat amygdala, caudate nucleus and hippocampus may result in kindled seizures[18]. The distribution of brain damage produced by either intrahippocampal, intraamygdaloid or systemic injections of cholinomimetics reflects well the areas within the projection fields of the basal forebrain cholinergic system, i.e. medial septum, nuclei of diagonal band and substantia innominata[3]. This observation demonstrates the potential of the cholinergic pathways to participate in the production of the brain damage in both experimental and human epilepsy. The moderate damage in the amygdala and hippocampus observed at the injection site of high doses of carbachol and bethanechol probably occurs by seizure-related mechanism since both hippocampal and amygdaloid neurons constitute a part of the limbic circuitry in which seizure activity is propagated. A plausible explanation for the local damage after intraamygdaloid and intrahippocampal cholinomimetics seems to be the restricted self-destruction of brain, a mechanism terminating protracted focal status epilepticus[19].

Calcium dependence of cell death has been implicated in ischemic[20], hypoglycemic[21] and epileptic[22] brain damage. Muscarinic cholinergic excitation in mammalian CNS occurs as a result of blockade of K^+ conductance and is mediated by voltage-dependent Ca^{++} and Na^+ conductances[7]. Calcium entry is primarily responsible for the prolonged depolarizations associated with ACh excitation[7]. The stimulation of glutamate receptors gates the influx of Ca^{++} and Na^+ causing an ionic imbalance which may result in cellular damage[23]. Thus, one may infer that cholinomimetics might display a direct neurotoxic action on CNS neurons as well. Finally, a potent convulsant and brain damaging action of cholinomimetics in rats may indicate a valid role for cholinergic mechanisms in the pathogenesis of epileptic brain damage in man. Moreover, it should be emphasized that cholinergic agents, which have received extensive application in human medicine and agriculture may be dangerous for man and therefore these compounds should be used with care.

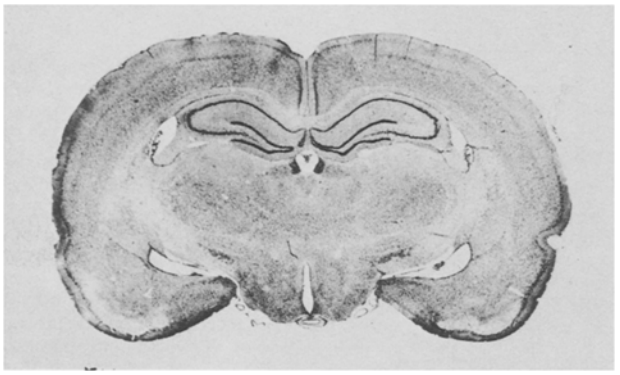

Figure 2. Low-power photomicrograph demonstrating neuropathological alterations in the rat brain produced by 400 mg/kg pilocarpine. Note the nearly total disruption of the pyriform cortex and parts of amygdala on both sides. Prominent neuronal degeneration is apparent bilaterally in dorsolateral, mediodorsal and reuniens thalamic nuclei, while ventral thalamic nuclei are only marginally affected. Note marginal alterations in the CA 4 subfield of the dorsal hippocampus. ×8. Cresyl violet stain. Survival time: 24 h.

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