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LIMBIC SEIZURES PRODUCED BY PILOCARPINE IN RATS: BEHAVIOURAL, ELECTROENCEPHALOGRAPHIC AND NEUROPATHOLOGICAL STUDY

WALDEMAR A. TURSKI[1], ESPER A. CAVALHEIRO[2], MICHAEL SCHWARZ[1,*], STANISŁAW J. CZUCZWAR[1], ZDZISŁAW KLEINROK[1] and LECHOSŁAW TURSKI[1,*]

[1]*Department of Pharmacology, Institute of Clinical Pathology, Medical School, Jaczewskiego 8, PL 20-090 Lublin (Poland) and* [2]*Departamento Fisiologia, Escola Paulista de Medicina, R. Botucatu, 862-2° Andar, 04023 Sao Paulo, SP (Brazil)*

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SUMMARY

Behavioural, electroencephalographic and neuropathological responses to increasing doses of pilocarpine (100–400 mg/kg) administered intraperitoneally to rats were studied. At the dose of 400 mg/kg pilocarpine produced a sequence of behavioural alterations including staring spells, olfactory and gustatory automatisms and motor limbic seizures that developed over 1–2 h and built up progressively into limbic status epilepticus. Smaller doses showed different threshold for these behavioural phenomena but a similar time course of development.

The earliest electrographic alterations occurred in the hippocampus and then epileptiform activity propagated to amygdala and cortex. Subsequently electrographic seizures appeared in both limbic and cortical leads. The ictal periods recurred each 5–15 min and were followed by variable periods of depression of the electrographic activity. The sequence of electrographic changes correlated well with the development of behavioural phenomena.

Histological examination of frontal forebrain sections revealed disseminated, apparently seizure-mediated pattern of brain damage. Neuropathological alterations were observed in the olfactory cortex, amygdaloid complex, thalamus,

* Present address: Department of Biochemical Pharmacology, Max-Planck Institute for Experimental Medicine, Hermann-Rein Str. 3, D-3400 Göttingen, F.R.G.

neocortex, hippocampal formation and substantia nigra. Pretreatment of animals with scopolamine (20 mg/kg) and diazepam (10 mg/kg) prevented the development of convulsive activity and brain damage.

These results show that systemic pilocarpine in rats selectively elaborates epileptiform activity in the limbic structures accompanied by motor limbic seizures, limbic status epilepticus and widespread brain damage. It is suggested that a causative relationship between excessive stimulation of cholinergic receptors in the brain and epileptic brain damage may exist.

INTRODUCTION

The functional role of acetylcholine (ACh) in the central nervous system is of considerable neurobiological interest. ACh, acetylcholinesterase inhibitors and ACh analogues are effective epileptogenic agents when applied intracerebrally [9, 10, 24, 36, 38, 40, 41, 42] or systemically [18, 39]. Intraamygdaloid [24, 38] and intrahippocampal [9, 36, 38] injections of muscarinic cholinergic agonists in rats produce electrographic and behavioural occurrence of limbic seizures [36] accompanied by widespread brain damage topographically resembling that produced by kainic acid [5, 6, 32] and folates [26], and frequently observed in autopsied brains of human epileptics [11]. These results raise the possibility that the disturbed function of cholinergic system in the CNS may elaborate epileptiform activity in the limbic structures. Therefore, we have tried whether pilocarpine, a potent cholinergic agonist originally isolated from the leaflets of South American shrubs and until recently commonly used in the treatment of acute glaucoma in man [35], is capable of inducing convulsive disorders and seizure-related brain damage after systemic administration in adult rats.

METHODS

Animals

Adult male Wistar rats, weighing 200–220 g were used. For at least 1 week prior to experiments animals were housed in groups of 2–5 on a standard light–dark cycle (light on 06.00–18.00 h) with rat chow pellets and tap water continuously available. The colony room had a temperature of 20–22 °C and humidity of 45–55 %. Rats were assigned to experimental groups by means of a completely randomized method. To determine the subsequent time of behavioural and electrographic testing for different treatment groups, a completely randomized schedule was likewise employed. Experimental groups consisted of 5–10 animals. All behavioural assessments took place between 08.30 and 16.00 h. For determination of behavioural changes each rat was individually placed in a Plexiglas compartment (40 × 25 × 17 cm). Prior to administration of the drug solutions

each animal was habituated for 20–30 min. After the habituation animals were removed, injected s.c. and i.p. with the appropriate drug and rapidly returned to the experimental cage. A total of 51 rats was studied with different doses of pilocarpine. Twenty-two animals were used in determining occurrence and time–course of behavioural alterations after pilocarpine. Eleven animals were used in determining scopolamine- and diazepam-reversibility of pilocarpine effects. Eighteen rats were used for EEG recordings. Fourteen additional rats served as controls.

Surgery and EEG recording procedure

For EEG recordings, bipolar twisted wire electrodes (tip diameter 100 μm, interelectrode distance 500 μm) were positioned stereotaxically under pentobarbital anesthesia (50 mg/kg i.p.) and fixed to the skull with dental acrylic cement. Depth recording sites included the dorsal hippocampus (CA1 subfield) (A 3.2, L 2.6, H 3.4) and amygdala (A 6.0, L 4.5, H 8.5) [1]. Surface recordings were made from jeweler screws placed bilaterally over the sensorimotor cortex. Signals under investigation were amplified by a standard Van Gogh EEG apparatus (time constant 0.03 sec, high cutt off filter 15). EEG recordings were performed 5–7 days after surgery. Recordings were carried out in a Plexiglas observational compartment (30 × 30 × 45). Before EEG recording session the animals were individually placed in the recording compartment, connected to the recording plug, and given 20–30 min for habituation to the experimental setup. Following the period of habituation, baseline EEG recordings were made for at least 15–30 min. EEG recordings were made continuously and behaviour noted for periods ranging from 5–7 h following pilocarpine injection. Additional recordings were made between 24–27 h following injection. The correct position of the implanted deep electrodes was histologically determined in cresyl violet stained serial sections.

Drugs

Pilocarpine hydrochloride (Sigma, St. Louis, MO, U.S.A.) was freshly dissolved in saline and administered intraperitoneally in doses of 100, 200 and 400 mg/kg. Methyl scopolamine nitrate (Sigma), was injected subcutaneously in the dose of 1 mg/kg 30 min prior to all dosages of pilocarpine in order to minimize the peripheral cholinergic effects [2]. Scopolamine hydrochloride (Sigma) was dissolved in saline and administered s.c. in the dose of 20 mg/kg. Diazepam (Polfa, Poznań, Poland) was suspended in a 3% solution of Tween 81 (Loba Chemie, Wien, Austria) and given i.p. in the dose of 10 mg/kg. Scopolamine and diazepam were administered 30 min prior to injection of 400 mg/kg pilocarpine.

Histology

For histological examination by light microscopy brains were processed 24–27 h after administration of pilocarpine. By this time, neurotoxic manifes-

tations are normally evident in histological sections [26, 31]. 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. Extensive histological changes after i.p. pilocarpine were identified by the presence of widespread degeneration of neurons and an extensive disruption of the surrounding neuropil. Degenerating neurons were found scattered throughout the affected areas, some of these degenerating cells appeared dark and shrunken, while others appeared swollen and disrupted. Moderate cellular degeneration was identified by the presence of the majority of shrunken and dark staining cells, while surrounding neuropil appeared relatively unaffected. In areas less severely damaged, cellular degeneration was largely restricted, neurons were shrunken and pyknotic compared with control tissue and nucleus often displaced to the periphery. Consequently, 3 types of histological changes were noted, a *mild form* with a dropout of neurons and gliosis, *severe form* where entire nuclear groups or areas underwent necrosis, and *moderate form* where intensity of changes was restrained and less severe. Fig. 6C shows an example of mild changes within the pyramidal layer of the CA3 subfield of the dorsal hippocampus. Fig. 7B illustrates an example of severe changes with extensive neuronal degeneration, edema and disruption of the neuropil in the posterior pyriform cortex. Fig. 6F illustrates moderate changes in the lateral thalamic nucleus. Mild, moderate and severe changes could occur in the same animal.

RESULTS

Behaviour

Behavioural alterations produced by i.p. pilocarpine were dose- and time-dependent. At 100 and 200 mg/kg of pilocarpine, immediately after injection animals were motionless, they crouched on all limbs and their ears cocked back. This period preceded the second phase in which olfactory and gustatory automatisms predominated. This phase started 10–15 min after injection of 100 and 200 mg/kg of pilocarpine and 5–10 min after 400 mg/kg. Gustatory and olfactory automatisms included mouth movements with prominent chewing and teeth chattering, which were frequently associated with mild salivation, eye blinking, vibrissae twitching and yawning. This activity persisted up to 60–90 min after 100 mg/kg of pilocarpine, and lasted up to 2 h after 200 mg/kg. At the high dose of pilocarpine (400 mg/kg) these motor activities persisted only 18–45 min after injection and developed into motor limbic seizures with intense salivation, rearing, upper extremity clonus and falling. Motor limbic seizures commenced after 30 min (mean 30.3 ± 10.5 min, n = 10), recurred every 5–15 min and reached maximal frequency by 13/1–2 h. This type of convulsive behaviour lasted up to 90–150 min after

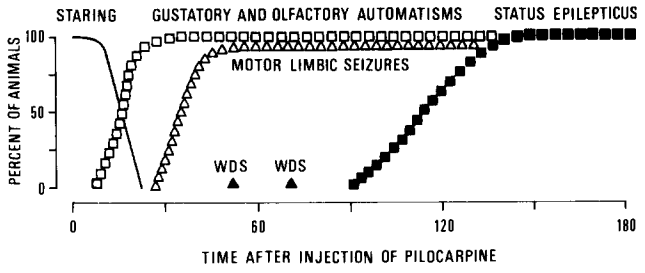

Fig. 1. Time-dependence of behavioural alterations produced in rats by intraperitoneal injection of pilocarpine in the dose of 400 mg/kg. Abscissa: time (min) after injection of pilocarpine. Ordinate: the percent of animals (n = 10) showing a particular behaviour. WDS, wet dog shakes.

injection. At this time (mean 107.7 ± 21.2 min, n = 10) limbic status epilepticus was reached. Wet dog shakes were infrequently observed in the phase of motor limbic seizures. Wet dog shakes appeared only after episodes of motor limbic seizures, and never preceded them. The time-dependence of behavioural responses after i.p. pilocarpine at the dose of 400 mg/kg is shown in Fig. 1. The dose-dependence of behavioural responses after pilocarpine is illustrated in Table I. Pretreatment with 20 mg/kg of scopolamine (n = 6) and 10 mg/kg of diazepam (n = 5) prevented the development of behavioural alterations produced by 400 mg/kg of pilocarpine.

Electroencephalography

Electrographic alterations induced by i.p. injections of pilocarpine were dose- and time-dependent. Pilocarpine produced both ictal and interictal epileptiform activity in the EEG, which correlated well with the sequence of behavioural alterations. Immediately (2–3 min) after injection of pilocarpine in doses of 100, 200 and 400 mg/kg the normal background activity was replaced with low voltage fast activity in the cortex and amygdala (Fig. 2B), while significant theta rhythm (6–7 Hz) appeared in the hippocampus. By 15–20 min, high voltage fast activity superposed over the hippocampal theta rhythm and isolated high voltage spikes were initially registered exclusively in hippocampal leads without substantial changes in cortical and amygdaloid recordings (Fig. 2C, D). Animals receiving pilocarpine in doses of 100 and 200 mg/kg continued to show high voltage fast activity and spiking for at least 1–2 h. Spiking activity, which appeared at variable frequencies and was initially restricted to the hippocampus usually spread to the cortex and amygdala (Fig. 2E), but fully developed electrographic seizures did not appear with these doses. These patterns of EEG changes were often associated with behaviourally encountered staring and gustatory automatisms. By 24 h after 100 and 200 mg/kg pilocarpine EEG records were indistinguishable from the pre-drug background activity.

TABLE I

Severity of convulsive disorders in rats produced by intraperitoneally administered pilocarpine

In order to minimize peripheral cholinergic effects methyl scopolamine nitrate in the dose of 1 mg/kg was administered s.c. to all rats 30 min prior to pilocarpine. Intensity of behavioural alterations: −, no; +, mild to moderate; + +, moderate to severe; + + +, severe to intense.

Treatment i.p.	*Staring*	*Gustatory and olfactory automatisms*			*WDS*	*Episodes of motor limbic seizures*		*Limbic status epilepticus*	*Post seizure death*
		Chewing	*Teeth chattering*	*Eye blinking*		*Number*	*Onset (min)*	*Onset (min)*	
Pilocarpine (100 mg/kg)									
Rat No. 1	+	+	−	+	−	−	−	−	−
2	+	+	−	−	−	−	−	−	−
3	+	+	+	−	−	−	−	−	−
4	+	+	−	−	−	−	−	−	−
5	+	+	+	−	−	−	−	−	−
6	+	+	−	−	−	−	−	−	−
Pilocarpine (200 mg/kg)									
Rat No. 1	+	+ +	+	+	−	−	−	−	−
2	+	+ +	+	+	−	−	−	−	−
3	+	+ +	+	+ +	−	3	58	−	−
4	+	+	+	−	−	−	−	−	−
5	+	+	−	−	−	−	−	−	−
6	+	+	+	−	−	−	−	−	−
Pilocarpine (400 mg/kg)									
Rat No. 1	+ +	+ + +	+	+	+ [§]	9	37	105	−
2	+ +	+ + +	+	+	−	11	27	89	−
3	+ +	+ + +	+	+	−	7	18	86	+
4	+ +	+ +	+	+	−	6	48	120	−
5	+ +	+ +	+	+	+ [§]	8	32	96	−
6	+ +	+ + +	+	+	+ [§]	13	24	124	−
7	+ +	+ + +	+	+	−	4	16	78	+
8	+ +	+ + +	+	+	−	10	26	106	−
9	+ +	+ + +	+	+	−	8	30	128	−
10	+ +	+ + +	+	+	−	8	45	145	

[§] Wet dog shakes (WDS) were observed only after episodes of motor limbic seizures.

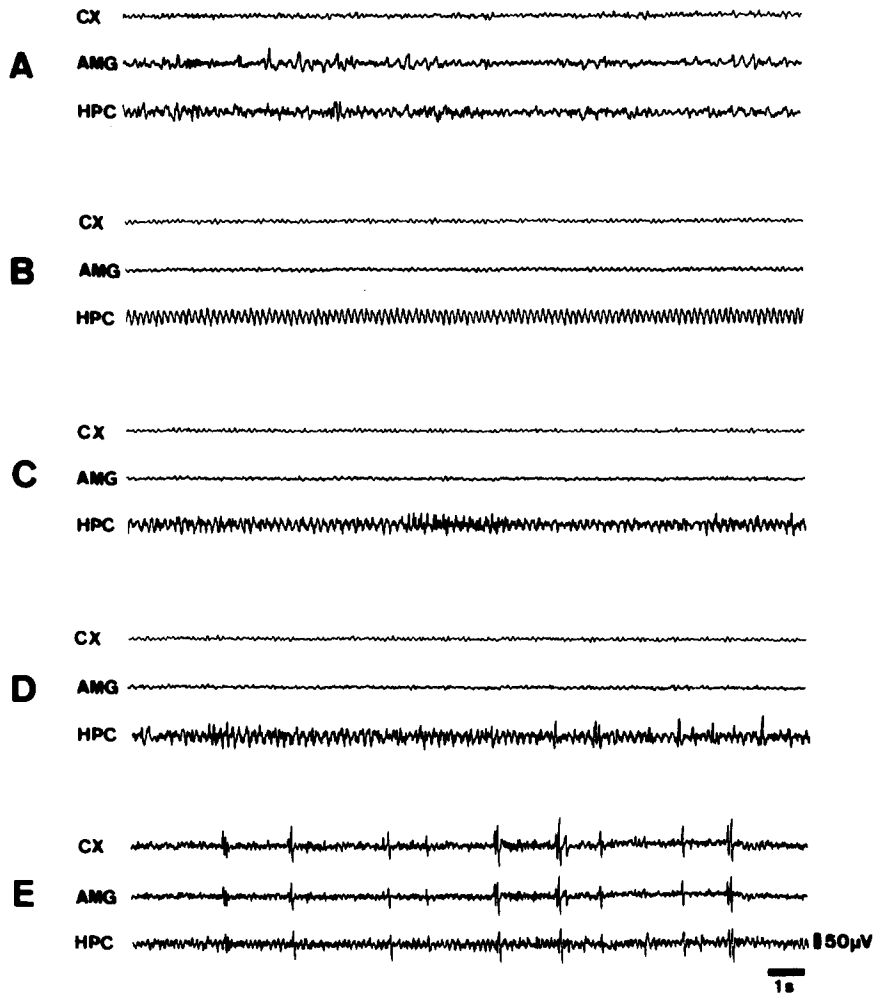

Fig. 2. Electroencephalographic recordings illustrating the sequence of alterations observed after the intraperitoneal injection of pilocarpine in rats. A: pre-injection control recordings. B: electroencephalographic correlates 5 min after injection of pilocarpine in the dose of 400 mg/kg. Note the low voltage fast activity in the amygdaloid and cortical recordings and the significant hippocampal theta rhythm. C and D: 20–30 min after injection of pilocarpine. High voltage fast activity (C) and spikes (D) superpose over the hippocampal theta rhythm. Isolated high voltage spikes are initially registered exclusively in hippocampal leads (D), while amygdaloid and cortical recordings display no substantial changes. E: high voltage spiking similar to that shown in panel D spreads to the other leads 30-50 min after pilocarpine.

Animals receiving pilocarpine in the dose of 400 mg/kg developed typical electrographic seizures, which started 40–45 min after injection. High voltage fast activity and prominent high voltage spiking preceded the development of a seizure. Characteristically, these activity changes usually originated in the hippocampus and rapidly spread to amygdala and cortex. Fully developed electrographic seizure illustrates Fig. 3A–D. The ictal periods lasted 1–2 min, recurred every 5–15 min,

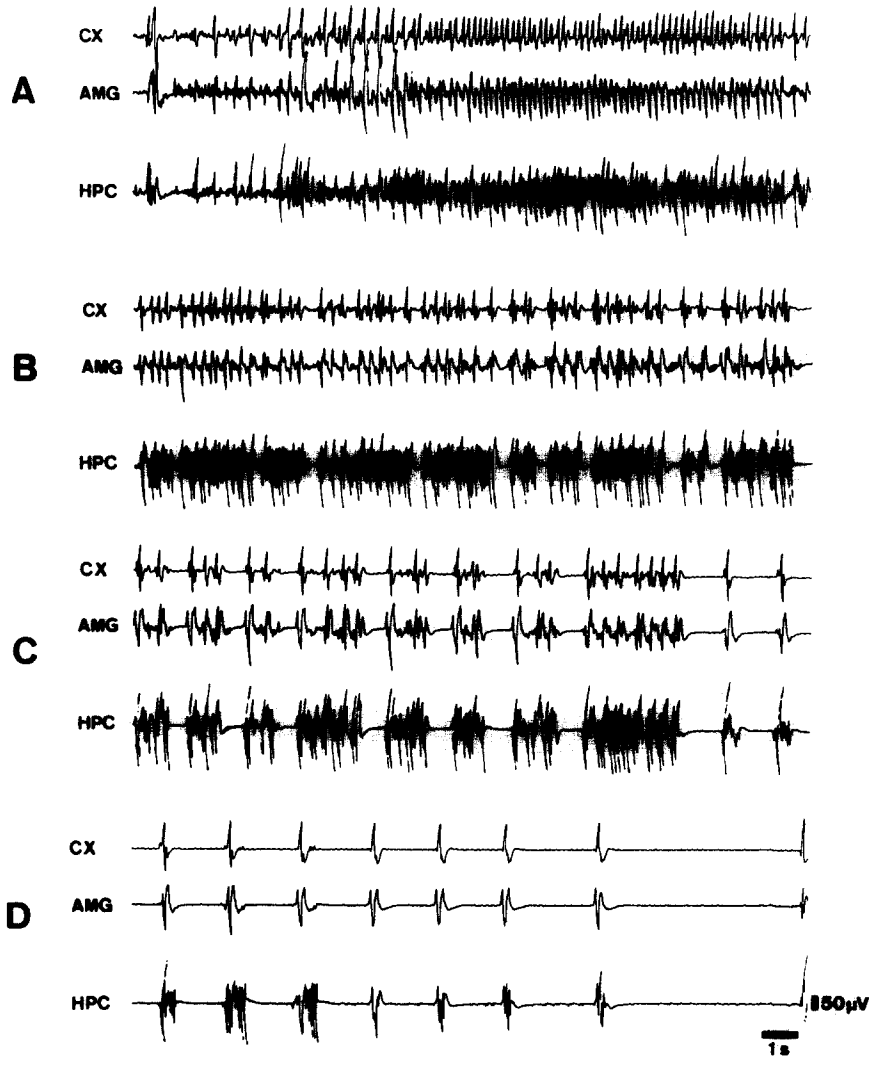

Fig. 3. Electrographic correlates of pilocarpine-induced behavioural seizures. A: electrographic seizure observed 45–50 min after injection of 400 mg/kg pilocarpine. The fast activity develops into high voltage spiking resulting rapidly in seizure activity. This activity is followed by post-ictal depression with highly synchronized slow spiking that occurs in all leads. Note the gradual evolution (A–D) of the seizure as well as the progressive increasing of electrographic depression periods (C and D).

and were followed by variable periods of depression of the electrographic activity. By 1–2 h electrographic activity progressively built up into a status epilepticus (Fig. 4). This pattern of EEG changes lasted for 5–6 h and was followed by a progressive normalization of the electrographic activity. By 24 h after 400 mg/kg pilocarpine EEG could be considered as normal, although poor theta rhythm was observed at the hippocampal level, even when the animal performed orientation or voluntary movements (walking). Pretreatment with both scopolamine

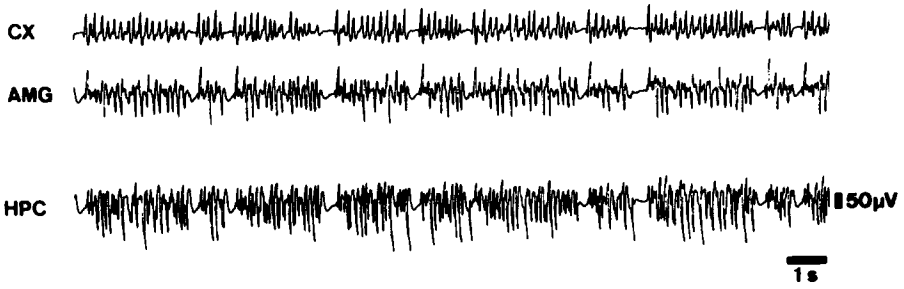

Fig. 4. Electroencephalographic recordings illustrating the electrical alterations observed 2–3 h after injection of 400 mg/kg pilocarpine during the status epilepticus.

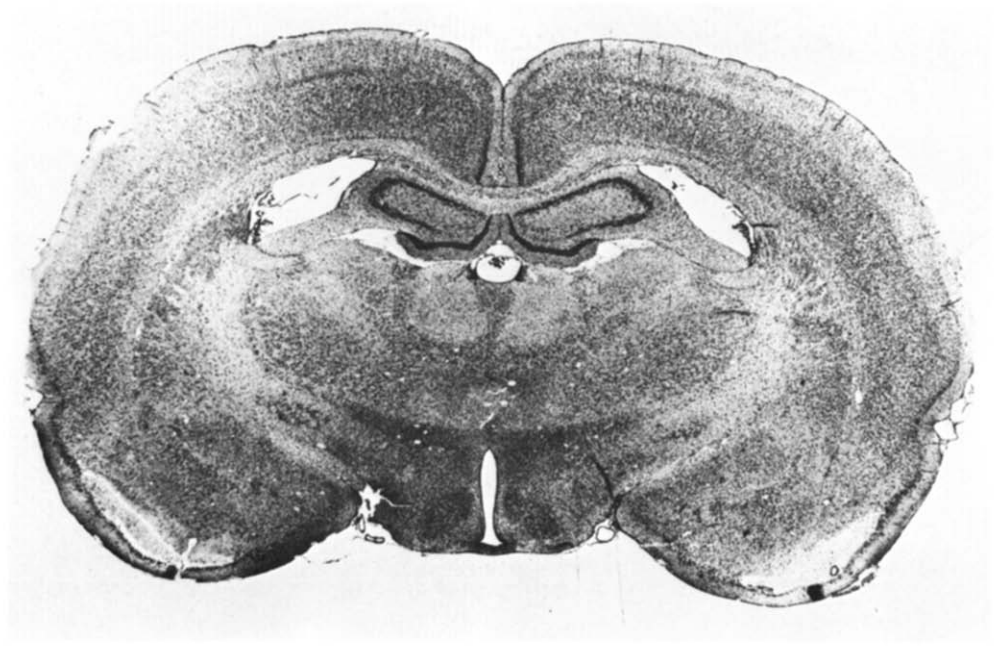

Fig. 5. Low-power photomicrograph demonstrating neuropathological alterations in the rat brain produced by 400 mg/kg pilocarpine. Note the total disruption of the pyriform cortex and parts of amygdala on both sides. Prominent cellular degeneration is apparent bilaterally in mediodorsal, dorsolateral, medioventral and reuniens thalamic nuclei, while ventral thalamic nuclei are only marginally affected. Cellular degeneration is also apparent bilaterally in the temporal cortex dorsal to the sulcus rhinalis and in parietal cortex. Note marginal alterations in the CA4 subfield of the dorsal hippocampus. × 12. Cresyl violet stain. Survival time: 24 h.

(20 mg/kg, s.c.) and diazepam (10 mg/kg, i.p.) prevented the evolution of EEG alterations produced by pilocarpine in the dose of 400 mg/kg. The EEG changes in rats pretreated with diazepam and scopolamine were due to the depressant effects of both drugs. After the injection of diazepam in the dose of 10 mg/kg the normal background activity was replaced with the low voltage fast activity, which prevailed in all leads. Injection of 400 mg/kg pilocarpine in diazepam pretreated

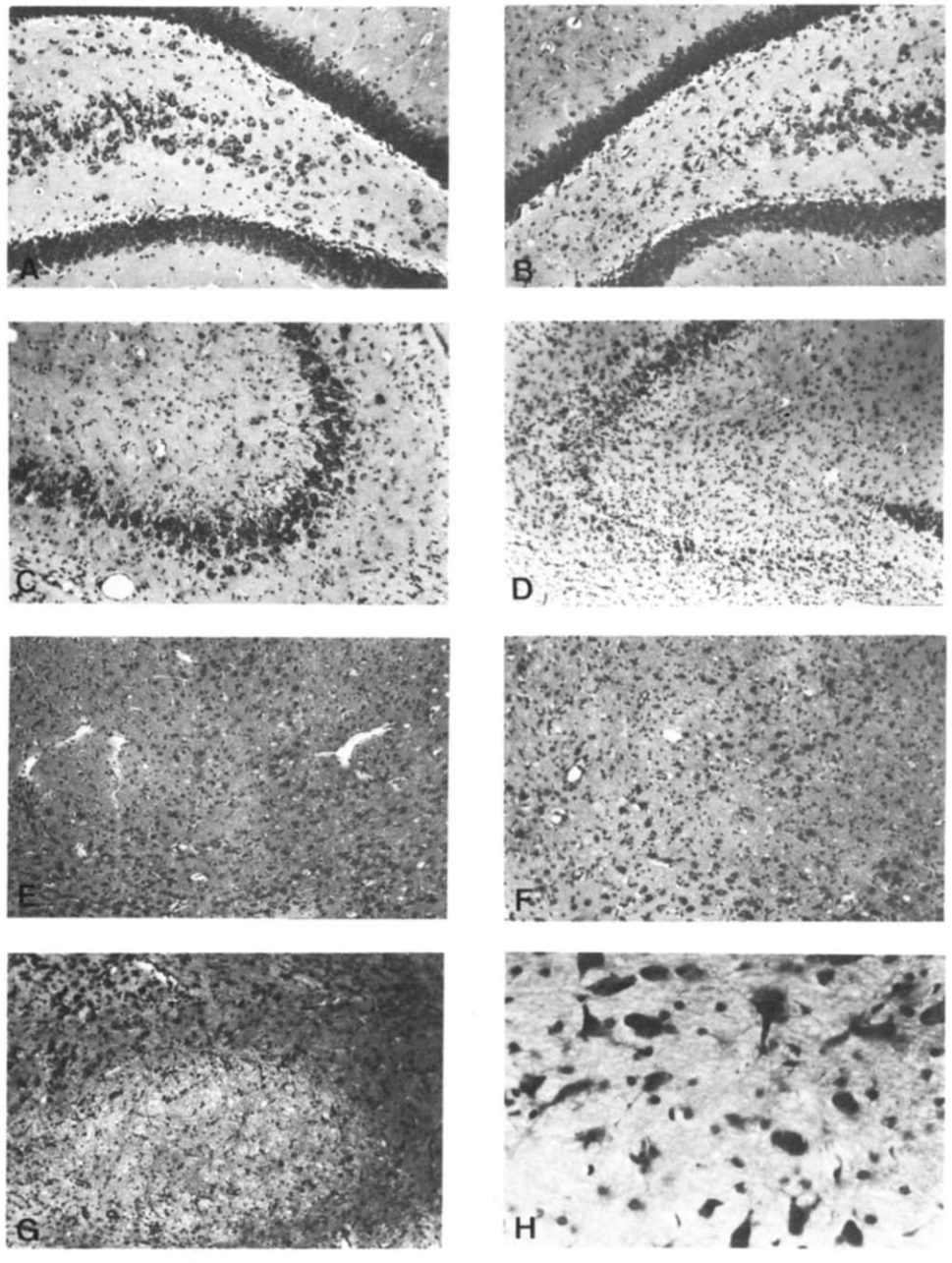

Fig. 6. Neuropathological alterations in the rat brain produced by systemic administration of pilocarpine. Cresyl violet stain. Survival time: 24 h. A: photomicrograph of dentate gyrus and hippocampal CA4 subfield of saline-treated control rat. × 31. B: representative section illustrating the destruction of the dentate gyrus after i.p. pilocarpine in the dose of 400 mg/kg. Note severely disrupted CA4 subfield with shrunken and dark staining pyramidal cells. Moderately swollen elements at granule cell-hilar border could be encountered as well. × 31. C and D: photomicrographs of CA3 hippocampal subfield demonstrating different types of tissue reaction observed within the hippocampus of rats treated i.p. with 400 mg/kg pilocarpine. C: note mild cellular degeneration within the pyramidal layer and mild glial infiltration. Shrunken and dark staining cells are visible

rats resulted in a decrease in the voltage of the EEG activity in the amygdaloid leads, and in a replacement or alternation of the regular theta activity with the low voltage fast activity in the hippocampal leads. Thus, no epileptiform activity could be recognized at any time point following the application of pilocarpine in diazepam-pretreated rats. Pretreatment of rats with 20 mg/kg scopolamine, which typically blocked hippocampal theta rhythm and resulted in the appearance of the high voltage slow activity in the amygdaloid leads, likewise completely prevented the gradual evolution of electrographic changes produced by 400 mg/kg pilocarpine.

Neuropathology

Histological examination of frontal forebrain sections revealed wide-spread brain damage in pilocarpine treated rats. Intraperitoneal injection of pilocarpine in the dose of 400 mg/kg resulted in consistent neuropathological alterations within the olfactory cortex, amygdaloid complex, thalamus, hippocampal formation and neocortex. In the olfactory system, i.e. anterior olfactory nucleus including its medial and lateral parts, pyriform cortex, periamygdaloid cortex and claustrum, extensive neuronal degeneration and complete disruption of neuropil were encountered. The pyriform cortex appeared severely swollen and edematous (Fig. 7B). The majority of cells in layers II and III [27] were dark and shrunken, and were interspersed with large vacuoles in the tissue (Fig. 7C). This pattern of damage resembles that observed after peripheral kainic acid [31] and has been described ultrastructurally as dendritic or glial swelling [25]. The anterior olfactory nucleus and the entorhinal cortex (Fig. 7D) were severely damaged as well. Severely shrunken and darkened neuronal somata with extensive disruption of surrounding neuropil were typically observed within both areas. The olfactory tubercle was relatively insensitive to pilocarpine (Table II) and neuronal degeneration has not been found in this area. Regardless of variance in the location of damage in amygdala, all nuclei within the amygdaloid complex may be damaged by systemic injection of pilocarpine (Table II, Figs. 5 and 8). The basal amygdaloid nucleus appears to be particularly sensitive to pilocarpine. Prominent edema,

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both in stratum pyramidale and oriens. Some changed cells contain narrowings which lead to fragmentation and disintegration. Dark staining and shrunken cells are interspersed among normal appearing neurons. × 31. D: extensive cell loss in the pyramidal layer of CA3 hippocampal subfield. Note extensive destruction of virtually all pyramidal neurons and infiltration of glial cells. × 31. E and F: photomicrographs illustrating destruction of the mediodorsal (E) and dorsolateral (F) thalamic nuclei. Almost complete or nearly complete destruction of the neuronal population within both nuclei is apparent. × 31. G and H: photomicrographs of the substantia nigra 24 h after i.p. injection of 400 mg/kg pilocarpine. Severe neuronal depletion accompanied by edema and disruption of the neuropil is apparent within the pars reticulata (G). × 31. Darkened and shrunken neurons appear likewise in the pars compacta (H). × 148.

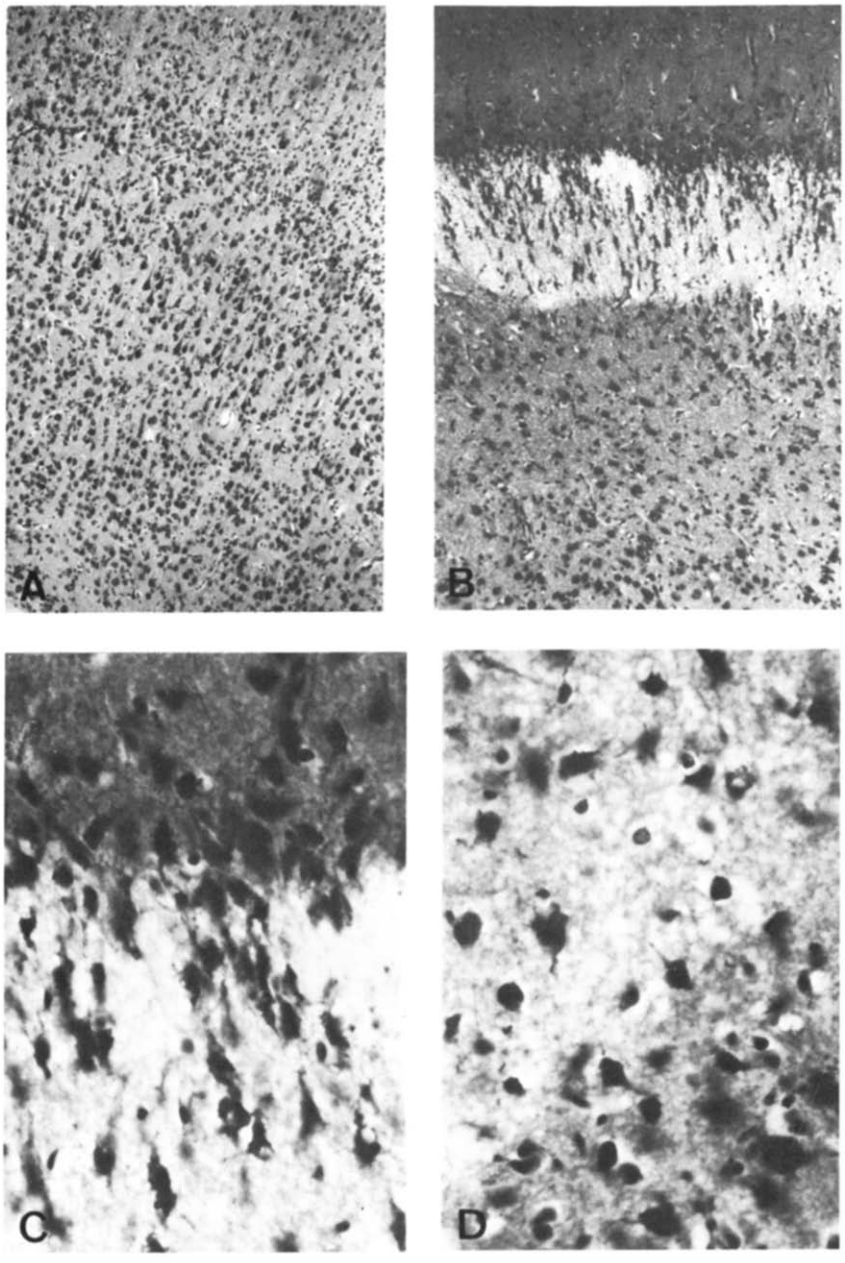

Fig. 7. Neuropathological alterations in the rat brain produced by systemic administration of pilocarpine. Cresyl violet stain. Survival time: 24 h. A: parietal cortex in the rat injected systemically with 400 mg/kg pilocarpine. Shrunken and darkened pyknotic pyramidal neurons are present in the layer V. Dark staining cells are also prominent in the layer II and III. Darkened and shrunken neurons are widely interspersed among normal appearing cells. × 25. B and C: photomicrographs illustrating the destruction of a portion of the posterior pyriform cortex of the rat injected i.p. with 400 mg/kg pilocarpine. B: note extensive edema and disruption of the neuropil in the layer II and III [27]. Dilated, clear profiles around the cell bodies in the layer I and II are prominent. Shrunken and darkened neuronal somata appear throughout all layers of the pyriform cortex. × 40. C:

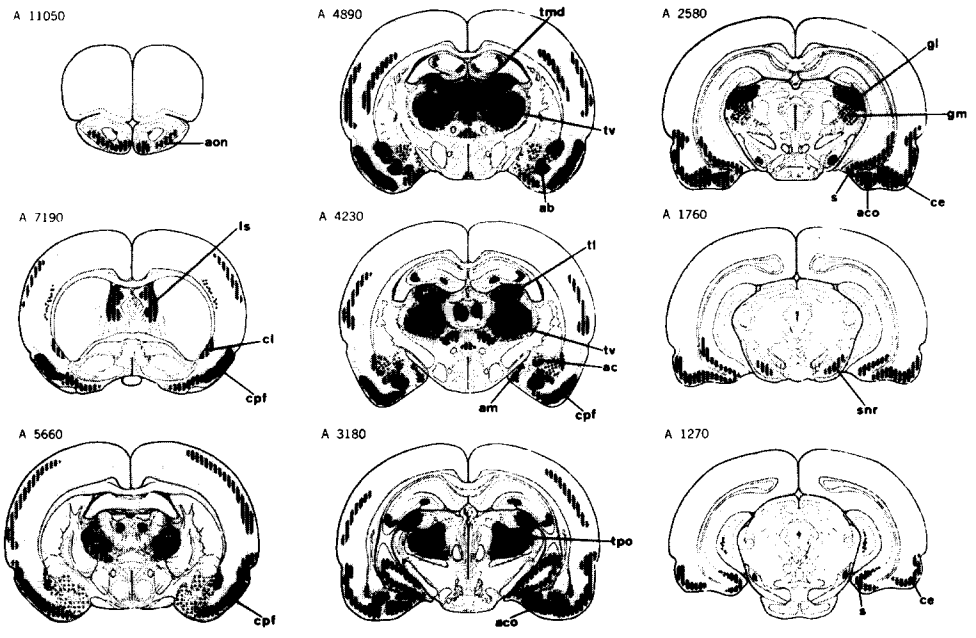

Fig. 8. Schematic reconstruction of the distribution of neuropathological alterations in the rat brain after intraperitoneal administration of 400 mg/kg pilocarpine relying on the atlas of König and Klippel [14]. The lesions were estimated in cresyl violet stained serial sections of the entire brain. Survival time: 24 h. Dark areas represent nearly total destruction of neurons accompanied by edema and disruption of the neuropil. Hatched areas represent moderate cellular destruction with partial neuronal loss apparent as numerous shrunken and dark staining neurons. Dotted areas represent mild cellular destruction with more isolated degenerating neurons which were found in several scattered locations. Abbreviations for anatomical structures, cf. Table II.

degeneration and disappearance of virtually all neuronal somata with disruption of the neuropil were observed within this area. Posterior part of the cortical nucleus and the amygdalo-hippocampal area were also extremely sensitive and severely damaged. The bed nucleus of the stria terminalis was frequently affected as well. In the dorsal hippocampus degeneration of pyramidal cells was observed in the CA4 and CA3 subfields (Fig. 6B–D). Round profiles (presumably dendrites) were noted in stratum lacunosum-moleculare and stratum lucidum of CA3 subfield. In the area dentata, degenerated neurons were observed in the hilus and at the granule cell-hilar border. Extensive edema and destruction of virtually all neurons were encountered in the ventral hippocampus of all rats treated with

high-power photomicrograph illustrating the destruction of layer II of the pyriform cortex. Note the nearly total disruption of the pyramidal neurons in the layer II. The cells become dark and severely shrunken. Some neurons contain narrowings which lead to fragmentation and disintegration. The destruction of neuronal population is accompanied by edema and disruption of the neuropil. × 196. D: high-power photomicrograph of the entorhinal cortex of the rat injected i.p. with 400 mg/kg pilocarpine. Note the nearly total disruption of the cytoarchitecture of the entorhinal cortex. Neurons stain darkly and appear shrunken. Note marked edema and disruption of the neuropil. × 196.

TABLE II

Distribution and extent of cellular degeneration in the rat brain following intraperitoneal administration of 400 mg/kg pilocarpine

−, no damage; +, mild cellular destruction; + +, moderate cellular destruction; + + +, near total destruction of neurons accompanied by disruption of the neuropil.

	Amygdaloid complex					*Thalamus*					*Hippocampal formation*							*Neocortex*	*Olfactory cortex*		*Substantia nigra*	
	ac	am	aco	ab	al	tmd	tv	tl	tpo	gl	CA3	CA4	CA1	s	ce	cpf	ls	tcx	aon	to	snr	snc
Rat no. 1	+	+	+ +	+ + +	+	+ + +	+ +	+	+ +	+ +	+	+	−	+	+ +	+ + +	+ +	+	+ +	−	+ +	+
2	−	+	+ +	+ + +	+	+ + +	+	+ +	+	+ +	+	−	−	+	+	+ +	+	+	+ +	−	+	−
3	not examined																					
4	−	+	+ +	+ + +	+	+ + +	+ +	+	+	+	+	−	−	+	+ +	+ + +	+ +	+ +	+ + +	−	+ +	+
5	+	+ +	+ + +	+ + +	+	+ + +	+ +	+ +	+	−	+ + +	+ +	−	+ +	+ + +	+ + +	+ +	+	+ + +	+	+ + +	+
6	+	+	+ +	+ + +	+	+ +	+	+	+	+	+	+	−	−	+ +	+ +	+	+ +	+ + +	−	+ +	−
7	not examined																					
8	+	+	+ +	+ + +	+	+ + +	+ +	+ +	+ +	+ +	+	−	−	+	+ +	+ + +	+	+ +	+ + +	−	+ +	+
9	+	+	+ + +	+ + +	+	+ + +	+ +	+	+	+	+ +	+	−	−	+	+ + +	+ +	+ +	+ + +	−	+	−
10	+	+	+	+ + +	−	+ + +	+ +	+	+ +	+ +	+	−	−	+	+ +	+ + +	+ +	+ +	+ +	−	+ +	−

List of abbreviations

ac	central amygdaloid nucleus
am	medial amygdaloid nucleus
aco	cortical amygdaloid nucleus
ab	basal amygdaloid nucleus
al	lateral amygdaloid nucleus
tmd	mediodorsal thalamic nucleus
tv	ventral thalamic nucleus
tl	lateral dorsal thalamic nucleus
tpo	posterior thalamic nucleus
gl	lateral geniculate nucleus
s	subiculum
ce	entorhinal cortex
ls	lateral septum
cpf	pyriform cortex
tcx	temporal cortex
aon	anterior olfactory nucleus
to	olfactory tubercle
snr	substantia nigra pars reticulata
snc	substantia nigra pars compacta

400 mg/kg pilocarpine. Most severe damage was identified in the ventral hippocampus, where subfields CA3 and CA1 were affected. Degeneration of pyramidal cells has not been observed in the CA1 subfield of the dorsal hippocampus. The ventral part of the subiculum was severely damaged as well. The lateral septal nucleus was also consistently affected. The extent and location of damage in the thalamus vary among rats treated with 400 mg/kg pilocarpine. However, neuronal degeneration with complete disruption of neuropil usually occurs in the dorsomedial and ventromedial nuclei (Fig. 5, Fig. 6E). Lateral nucleus (Fig. 6F) and the posterior group of thalamic nuclei were less severely damaged. In 7 rats out of 8, the dorsal part of the lateral geniculate nucleus was extensively damaged. Severe damage was present in the neocortex. The damage typically consists of dark cells in outer molecular layers II and III, but also in the layer V (Fig. 7A). Temporal cortex dorsal to the rhinal sulcus and cingulate cortex were particularly sensitive to pilocarpine. Somatomotor, perirhinal, retrosplenial and visual cortices were frequently affected. In addition, nearly complete destruction of the neuronal population with edema and disruption of the neuropil were observed in the substantia nigra pars reticulata (Fig. 6G). Shrunken and dark staining cells were likewise observed in the substantia nigra pars compacta (Fig. 6H). Little neuronal degeneration was encountered in the hypothalamus. Neuropathological alterations were never observed in the corpus striatum. Typical pattern and distribution of pilocarpine-induced damage within the rat brain is illustrated in Figs. 5–8. Whether or not some of these alterations might eventually recover [31] requires further extensive studies utilizing different survival times after injection of pilocarpine.

Intraperitoneal injection of pilocarpine in the dose of 200 and 100 mg/kg resulted in mild neuropathological alterations. At 200 mg/kg, destructions included pyriform cortex, anterior olfactory nucleus and amygdaloid cortical and basal nuclei. The rat, which displayed severe motor limbic seizures after 200 mg/kg pilocarpine showed additionally destructions in mediodorsal thalamic nuclei and in neocortex. The extent and location of brain damage in rats injected with 100 mg/kg pilocarpine was largely confined to pyriform cortex and anterior olfactory nuclei.

The animals pretreated with either 20 mg/kg of scopolamine (n = 6) or 10 mg/kg diazepam (n = 5) 30 min before i.p. injection of 400 mg/kg pilocarpine displayed no epileptiform activity and the neuropathological alterations were not detected in their brains.

DISCUSSION

The main finding of this study is that systemically administered pilocarpine, acting via scopolamine-reversible mechanism, can selectively initiate a sequence of convulsive disorders and electrographic alterations in the limbic structures

accompanied by widespread brain damage. The electrographic findings indicate that one of the most sensitive structures to the convulsant effect of pilocarpine is the hippocampus. The earliest electrographic responses to i.p. administered pilocarpine occur in the hippocampal recordings, while other structures were not, or slightly affected. Furthermore, high dose of pilocarpine produced electrographic alterations initially restricted to the hippocampus. A replacement of the normal background rhythm with high voltage fast activity and prominent high voltage spiking was noted in hippocampal recordings without substantial changes in amygdaloid and cortical leads. The changes in the electrical activity in the hippocampus correlated well with behaviourally encountered staring and periods of decreased responsiveness to external stimuli such as prodding or tapping on the cage.

Iontophoretic studies of the action of ACh in the hippocampus [3, 4] have emphasized that bursts elicited by ACh in pyramidal cells are similar to those seen during hippocampal epileptogenesis [30]. ACh may produce long-lasting muscarinic excitatory effect on hippocampal pyramidal cells [3], thus activating a powerful positive feedback mechanism within the septo-hippocampal-septal circuitry [12, 19, 21, 33]. This allows a development of long-term changes in excitability within the hippocampal subfields comparable to that which characterizes the kindling model of epilepsy [8, 40, 41, 42]. Furthermore, modulatory actions of ACh might account for increase in the effectiveness of other excitatory inputs, particularly in situations where epileptiform activity would give rise to more ACh release and facilitate the development of excitation that characterizes ictal discharges [8]. Thus, there is good electrophysiological basis for the assumption that the excessive stimulation of muscarinic cholinergic receptors located on hippocampal pyramidal cells [29] can easily lead to the initiation of the seizure activity in limbic system and consequently, it may be inferred that cholinomimetics may elaborate limbic status epilepticus and produce seizure-related brain damage.

In fact, neuropathological examinations of frontal forebrain sections in rats treated with systemic pilocarpine have shown widespread brain damage. The pyramidal neurons of the hippocampal formation were considerably sensitive to pilocarpine neurotoxic activity. Neuronal degeneration was more intense and widespread in the ventral hippocampus, and the changes in the ventral CA3 subfield were greater than in CA1. Surprisingly, dorsal hippocampus was less severely damaged. Neuronal degeneration was detected in the subfield CA4 and CA3 of the dorsal hippocampus, but has not been observed in the subfield CA1. This represents a clear difference from the studies with systemic kainic acid in rats [17, 31]. The latter finding may indicate that hippocampal mechanisms are differently involved in the convulsant effects of both kainic acid and pilocarpine. On the other hand, it is apparent that degeneration of pyramidal cells in the CA1 subfield is more prominent in rats with survival times longer than 24 h after injection of kainic acid [31]. However, it should be stressed that our efforts to

demonstrate neuronal degeneration in the CA1 subfield in rats with survival times as long as 30 days after injection of 400 mg/kg pilocarpine have failed so far [37]. Furthermore, the distribution and extent of damage caused by pilocarpine in hippocampus differs from that induced by cerebral ischemia [28]. The occurrence of an extensive neuronal degeneration in the CA1 subfield of the dorsal hippocampus is typically encountered in rats with experimental ischemia [28, 32], which is in apparent contrast with the findings in brains of rats treated with pilocarpine. Accordingly, one may infer that an ischemic factor is of minor importance in the genesis of pathological alterations caused by pilocarpine. In fact, blood glucose and oxygen were well maintained in animals with limbic status epilepticus produced by pilocarpine [37].

Further, from the neuropathological studies with systemic pilocarpine in rats, it is apparent that neuronal degeneration is consistently present in axonally interconnected areas. Dramatic histological changes were evident in mediodorsal thalamic nuclei, basolateral amygdaloid nuclei and neocortex which are reciprocally interconnected [15, 16] and which receive a massive cholinergic input from the substantia innominata [7, 13, 43, 44]. These findings suggest a valid role for substantia innominata and areas within its projection field in the amplification and spread of seizure activity caused by pilocarpine. Accordingly, studies of the effect of kainic acid in the substantia innominata have emphasized an extreme sensitivity of cholinergic neurons within this area to the neurotoxin [22], which may indicate that they receive glutamatergic input, perhaps as a feedback loop from the cortical areas to which they project [22].

The local administration of cholinomimetics into the rat amygdala may result in a sequence of seizure disorders accompanied by widespread brain damage [24, 38] resembling that observed after intraamygdaloid injections of kainic acid [5] and folates [26]. These observations may indicate a role for the amygdala in the pathogenesis of brain damage produced by pilocarpine and reveal that initiation of abnormal seizure activity elsewhere in the limbic system may result in very similar pathology. From this work, it appears likely that a massive cholinergic input from the substantia innominata [13, 23, 44] to amygdala may be in part responsible for mediating the extremely low seizure threshold of this area.

The distribution of the brain damage produced by pilocarpine 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 complex [7, 13, 21, 43, 44]. In this situation, one would expect that brain damage observed after i.p. pilocarpine would be a direct consequence of cholinergic overstimulation. Two findings argue against this hypothesis. Firstly, scopolamine, a cholinergic muscarinic antagonist, prevented the development of but failed to disrupt pilocarpine-induced limbic status epilepticus [37]. Conversely, diazepam, a potent anticonvulsant, prevented the development of status epilepticus and reduced the convulsive activity when administered 2 h after pilocarpine [37]. Secondly we provided

evidence that bethanechol administered intrastriatally and intranigrally in the dose as high as 50 μg did not result in any local degeneration [9, 38]. While these experimental findings appear to be convincing, the interpretation that cholinomimetics do not show neurotoxic properties in the brain should be premature. These findings may only suggest that striatum and substantia nigra are less sensitive to cholinergic neurotoxicity, but did not exclude direct neurotoxic properties of cholinomimetics. Indeed, cholinergic agents when injected intrahippocampally and into the amygdala produced local damage within both structures [9, 24, 36, 38]. In addition, McGeer et al. [22] showed that injection of folic acid into the substantia innominata resulted in the widespread brain damage which paralleled the density of cholinergic innervation from this structure. Given these uncertainties the elucidation of mechanisms involved in the genesis of the brain damage produced by pilocarpine should be made with caution.

The demonstration of potent brain damaging properties of pilocarpine should be taken into account when considering the possible involvement of cholinergic mechanisms in human pathology [20]. The topography of brain damage we describe after intraperitoneal pilocarpine and that previously reported after intrahippocampal [9, 36, 38] and intraamygdaloid [24, 38] injections of cholinomimetics in rats conforms closely to that observed in autopsied brains of human epileptics [11]. This observation indicates that cholinergic mechanisms may have crucial importance for the initiation and development of the epileptic brain damage in man.

It seems likely that systemic application of pilocarpine in rats may provide a convenient and useful animal model for studying mechanisms of and therapeutic approaches to temporal lobe epilepsy. The relevance of this animal model for human neurological disease is stressed by the occurrence of spontaneous recurrent seizures in rats injected intraperitoneally with pilocarpine [37]. This finding strengthens the clinical importance of the model since spontaneity is one of the prominent 'signs' of human epilepsy. Moreover, systemic administration of pilocarpine in mice produces severe limbic seizures and widespread damage to forebrain structures, which provides additional evidence for the common usefulness of this treatment as a model for epilepsy, and in particular, for evaluation of treatments specifically designed to alleviate convulsant activity and neuronal degeneration [39].

Cholinomimetics are widely used in human medicine and agriculture [34, 35]. Repeated instillation of pilocarpine and physostigmine into the eyes is commonly recommended in the treatment of acute glaucoma in man [35]. The extreme toxicity of 'irreversible' acetylcholinesterase inhibitors, such as sarin, soman and tabun has been well recognized and these agents have received extensive application as chemical-warfare poison gases [34]. 'Reversible' acetylcholinesterase inhibitors are widely used as agricultural insecticides [34], which are generally dispersed as aerosols or as dusts. Accordingly, these compounds are easily and

effectively absorbed by mucous membranes, skin, gastrointestinal tract and lungs. In this regard, it is important to ask whether acetylcholinesterase inhibitors may produce brain damage. In fact, microinjections of physostigmine into the rat amygdala have been found to induce sustained limbic seizures and widespread brain damage [24]. In this situation, one would emphasize, that a potent convulsive and brain damaging action of cholinomimetics may be extremely dangerous for man and these compounds should be used with care.

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