Inhibition of cerebral protein synthesis impairs long-term habituation

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When brain protein synthesis is inhibited shortly before or after training, retention is normal for a few hours but subsequently is severely impaired\(^1,7,10\). When inhibition is initiated 30 min or longer after training, memory is not affected. These findings have been obtained with rodents, chicks and fish, using a variety of learning tasks including discrimination problems, passive avoidance, and active avoidance. Taken together, these results suggest that brain protein synthesis, during or shortly after training, is required for the formation of ‘long-term’ memory. Recently, interest has developed in the study of simpler forms of learning which, in suitable preparations, might be amenable to relatively direct neurophysiological and biochemical analysis. Habituation, the gradual waning of a response to repeated presentations of the same stimulus, is exhibited throughout the animal kingdom and is frequently considered to be the simplest form of learning\(^5\). Under favorable circumstances habituation can persist for days or weeks\(^8,9\).

Recently, it was shown that the electrophysiological correlates of short-term habituation recorded from motoneuron L-7 of \textit{Aplysia} proceeded normally during stimulation of the siphon nerve in spite of marked inhibition of ganglionic protein synthesis\(^8\). This result suggested that protein synthesis is not required for short-term habituation, and is in keeping with evidence from fish, chicks, and rodents that memory can persist normally for many minutes and sometimes for hours when training occurs during profound inhibition of brain protein synthesis\(^1,7,10\). We now report that inhibition of cerebral protein synthesis by anisomycin impairs long-term habituation in mice.

Male hybrid mice (C3H × Balb/C) were obtained from Simonson Laboratories (Gilroy, California) at 8 weeks of age and introduced to the experimental situation at 12 weeks of age when they weighed 23–25 g. Habituation was studied with an innate suppression technique\(^6,16\). This technique assessed the responsiveness of mice to presentations of loud auditory stimuli (conspecific distress cries), as indicated by the capacity of these stimuli to interrupt drinking. Typically, stimuli given in a short session become progressively less effective in interrupting drinking, and this reduction in interruptability constitutes short-term habituation. Long-term habituation is evi-
enced by the fact that this reduction in responsiveness to auditory stimuli can persist for several days.

Mice were fluid deprived for 48 h and then for the duration of the experiment were permitted each day to drink water for 3 min in 12 cm × 13 cm × 19 cm high chambers constructed of 3 mm white Plexiglas. To maintain body weight, mice were given an additional 7 min access to water in their home cages at the end of each daily 3-min drinking period. Each lick of water emitted during the 3-min drinking period activated a pen on an event recorder. The total fluid volume consumed during each drinking period was also noted. A recording of a distress cry was obtained by exposing a male hybrid mouse (C3H × Balb/C) to inescapable footshock (0.4 mA). For presentation, the cries were amplified to a volume of 106 dB (measured 3 cm above the floor of the closed drinking chamber with a Brüel and Kjaer sound level meter, Type 2209, C scale, slow ballistic response).

The mice drank undisturbed for 7 days while the fluid volume consumed during each drinking period stabilized. Distress cries were first presented on the following day (Day 1). The stimuli were 4 repetitions of a 4-sec conspecific distress cry, delivered through a 10 cm round loudspeaker (Oaktron AA3C) mounted in the top of each drinking chamber. The first distress cry occurred 15 sec after the beginning of the 3-min drinking period, and the 3 repetitions of this stimulus occurred at 15-sec intervals. To assess long-term habituation, mice heard the same recorded distress cry during their daily drinking period 3 days later (Day 4). No distress cries were presented during the intervening days. On these days, mice drank for their usual 3-min period without disturbance.

Anisomycin (30 mg/kg) and lithium chloride (150 mg/kg) were injected subcutaneously and intraperitoneally, respectively, in saline solution (0.3 ml/25 g body weight). Statistical comparisons between groups were made with Mann-Whitney U-tests.

Mice exhibited considerable within-session habituation to the 4 distress cries presented on Day 1. Fig. 1A indicates the median number of licks of water emitted by mice during each distress cry. Mice not drinking during the 4 sec prior to the first distress cry on Day 1 were eliminated from the experiment (N = 18/166 = 10.8%). Mice emitted a significantly greater number of licks during the second and third stimuli than during the first one (P < 0.01). The low number of licks emitted during the fourth stimulus was due to the fact that by this time many mice (54/148) had already stopped drinking. Since the effect of a stimulus on drinking could not be determined for mice not drinking at stimulus onset, including the data for all mice probably underestimates the extent of within-session habituation. Habituation was decidedly more pronounced if only those mice were considered that emitted > one lick of water during the 4 sec prior to stimulus presentation (Fig. 1A).

For the same reason, the best measure of long-term habituation was the number of licks of water emitted during the first distress cry of the retention test. Nearly all mice (93 %) emitted > 10 licks of water during the 4 sec prior to the first distress cry of the retention test. With each successive stimulus, however, the probability increased that a mouse had already stopped drinking well before stimulus onset. Long-term
Fig. 1. Thirsty mice heard 4 distress cries (106 dB for 4 sec) at 15-sec intervals, during a 3-min drinking period. Successive stimuli were progressively less effective in interrupting drinking (A, above). Data are shown for all mice tested, and also for those mice (percentages indicated) emitting ≥ one water lick during the 4 sec prior to each stimulus. The median number of water licks during the 4 sec prior to each stimulus was 34, 34, 29, 15. The distress cries on Day 1 were followed immediately by injections of saline, anisomycin (Ani-0 Hrs), lithium chloride (LiCl-0 Hrs), or by a delayed injection of anisomycin (Ani-8 Hrs.) Three days later (B, above), a distress cry interrupted the fluid intake of the Ani-0 Hrs group to a greater extent than it interrupted the fluid intake of each of the other 3 groups (P < 0.01). During the 4 sec prior to the distress cry on Day 1, the median number of water licks was 33, 34, 32, and 34 for mice given saline, LiCl, Ani-8, or Ani-0, respectively. During the 4 sec prior to the first distress cry on Day 4, the corresponding values were 34, 34, 34, and 32.

habituation was therefore assessed by comparing the number of licks emitted during the first distress cry on Day 1 with the number of licks emitted during the first distress cry on Day 4 (Fig. 1B).

Mice were given saline, lithium chloride, or anisomycin immediately after the drinking period on Day 1. Since anisomycin itself could possibly have produced a
TABLE I
MEAN FLUID INTAKE (ml) DURING DAILY 3-MINUTE DRINKING PERIODS

Mice were given the indicated drugs immediately or 8 h after the drinking period on Day 1. None of the differences between groups on Day 1 or Day 4 were significant (P > 0.1).

<table>
<thead>
<tr>
<th>Day</th>
<th>Saline-0 Hrs</th>
<th>LiCl-0 Hrs</th>
<th>Ani-8 Hrs</th>
<th>Ani-0 Hrs</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>1.0</td>
<td>1.0</td>
<td>1.0</td>
<td>1.0</td>
</tr>
<tr>
<td>2</td>
<td>0.9</td>
<td>0.7</td>
<td>0.6</td>
<td>0.5</td>
</tr>
<tr>
<td>3</td>
<td>1.0</td>
<td>0.9</td>
<td>0.9</td>
<td>0.7</td>
</tr>
<tr>
<td>4</td>
<td>1.2</td>
<td>1.2</td>
<td>1.1</td>
<td>1.0</td>
</tr>
</tbody>
</table>

Persisting effect on startle, another group was given anisomycin 8 h after the drinking period (Ani-8 Hrs) and tested again on Day 4. Mice given anisomycin immediately after the drinking period on Day 1 emitted significantly fewer licks during the first distress cry on Day 4 than each of the 3 control groups (P < 0.01). Taken together, these results indicate that long-term habituation to the distress cries persisted for at least 4 days; and that anisomycin given immediately after the distress cries on Day 1 impaired long-term habituation.

The lithium chloride groups were designed to evaluate the possibility that conditioned aversion to water was established by anisomycin on Day 1, and that this aversion was subsequently responsible for the observed suppression of drinking on Day 4. The present dose of lithium chloride (150 mg/kg) had been determined previously to produce as much conditioned gustatory aversion as the 30 mg/kg dose of anisomycin\textsuperscript{13}. Mice given lithium chloride immediately after the drinking period on Day 1 behaved on Day 4 similarly to control mice (P > 0.2 for each comparison), and also drank more water during the first distress cry of the retention test than the Ani-0 Hrs group (P < 0.01). These results suggest that conditioned aversion produced by anisomycin was not responsible for the impairment of long-term habituation.

Table I indicates the total fluid volume ingested by mice on Days 1-4. On Day 2 the saline group drank more than each of the other drug groups (P < 0.05), and the drug groups did not differ from each other (P > 0.05). By Day 4 fluid intake had returned to the pre-injection, Day 1 level for all groups and none of the between-group comparisons were significant (P > 0.1). An analysis of drinking during the 4 sec prior to the distress cries also suggested that by Day 4 mice had recovered from effects of the drugs on fluid intake. By Day 4 each group had returned to its Day 1 level and the 4 groups were drinking at the same median rate during the 4 sec prior to the first distress cry (saline = 34; LiCl = 34; Ani-8 = 34; Ani-0 = 32; P > 0.4).

Four conspecific distress cries produced within-session habituation, and between session habituation that lasted at least 3 days. Long-term habituation was partially disrupted by initiating a period of protein synthesis inhibition immediately after presentation of the distress cries. It seems unlikely that anisomycin caused suppression of fluid intake during the distress cry on Day 4 by causing greater conditioned gustatory aversion than the other drugs. First, during the 3-min retention sessions total
fluid intake was similar across drug groups. Second, during the retention sessions on Day 4, mice in each drug group drank at about the same rate during the 4 sec prior to the distress cry.

The present results differ from an earlier report that cycloheximide did not affect long-term habituation of exploratory activity in mice. Since overtraining is known to reduce markedly the susceptibility of mice to the amnesic effects of protein synthesis inhibitors, it seems possible that mice were relatively overtrained in the earlier study. It is also possible, of course, that there are other important, and as yet unidentified, differences between exploratory habituation and the present habituation paradigm.

Anisomycin produced only partial amnesia for long-term habituation (Fig. 1B). This finding is consistent with previous reports that anisomycin or cycloheximide can produce partial amnesia for discrimination training or passive avoidance training. In the present study, considerable protein synthesis required for long-term retention could have occurred during the habituation session on Day 1 before the establishment of inhibition by the post-session injection. Approximately 3 min elapsed between the first distress cry and the drug injection, and several more minutes must have elapsed before a substantial level of inhibition could have been achieved. It has been determined that the present dose of anisomycin (30 mg/kg) inhibits more than 90% cerebral protein synthesis during the 15–45 min period after injection, and that protein-synthesizing capacity gradually recovers during the subsequent 2–8 h (ref. 4).

The possible involvement of side effects of protein synthesis inhibitors has been considered repeatedly; and known effects of anisomycin on locomotor behavior, tyrosine hydroxylase activity and conditioned gustatory aversion have been dissociated from amnesia. In addition, as discussed previously, anisomycin, cycloheximide, and puromycin each inhibits cerebral protein synthesis and causes amnesia, but each has a different mechanism of action and exerts different side effects. All available evidence is, therefore, consistent with the hypothesis that anisomycin inhibits protein synthesis required for the formation of ‘long-term’ memory. Nevertheless, it is always difficult in behavioral pharmacological studies of this type to attribute definitively the behavioral effects of a drug to its major known mode of action.

Previous work has suggested that cerebral protein synthesis is required for ‘long-term’ memory of discrimination training, and active and passive avoidance learning. The present results suggest that this conclusion can be extended to habituation, a phylogenetically primitive form of long-lasting behavioral plasticity.

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