

Differential behavioral and biochemical effects of regional injection of cycloheximide into mouse brain

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Results of numerous experimental studies support the notion that antibiotic inhibitors which disrupt brain RNA or protein synthesis can selectively block the formation of long-term memory for various tasks in several species^{1-3,5,12,13}. There is, however, little evidence to suggest whether one can anatomically localize the site of the amnesic effect of these agents. While Flexner *et al.*⁹ compared the effects of injection of puromycin into frontal, temporal and ventricular regions of mouse brain on Y-maze retention, subsequent reports suggested that the resulting amnesia in mice may be unrelated to the effect of the drug on protein synthesis¹⁰. Consolidation, as evidenced by a rapidly growing insusceptibility of memory to subcutaneous or intracerebral injections of antibiotic^{3,4}, was not observed in Flexner's experiments. Subcutaneous injection of another protein synthesis inhibitor, cycloheximide, results in profound inhibition of protein synthesis throughout the body, including the brain, and is accompanied by amnesia³. The parenteral injection does not permit demonstration of putative brain regions that might mediate the amnesic effect. We recently demonstrated by means of radioautography that a zone of inhibition can be demarcated following stereotaxic intracerebral injection of small volumes of either puromycin or cycloheximide in the monkey⁷. The present report summarizes experimental results which suggest localization of the effects of small stereotaxically injected doses of cycloheximide (CXM) on protein synthesis and memory in the mouse.

Eight-week-old female CD-1 mice (Charles River) were housed individually 3 days before one-trial step-through passive avoidance training. Apparatus and training procedures were based on a procedure reported by Flood *et al.*⁸. A subject was placed into the small compartment of a two-chamber test box. After a 1 min habituation period, a sliding door separating the two chambers was opened, allowing the mouse to enter the large shock chamber. Five seconds after entry, the door was closed and a 0.15 mA scrambled shock was delivered through the grid floor. After a 2 sec

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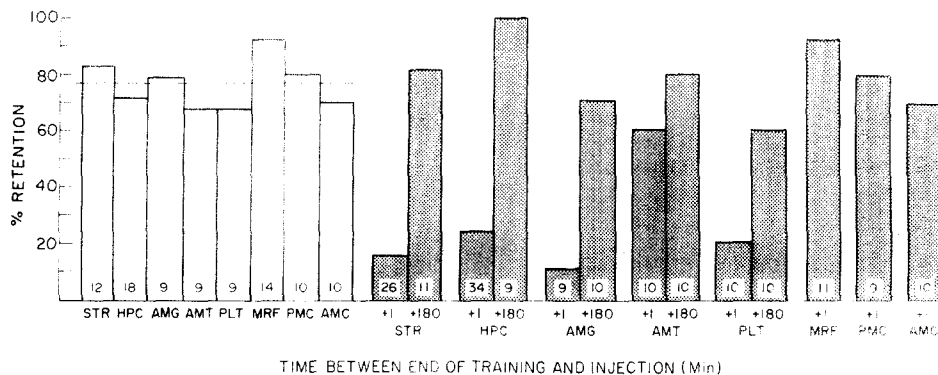


Fig. 1. Retention of groups injected bilaterally into various brain regions with saline immediately after training or CXM ($3 \mu\text{l}$, $10 \mu\text{g}/\mu\text{l}$) immediately or 180 min after training. Open bars indicate saline-treated groups. Dashed line indicates retention score of pooled saline-treated group. Injection sites included striatum (STR), hippocampus (HPC), amygdala (AMG), anterior medial thalamus (AMT), posterior lateral thalamus (PLT), midbrain reticular formation (MRF), posterior medial cortex (PMC), and anterior medial cortex (AMC). Numbers of subjects in each group are stated inside the bars.

minimum shock exposure, the mouse was allowed to escape into the start box. All training and testing was done with the observer unaware of the subject's experimental condition.

Either 2 min or 3 h after training, subjects were etherized and placed into a stereotaxic head holder¹⁵. A 1 cm scalp incision was made, holes were drilled in the skull and a specially designed cannula assembly was lowered into the appropriate position for injection in one of 8 brain regions (see Fig. 1). Bilateral injections of $3 \mu\text{l}$ of saline with or without $30 \mu\text{g}$ of cycloheximide (Actidione, Nutritional Biochemicals) were delivered simultaneously over a 1.5 min period by means of $10 \mu\text{l}$ Hamilton syringes. Following the injections, cannulas were removed and the incision was closed with a 9 mm wound clip. The entire procedure took less than 4 min.

Twenty-four hours after initial training, subjects were replaced in the training apparatus and retention step-through latencies (cut-off = 5 min) were measured by an automated device. In general, mice from all experimental groups either stepped through rapidly or did not step through in 5 min. Subjects were therefore classified as amnesic if the retention latency exceeded 2 min. Group scores were evaluated as per cent of subjects that showed retention.

Retention of all saline-injected groups (Fig. 1) was consistently high (67–93%). Comparison of saline groups with extreme scores (midbrain reticular formation, 93%, vs. posterior lateral thalamus or anterior medial thalamus, 67%) revealed no significant injection location effect among saline-treated groups (χ^2 analysis; $\chi^2 = 1.11$, N.S.), and in subsequent statistical analyses, saline group scores were therefore pooled. In contrast with the saline-treated group, subjects injected with CXM immediately after training showed retention deficits when injections were placed in striatum ($\chi^2 = 30.35$, $P < 0.001$), lateral hippocampus ($\chi^2 = 27.84$, $P < 0.001$), amygdala ($\chi^2 = 14.18$, $P < 0.001$), or posterior lateral thalamus ($\chi^2 = 11.62$, $P < 0.001$). No



AMT



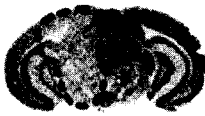
AMG



PLT



HPC



MRF



PMC



STR



AMC

Fig. 2. Unilateral injection of CXM into striatum caused protein synthesis inhibition in most of the striatum (excluding some anterior portions), some of anterior lateral thalamus, and an anterior portion of lateral hippocampus. CXM injected into lateral hippocampus inhibited protein synthesis in all of hippocampus, except the most ventral and anterior medial portions, and most of temporal and occipital neocortex. Protein synthesis inhibition from injection of CXM into amygdala was evident in all areas affected by the hippocampal injection, as well as in amygdala, posterior regions of pyriform cortex, and lateroventral striatum. Injection of CXM into posterior lateral thalamus produced a zone of protein synthesis inhibition including all nuclei in that area of thalamus and most of lateral hippocampus. The zone of protein synthesis inhibition produced by injection of CXM into anterior medial thalamus included virtually all thalamic nuclei including thalamic areas affected by CXM injection into posterior lateral thalamus. The anterior medial injection also produced inhibition in dorsal hippocampus and a small portion of the dentate gyrus. Injection of CXM into midbrain reticular formation produced protein synthesis inhibition in all of midbrain and pontine reticular formation and in substantia nigra and posterior lateral thalamic nuclei including most of nucleus posterior thalami. Posterior medial cortex injection of CXM produced protein synthesis inhibition in the dorsal hippocampus and overlying neocortex. Injection of CXM into anterior medial cortex produced a zone of protein synthesis inhibition including anterior cingulate cortex, all of frontal neocortex, and anterior striatum.

deficit in retention was observed when injections were placed in anterior lateral thalamus, midbrain reticular formation, posterior medial cortex, or anterior medial cortex. Furthermore, there were no significant deficits observed in any groups injected with CXM 180 min after training (Fig. 1). Significantly higher retention was observed in groups injected with CXM 180 min after training than in groups injected with CXM immediately after training for injections placed into striatum ($\chi^2 = 13.50$, $P < 0.001$), lateral hippocampus ($\chi^2 = 14.36$, $P < 0.001$), and amygdala ($\chi^2 = 4.54$, $P < 0.05$).

In additional statistical analyses, we found that CXM-injected groups that displayed amnesia compared to the saline-injected controls also generally showed significant retention deficits when compared with CXM-treated groups that were unimpaired relative to saline injected controls. There were no significant differences in degree of amnesia among groups impaired relative to saline-injected controls, nor were there any significant differences among groups unimpaired relative to saline-injected controls.

The behavioral results clearly demonstrate a regional difference in the resultant effects on retention of learned passive avoidance, with maximal amnesia following injection into striatum, hippocampus, amygdala, and posterior lateral thalamus. Further, the consolidation gradient following local injection is the same as that observed following subcutaneous injection⁶. In contrast, similar injections of CXM into regions bordering the above named areas, anterior medial thalamus, midbrain reticular formation, and neocortex, have no amnesic effect.

While CXM can enter the mouse brain following a parenteral injection, its intracerebral injection results in regional inhibition (Fig. 2), as has been shown in the monkey⁷. An injection of 30 μg of CXM was made as in the behavioral experiments, but on only one side, and 3 μl of saline was injected on the other. Immediately following CXM injection into a brain region, the mouse was injected subcutaneously with 50 μCi of L-methionine (methyl-¹⁴C, 10 mCi/mmole, New England Nuclear Corp.). After a 30 min incorporation period, the mouse was deeply etherized and perfused with 50 ml of saline and 50 ml of 10% formalin. The decapitated head was stored for 24 h in 10% formalin, then the brain was removed and stored for another 24 h in 10% formalin. Brains were sectioned at 40 μm and every fourth section was soaked in 10 ml of 10% formalin for 24 h, then dried onto a glass slide. Slides were stored in contact with Kodak No-Screen X-ray film in a light tight box for 2 weeks.

The 30 min incorporation period selected was somewhat arbitrary. The amnesic response might be best correlated with an as yet unknown combination of depth and duration of inhibition not apparent from the single time point. Also, while distinct regions of inhibition are apparent in the radioautograms, it cannot be ascertained whether or not there are also lesser but significant amounts of protein synthesis inhibition in the remainder of the brain. The region of readily apparent inhibition seen is large; whether smaller more punctate zones of inhibition would also result in amnesia and further localization of the antibiotic effects is presently unknown. The radioautographic results, taken together with the behavioral findings, proved nevertheless to be useful.

The zone of protein synthesis inhibition produced by hippocampal injection includes neither amygdala nor striatum. However, injection of CXM into either amygdala or striatum results in some protein synthesis inhibition in hippocampus. Since the hippocampal injection produces amnesia, memory impairments resulting from striatum or amygdala injections could be attributable to protein synthesis inhibition in hippocampus. A possible role of amygdala and striatum in passive avoidance memory is thus not clarified by this study. Since anterior medial cortex injections do not affect memory, yet cause protein synthesis inhibition in most of the striatum, it also appears that striatum is not involved. A role of inhibition in posterior thalamus is unlikely since injections of CXM into anterior medial thalamus and reticular formation do not produce amnesia, but do produce protein synthesis inhibition in posterior thalamus. Amnesia following posterior thalamus injection may thus be due to spread of protein synthesis inhibition into hippocampus. Injections in the hippocampus may have to be quite extensive to cause amnesia; anterior medial thalamic and posterior medial cortex injections which do not affect memory do inhibit protein synthesis in dorsal hippocampus as indicated on radioautography.

It has previously been proposed, on the basis of surgical lesion studies, that anterior limbic cortex is intimately associated with formation of memory of a passive avoidance task in the mouse¹¹. More generally, various lesions and associated electrophysiological studies have implicated posterior thalamus and pontine reticular formation^{14,16}. From experiments with reversible blocking agents, it has been inferred^{1,5,12} that the various blockers of protein (and RNA) synthesis selectively affect the storage of memory. We suggest further that in one-trial passive avoidance in the mouse, the hippocampus is implicated as a probable site of action.

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