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Acquisition and Retrieval of Conditioned Taste Aversion is Impaired by Brain Damage Caused by Two Hours of Pilocarpine-Induced Status Epilepticus

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Summary

The effect of Cavalheiro's pilocarpine model of epileptogenesis upon conditioned taste aversion (CTA), an important example of nondeclarative memory, was studied in adult Long Evans rats. Deterioration of CTA was studied during the silent period between pilocarpine-induced status epilepticus (SE) and delayed spontaneous recurrent seizures. SE was elicited by i.p. injection of pilocarpine (320 mg/kg) and interrupted after 2 hours by clonazepame (1 mg/kg i.p.). Peripheral cholinergic symptoms were suppressed by methylscopolamine (1 mg/kg i.p.), administered together with pilocarpine. CTA was formed against the salty taste of isotonic LiCl. In the experiment of CTA acquisition, the CTA was formed and tested during the silent period after SE. In the experiment of CTA retrieval, the CTA was acquired before SE and the retrieval itself was tested during the silent period. Retrieval of CTA acquired before SE was impaired more than the retrieval of CTA formed during the silent period. Our findings indicate that epileptic seizures can disrupt even non-declarative memory but that CTA formed by the damaged brain can use its better preserved parts for memory trace formation. Ketamine (50 mg/kg i.p.) applied 2 min after the onset of pilocarpine-induced status epilepticus protected memory deterioration.

Key words

Conditioned taste aversion • Epilepsy • Pilocarpine • Nondeclarative memory

Introduction

Epilepsy tends to result in alteration of various cerebral function including memory. Memory can be classified according to different aspects. Within the long-term memory, a declarative and a nondeclarative form can be distinguished. Declarative knowledge provides an explicit, consciously accessible record of previous, individual experience and sense of familiarity about this experience. Nondeclarative learning usually occurs in an incremental and automatic manner with no conscious awareness of exactly what had been learned. Both these types of memory can be modeled in animals. Place-navigation tasks, such as the Morris water maze, are

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suitable models of rat's cognitive behavior, which is analogous to declarative memory in human beings (Squire 1984). The hippocampus is considered to be a structure where the cognitive maps are processed (O´Keefe and Nadel 1978). The crucial role in the re-enforcement and retrieval of the declarative memory trace is played by hippocampal NMDA receptors (Fujikawa *et al.* 1994, Hort *et al.* 1999).

Conditioned taste aversion (CTA) is a suitable model of nondeclarative memory (Bureš *et al.* 1998). Rats are extremely proficient in associating the consequences of ingestion of a particular food with taste. If drinking of flavored fluid (conditioned stimulus) is followed within several hours by symptoms of poisoning (unconditioned stimulus) the rat develops a strong aversion to its taste (CTA) and rejects it on subsequent presentations. Since recognition of the aversive taste stops the consumption of flavored fluid, the volume of ingested aversive liquid can quantitatively characterize the efficiency of the process.

Relatively extensive research has been done to assess the effect of epilepsy and antiepileptic treatment on declarative memory. Long-term seizure activity leads to deterioration of some parts of the brain, particularly the area of hippocampus and related structures (mesial sclerosis). Hippocampal NMDA receptors are supposed to play a fundamental role in both declarative memory formation as well as in epileptogenesis. Memory deficits were described after lithium-pilocarpine-induced seizures (Harrigan *et al.* 1991, Kostakos *et al.* 1993), and during the "silent period" after pilocarpine-induced epileptic seizures (Hort *et al.* 1999).

Pilocarpine-induced status epilepticus (SE) in rats represents not only a model of severe seizures (Turski *et al.* 1983), but also a model of chronic epileptogenesis. After SE and a silent period lasting 2-3 weeks, spontaneous recurrent seizures (SRS) appear (Cavalheiro *et al.* 1991). The morphological changes observed a few days after were dominated by nerve cell death in the CA3 region of the hippocampus (Turski *et al.* 1983), but similar changes were found in many other regions of the brain, i.e. the amygdala (Clifford *et al.* 1987, Persinger *et al.* 1988, Fujikawa *et al.* 1994) and thalamus (Persinger *et al.* 1994).

The long term seizure activity also influences non-declarative memory. Several papers have proved the influence on CTA consolidation (Mikulka and Freeman 1984, Venugopal and Persinger 1988, Persinger *et al.* 1993, 1994). However, it is still not clear what is the actual course of attenuation of CTA after seizure activity.

The aim of the present paper was to determine the exact phase of CTA which is actually impaired by the pilocarpine epileptic seizures: the ability to develop CTA or to retrieve preictally-induced CTA. The second part of the experiment examined the role of NMDA receptors in nondeclarative memory impairment by assessing the ketamine-induced protection against the pilocarpine seizures. Histologically, we have paid attention to the structures which can be related either to the formation or the retrieval of CTA.

Methods

All experiments were performed in agreement with the Animal Protection Law of the Czech Republic and were approved by the Ethics Committee of the Second Medical Faculty of the Charles University.

Animals

Sixty male Long Evans hooded rats weighing between 250 and 330 g at the start of the experiments were obtained from the breeding colony of the Institute of Physiology. Animals were housed five per cage, after seizures each rat had to be housed individually in a single cage, in an animal room with constant temperature (22 ^{o}C) and natural lighting. They had free access to food and water except on the experimental days, when testing was preceded by 24-h water deprivation. This level of deprivation is well tolerated by rats and its use was approved by the Ethics Committee of the Second Medical Faculty of the Charles University to warrant constant motivation of animals.

CTA procedure

For the CTA formation, an isotonic solution of lithium chloride (LiCl) (0.64 %) can be used as a conditioned stimulus (CS) as well as an unconditioned stimulus (UCS). The rats do not have any neophobia to the LiCl solution, on the contrary, at the beginning of the experiments they prefer it to water because of the similarly salty taste of LiCl and NaCl (Scott 1973). The ingestion of LiCl leads to gastrointestinal distress (intestinal cramps, increased peristalsis, diarrhea, etc.) that serves as an UCS. Any subsequent contact with the LiCl solution is followed by its rejection. The test was performed with a single bottle technique providing the animal with no other choice than the liquid offered.

Before CTA acquisition, the rats were trained during two days to drink their daily portion of water within 10 min. On the third day, the rats were offered the

LiCl solution for 10 min. The ingestion in naive rats was similar to water ingestion but it was followed by gastrointestinal discomfort that caused the rejection of the LiCl solution at the end of the 10-min period. On the next day (4th day of the learning schedule), elaboration of CTA to LiCl solution is manifested by decreased intake of all salty solutions. In case of well trained intact rats, the contact with LiCl solution leads to its immediate rejection.

Status epilepticus

The Cavalheiro's pilocarpine (PIL) model of chronic spontaneous recurrent seizures (Cavalheiro *et al.*, 1991) was used. Uninterrupted severe tonic and clonic epileptic seizures (status epilepticus, SE) were induced by administration of pilocarpine (320 mg/kg i.p.). Together with PIL we have administered methylscopolamine (1 mg/kg i. p.) to attenuate the peripheral cholinergic activity of PIL. Seizures appeared approximately 15 min (range 5-30 min.) after administration of PIL. Half of the animals which had received pilocarpine entered the status epilepticus, which was interrupted by clonazepame (CZP) (1 mg/kg i. p.) after 2 h of continuous seizure activity. In the test for NMDA receptor protection, the chosen group that entered SE was given ketamine (50 mg/kg i.p.) 2 min after the onset of seizures. The animals were observed during this two- hour period, and their behavior was recorded. This group of rats was used to assess the influence of PIL-induced SE on CTA. About 50 % of rats did not develop any seizures after administration of PIL (320 mg/kg, Hort *et al.* 1999, 2000). Rats, which had received pilocarpine but did not develop motor SE, were given CZP 135 min after the injection of PIL (n=10) and were used as the control group. The rats after SE stayed lethargic for the next 24 h not being able to eat or drink. The rats that did not start to drink within the next 24 h received 3 ml of 10 % glucose i. p.

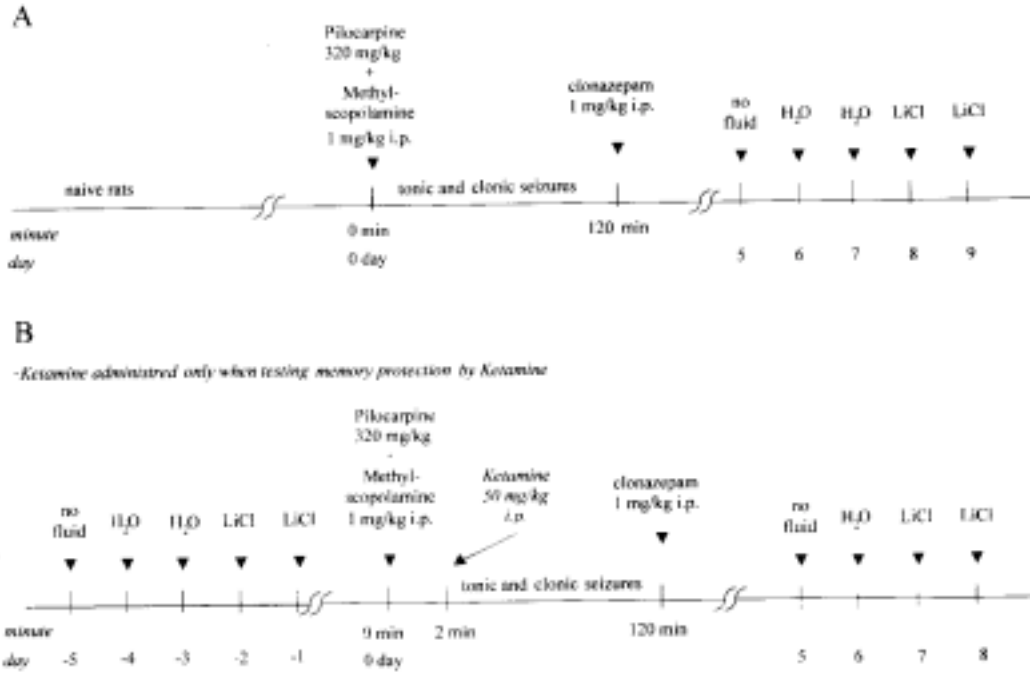

Fig. 1. ***A.*** *The time diagram of the experimental arrangement for testing of CTA formation impairment after pilocarpine-induced status epilepticus.* ***B****. The time diagram of the experimental arrangement for testing of CTA retrieval impairment after pilocarpine-induced status epilepticus.*

Procedures

CTA acquisition. A group of naive rats underwent SE and subsequently the CTA acquisition was tested during the "silent period". Control group of rats received the same drugs, but did not develop SE (Fig. 1a).

CTA retrieval. CTA was induced in the group of intact rats before pilocarpine injection. One subgroup of rats was protected by ketamine (50 mg/kg i.p.) administered 2 min after the appearance of seizures. The second subgroup underwent SE without ketamine. The

control group consisted of rats which had received all drugs but ketamine and did not exhibit behavioral seizures (Fig. 1b).

Data analysis

The daily liquid consumption was analyzed by two-way ANOVA with repeated measures followed by *post hoc* Newman-Keuls multiple comparisons.

Histology

When the testing was completed, the animals were perfused transcardially under deep pentobarbital anesthesia with heparinized physiological saline and subsequently with the fixation solution (4 % paraformaldehyde in a phosphate buffer, pH 7.4, both solutions at room temperature). The brains were removed, postfixed in the same solution for 24 h in a refrigerator, dehydrated, saturated with toluene and embedded in paraffin. Serial sections 10 μm thick were cut in the frontal plane and stained. The stained sections were dehydrated, cleaned with xylene and mounted in DPX (Fluka, Buchs). Sections were observed under an optical microscope (Olympus VANOX-S, Olympus, Hamburg). Attention was focused on the hippocampus and amygdala.

Results

Status epilepticus started with clonic seizures of the head and forelimb muscles. It was often accompanied by rearing and falling, but the animals after that assumed a normal position again immediately. These episodes were followed by uninterrupted SE formed by tonic and clonic seizures, which were arrested by clonazepame administered 2 h later. Even after interruption of the status, the animals were in poor shape and intensive care had to be taken to provide adequate drinking and feeding behavior. Therefore, the rats could not be tested earlier than on the sixth day after the SE.

In the experiment of CTA acquisition, the rats underwent SE and then acquired CTA to the LiCl solution. The experimental (n=10) and control (n=10) rats drank 8.7±0.6 ml and 8.1±0.4 ml of water on day 6, 11.2±0.6 and 12.0±0.5 ml of water on day 7, 10.0±1.0 ml and 10.0±0.7 ml of LiCl on day 8. The lithium ingestion lead to severe gastrointestinal distress within several minutes. On day 9, the experimental and control groups drank 6.5±1.2 ml and 1.0±0.3 ml of LiCl., respectively. The experimental rats were drinking with apparent hesitation, the controls rejected the offered liquid after the first lick. Two-way ANOVA (conditions x days) with repeated measures on various days yielded significant main effect of groups ($F(1,18)=12.36$, $p=0.0025$) and of days ($F(1,18)=44.7$, $p<0.0001$) as well as significant interaction ($F(1,18)=8.64$, $p=0.009$). Subsequent *post hoc* multiple comparisons showed that the CTA after SE was significantly ($p<0.01$) weaker in comparison with the control group (Fig. 2). Decrease of LiCl consumption on day 9 against day 8 ($p<0.05$) indicates that the SE rats acquired a weak CTA.

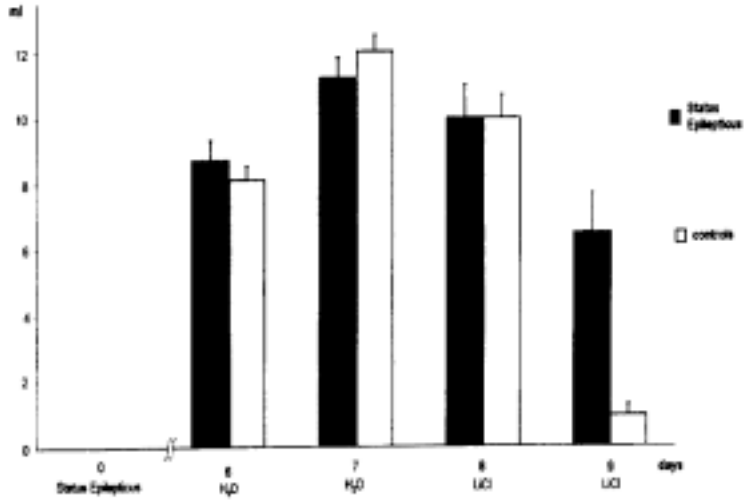

Fig. 2. *Impairment of CTA formation after pilocarpine-induced status epilepticus. Ordinate: fluid consumption (mean ± S.E.M.) during 10 min. Abscissa: days after status epilepticus.*

In the experiment of CTA retrieval, the rats initially acquired CTA and later underwent SE. The subgroup that was not protected by ketamine during SE (n=10) drank 10.5±0.7 ml of water on the first experimental day (sixth day after the seizures), the rats protected by ketamine (KET) (n=10) drank 9.5±1.2 ml and the control group (n=10) drank 9.0±1.1 ml. On day 7, rats in the experimental group drank 10.8±1.2 ml (KET 1.9±0.7 ml, controls 1.5±0.5 ml) of the LiCl solution. The experimental group did not exhibit any hesitation in

drinking the LiCl solution. The KET and control groups rejected the LiCl solution immediately. LiCl ingestion resulted in the gastrointestinal distress in the experimental group and the extinguished CTA began to be relearned.

On day 8, rats in the experimental group drank 4.3±1.7 ml of LiCl solution (KET 0.8±0.4 ml, controls 0.4±0.25 ml). The experimental rats started to drink very rapidly at the beginning, but after several minutes they rejected the LiCl solution. The KET and control group rejected the offered LiCl instantly (Fig. 3). Two-way ANOVA (conditions x days) with repeated measures on various days yielded a significant main effect of groups ($F(2,27)=17.57$, $p<0.0001$) and of days ($F(2,54)=72.17$, $p<0.0001$) as well as significant interaction ($F(4,54)=8.07$, $p<0.0001$). The subsequent Newman-Keuls *post hoc* multiple comparisons showed significantly higher LiCl intake in the experimental group on day 7 ($p<0.01$) and on day 8 ($p<0.05$). Differences between the control group and the group protected by ketamine on day 7 and day 8 were not significant.

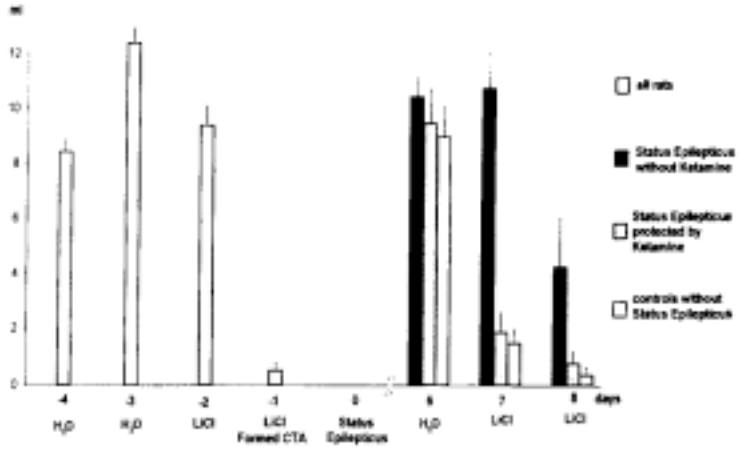

Fig. 3. *Impairment of CTA retrieval after pilocarpine-induced status epilepticus. For legend see Fig. 2.*

Histological examination showed a marked expansion of the lateral ventricles in all SE rats. The density of the pyramidal cells in CA1 and CA2 hippocampal fields was distinctly decreased, especially at the CA1-CA2 border (Fig. 4a). Part of the CA1 region was relatively well preserved. A remarkably low density or almost total disappearance of neurons (mainly pyramidal cells) was found in the CA3b region.

Prominent bilateral loss of cells in the amygdala was identified in all experimental animals. The neuronal population of nucleus amygdalaris centralis (Fig. 4b) and area amygdalaris anterior was destroyed and replaced by numerous glial cells. Necrosis and gliosis were also detected in the globus palidus, nucleus caudatus, putamen and pyriform cortex. Such findings indicated that acute degenerative changes were in progress at the time of the perfusion of the animals. (i.e. 9-19 days after the administration). Histological data were similar in all six rats. No quantification was performed in this study because of the nearly total extinction of neurons in some subfields.

Discussion

Impairment of cognitive learning during the silent period between pilocarpine-induced status epilepticus and appearance of spontaneous recurrent seizures was discussed in preceding papers (Hort *et al.* 1999, 2000). Place navigation in the Morris water maze consists of two distinct components: declarative place representations (cognitive maps) as well as more general (procedural) learning (Morris *et al.* 1990). The procedural aspects include learning to inhibit inborn nonadaptive behavior, such as swimming along the wall (Paylor and Rudy 1990, Whishaw and Mittleman 1986), while selecting appropriate behavioral strategies, such as swimming across the pool or uniformly searching its surface. Other procedural components involved skills such as improved distance and angle judgment that are a necessary prerequisite for the cognitive demands of the task. The hippocampal formation is critical for computing place representations but is believed to be dispensable for procedural memories (O´Keefe and Nadel 1978).

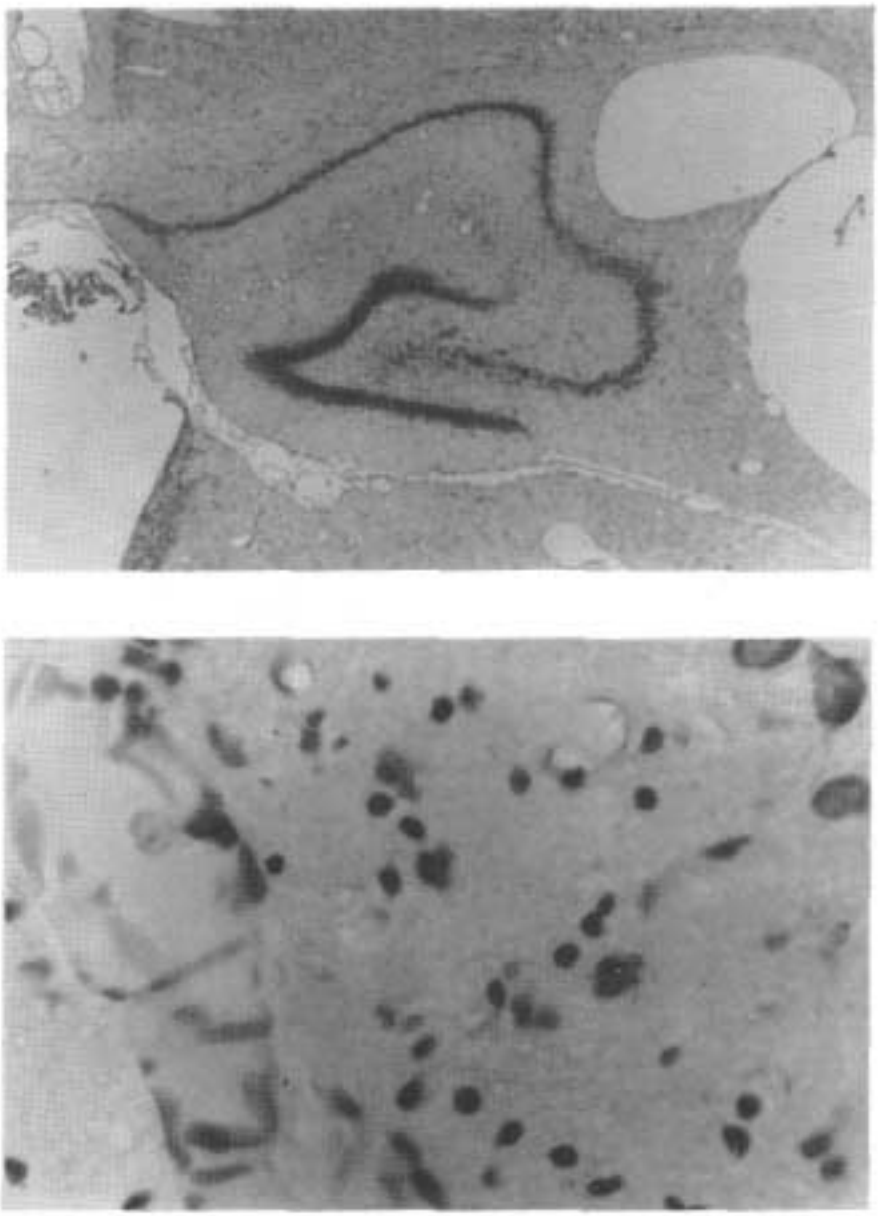

Fig. 4. ***A.*** *Transversal section of the right hippocampus. Nissl staining, magnification 30x. Destruction of CA1, CA2 and CA3 hippocampal fields. Expansion of the cerebral ventricles.* ***B.*** *Transversal section of the right nucleus amygdalaris centralis. Nissl staining, magnification 300x. Neuronal necrosis and gliosis in nucleus amygdalaris centralis.*

We previously reported that declarative memory is seriously impaired by pilocarpine-induced SE (Hort *et al.* 1999). Some impairment of procedural components, in addition to cognitive mechanisms, is very probable. Persinger *et al.* (1994) described the deterioration of the declarative (radial-maze acquisition) and non-declarative (conditioned taste aversion) form of memory after seizures induced by a systemic injection of lithium-pilocarpine. By means of a multivariate analysis, these authors demonstrated that the amount of damage within the CA1 and the basolateral amygdala was most strongly associated with attenuated CTA, whereas damage within the mediodorsal thalamus was primarily associated with deterioration of radial maze acquisition.

Investigation of neuroanatomical substrate of CTA yielded contradictory results in terms of the role of the hippocampus. Functional ablation studies with CTA and neuroanatomical findings especially emphasized the role of the pontine parabrachial nucleus, amygdala, hypothalamus, entorhinal, prefrontal and insular cortex, as well as the area postrema, thalamus and the globus pallidus (for review see Bureš *et al.* 1998).

We did not inspect the insular cortex histologically. This structure is known to be necessary for CTA acquisition and during its bilateral inactivation rats extinguish the CTA instead of learning (Bureš *et al.* 1998). Our rats after pilocarpine SE were able to relearn CTA partially so that the insular cortex was apparently partially functional.

There is a consensus that the parabrachial nuclei play a fundamental role in CTA (Bureš *et al.* 1998, Ivanova and Bureš 1990). Although we did not make a histological investigation of these nuclei, they were probably not destroyed, because the rats in our experiments were able to relearn CTA again after SE.

Taste aversion only slightly depends on the hippocampal function (Bureš *et al.* 1998). Yamamoto *et al.* (1995) showed that lesions of the whole hippocampus

had no effect on acquisition, but attenuated the neophobic response to the saccharin CS, and showed a significant disruptive effect on CTA retrieval. Murphy and Brown (1970) found that large aspiration of the hippocampus did not affect the speed of acquisition, strength or resistance to extinction of CTA to sucrose paired with LiCl illness. Miller *et al.* (1975) reported that extensive aspiration lesions of the hippocampus did not affect the acquisition or strength of aversion to saccharin paired with cyclophosphamide, although the lesioned rats extinguished the aversion more rapidly than control rats. Reilly *et al.* (1993) showed that ibotenic acid lesions of the hippocampus had essentially no effect on the acquisition of CTA, but significantly impaired maze learning. These results suggest that the hippocampus does not play an important role in the acquisition of CTA or in the association of CS with UCS. Nevertheless, it appears to be involved with previous experience classifying the stimulus as novel or familiar, as safe or dangerous. In this respect, it is of interest to note that Gallo and Candido (1995a,b) showed that dorsal hippocampal lesions impaired blocking, but not latent inhibition of taste aversion learning in rats.

Some conflicting results of the limbic lesion studies are probably due to different combinations of tastes and flavors used to induce CTA. A number of experiments comparing the involvement of the hippocampus in CTA and passive avoidance learning lead to the conclusion that it is easier to disrupt the latter than the former (Gaston 1978). Several studies have failed to find deficits in the acquisition of taste aversion after hippocampal lesions (Murphy and Brown 1970, Miller *et al.* 1971, 1986, Bermúdez-Rattoni *et al.* 1987). Kesner *et al.* (1975) reported that neither post-CS nor post-UCS stimulation of the hippocampus had any disruptive effect on CTA learning. Nevertheless, when irradiation rather than a poisonous drug was used as the toxic agent, rats with large hippocampal lesions failed to acquire saccharin aversion (Miller *et al.* 1971).

Our results are probably due to damage of the amygdala. Participation of the basolateral amygdala in CTA acquisition and retrieval was demonstrated in lesion experiments (Rolls and Rolls 1973a,b, Nachman and Ashe 1974). Kesner *et al.* (1975) and Arthur (1975) demonstrated that amygdalar stimulation interferes with CTA acquisition when applied shortly after poisoning or in the CS-UCS interval. Lick-triggered stimulation of the amygdala interferes with retrieval of CTA (Brožek *et al.* 1979). Serious damage but not total destruction of the amygdala found in our experiments can explain the fact that CTA acquired before SE is attenuated but can be relearned.

When the NMDA receptors were protected by ketamine, the CTA was preserved. The most probable explanation is that ketamine blocked NMDA receptors during SE in the whole brain and this decreased the intensity and extent of seizures and therefore protected all brain regions against the damage. It is also possible that ketamine protected amygdalar NMDA receptors during SE. Probably both effects participated synergically.

We can thus conclude from our results that epileptic seizures have a great influence not only on declarative but also on nondeclarative memory, the retrieval of which seems to be more affected than its acquisition.

Acknowledgements

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Reprint requests

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