

## Modulation of Long-Term Memory by Delayed Administration of the Amide of L-Pyroglutamyl-D-alanine, a Nootropic Agent, in Spaced and Massed Training in Rats

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The effects of the nootropic agent L-pyroglutamyl-D-alanine amide (given at a dose of 0.5 mg/kg i.p., 24 h after training) on long-term memory were studied in rats with contextual and conditioned reflex freezing. Spaced and massed training protocols were used; habit formation was tested 72 h after training. In massed training, which led to lower levels of learning as compared with spaced training, the dipeptide improved contextual memory, while in spaced training, which led to higher initial learning levels, dosage with dipeptide improved reproduction of the habit. Testing of responses to the conditioned signal (a sound) 96 h after training, revealed no significant differences between groups. This is evidence for the selectivity of the effect of L-pyroglutamyl-D-alanine amide for contextual memory.

**KEY WORDS:** Learning, memory, dipeptide, nootrope.

Training is termed spaced when two or more repeated training acts are separated by a time interval rather than following immediately one after the other, as in massed training [15].

Spaced repetition of training sessions produces better long-term memory than massed learning in a great diversity of conditions [9, 15, 24]. This phenomenon is general in biological systems, and is seen in both humans and animals [15].

However, despite numerous experimental studies [13, 14, 16, 24, 25], the exact causes of differences in the properties of memories formed in massed and spaced training remain to be determined. Only recently have a number of indications appeared which suggest that these differences may have their basis in processes associated with the dynamic regulation of gene expression during learning. Thus, Tully's

group have obtained data in a model of associative olfactory learning in *Drosophila* showing that memory formed in spaced training depends on protein synthesis and on activation of the CREB gene, which encodes a constitutive transcriptional factor, while memory formed during massed training is independent of these processes [27, 28]. A similar relationship between expression of the CREB gene was also found in mouse models of learning [20]. Mice bearing a directed deletion in the CREB gene had disrupted long-term memory in a series of massed-training tasks, and the genetic defect was overcome by spaced training, which led to the formation of normal long-term memory. A similar phenomenon has been seen in three training models—spatial training, social transmission of taste preference, and training to contextual and conditioned-reflex freezing [20].

The latter model is of special interest for studies of memory mechanisms. The freezing state is an indicator that an animal is expecting a biologically dangerous event [12]. We believe that the freezing reaction quite clearly shows that an animal has the apparatus for foreseeing a future outcome, the

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apparatus for anticipating a reflection of reality—i.e., a result-of-action acceptor, one of the key components of the central architecture of the functional systems underlying voluntary behavior [1, 8]. This leads to the possibility of performing experimental analysis of the processes of formation and storage of result-of-action acceptors and the mechanisms of their extraction from memory in response to trigger and contextual stimuli. A particular advantage of the contextual and conditioned-reflex freezing model is that in this model, the animal can be induced to freeze by contextual afferentation and in response to a trigger signal [12]; studies performed in recent years have demonstrated that different molecular and anatomical substrates are involved in these two mechanisms.

In particular, lesioning of a variety of rat brain structures was used in [23] to show that the amygdala is involved in forming both contextual and conditioned-reflex freezing, while the hippocampus is associated only with contextual freezing. The role of the hippocampus in processes specifically associated with freezing in contexts in which pain had previously been encountered was also demonstrated in [19]. Recent studies have also shown that mice with directed mutations in the CREB gene have significantly greater disruption in memory for contextual than for conditioned-reflex freezing [10].

In view of these points, we used a model of training to contextual and conditioned-reflex freezing in rats to study possible mechanisms of the difference between massed and spaced training.

One approach to analyzing the characteristics of memory formation in spaced and massed training conditions consists of using nootropic agents. These substances, which stimulate higher integrative brain functions, can act on memory formation [5, 6, 26]. In terms of the objectives of these studies, it was particularly important that their effects should be associated with the quite selective potentiation of long-term memory [21], at the same time as characterizing massed and spaced forms of learning. In the present work we used L-pyroglutamyl-D-alanine amide, which was constructed as a peptide analog of piracetam on the basis that piracetam has a peptidergic mechanism of action [4]. This compound has been shown to be able to increase the rate of learning in passive and active escape tests at doses 500-1000 times lower than the effective doses of piracetam, and has been shown to be active when given by the i.p. and p.o. routes [7]. An important criterion for selecting this compound for the present studies was the fact that it eliminates deficits in long-term hippocampal potentiation due to alcohol [11], which

shows that its effects may be mediated by actions on the mechanisms of hippocampus synaptic plasticity.

Thus, the aim of the present work was to study the effects L-pyroglutamyl-D-alanine amide on memory induced by spaced and massed training in a model with contextual and conditioned-reflex freezing in rats.

Functional systems theory suggests that the action acceptor can be actualized at the time the external event is most likely to occur. This concept is in agreement with experiments on various animal species.

For example, P. K. Anokhin's studies on dogs [1] provided experimental evidence for cyclical changes in the defensive dominant between sequential reinforcements associated with conditioned stimulation with an electric shock, as indicated by withdrawal of the stimulated limb in the absence of a shock. Withdrawal became more frequent as the time for applying the next painful stimulus approached; this is evidence that the animal's central nervous system approached a state of a defensive dominant, increasing the level of excitability of all those nervous structures and their working machinery which keep the animal in a state of anxious readiness for the stimulatory event [1].

Rat experiments using models of training to passive and active escape showed that there are time "windows" for optimal reproduction of the acquired habit at 12, 24, 36, 48, and 60 h after training, with this effect being expressed at the highest level at 24 and 48 h [17, 18]. This phenomenon was shown to be independent of the time of day at which training was performed [18], and the effect disappeared some 72 h after training [17]. Such periods for the best production of a habit associated with endogenous actualization of the corresponding result-of-action acceptor might also exist in the conditions applying to a model of contextual and conditioned-reflex freezing. Thus, L-pyroglutamyl-D-alanine amide was given 24 h after training in the present experiments.

## METHODS

Studies were performed using Wistar rats weighing 300-450 g. Animals were kept in cages (type T4) in groups of 6-7 animals per cage, with free access to standard feed and water. Before training, rats were handled once daily for five days, by picking them up as though to give i.p. injections. L-Pyroglutamyl-D-alanine amide and isotonic NaCl were given i.p. L-Pyroglutamyl-D-alanine amide was dissolved

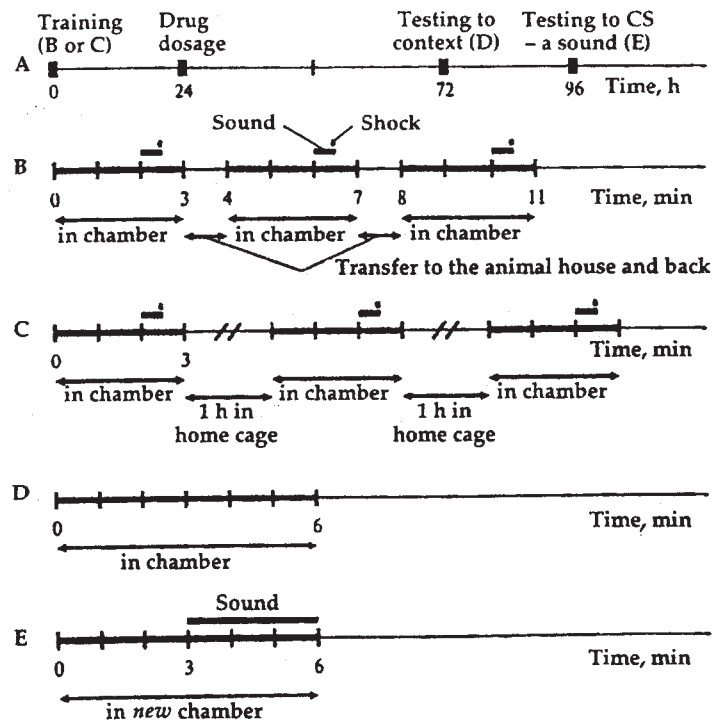


Fig. 1. Overall diagram of the experiment (A), diagrams of massed training scheme (B), the spaced training scheme (C), testing to context (D), and testing to the conditioned stimulus (CS, a sound) (E).

in isotonic saline immediately before use for injections. The dose used was 0.5 mg/kg.

Two chambers were used in these experiments: a Biotest RK-5301 and a round chamber prepared in the laboratory. The Biotest RK-5301 was illuminated with a daylight lamp, and was a rectangular chamber of 25 × 30 × 25 cm with an electrically conducting floor made of metal rods 3 mm in diameter and located 10 mm apart; a loudspeaker was mounted one of the chamber walls. The round chamber was a Plexiglas cylinder of size 20 × 32 cm, lying on its side.

Animals were transferred from the animal house to the experimental room and back using a standard cage when animals were to be trained and tested to contexts, and a closed cage when animals were to be tested with the conditioned signal.

Figure 1, A shows the overall experimental scheme. The experiment started with massed (group M,  $n = 19$ ) or spaced (group S,  $n = 17$ ) training (Fig. 1, B and C respectively). Animals of each group received either test compound (groups MT,  $n = 10$ , and ST,  $n = 9$ ) or isotonic saline (groups MC,  $n = 9$ , and SC,  $n = 8$ ) 24 h after training. Testing to context was performed 72 h after training (Fig. 1, D), and testing to the conditioned signal was performed 96 h after training (Fig. 1, E). Thus, each animal was

tested twice—72 and 96 h after training. The two test sessions were separated by a time interval of 24 h, to reduce the effect of the first on the second.

Training and testing to context were performed using the Biotest RK-5301 chamber; testing to the conditioned signal was performed using the round plastic chamber.

Training consisted of three sessions. In the case of massed training (Fig. 1, B), the interval between sessions was 1 min; in spaced training (Fig. 1, C), the interval was 1 h. Rats were placed in the chamber for 3 min in each sessions. A sound of 2400 Hz (the conditioned signal), lasting 30 sec, was presented when the rats had completed 2 min in the chamber. Rats were presented with an electric current (0.28 mA, 50 Hz) 2 sec before the sound ended; sound and shock were switched off together. Rats were left in the chamber for a further 30 sec after the sound and shock had ended.

For testing to context, rats were placed in the chamber in which they had been trained, and were left there for 6 min (Fig. 1, D). For testing to the conditioned signals, rats were placed in a new chamber (round), again for 6 min (Fig. 1, E). The sound was presented for 3 min, starting at the end of the third minute.

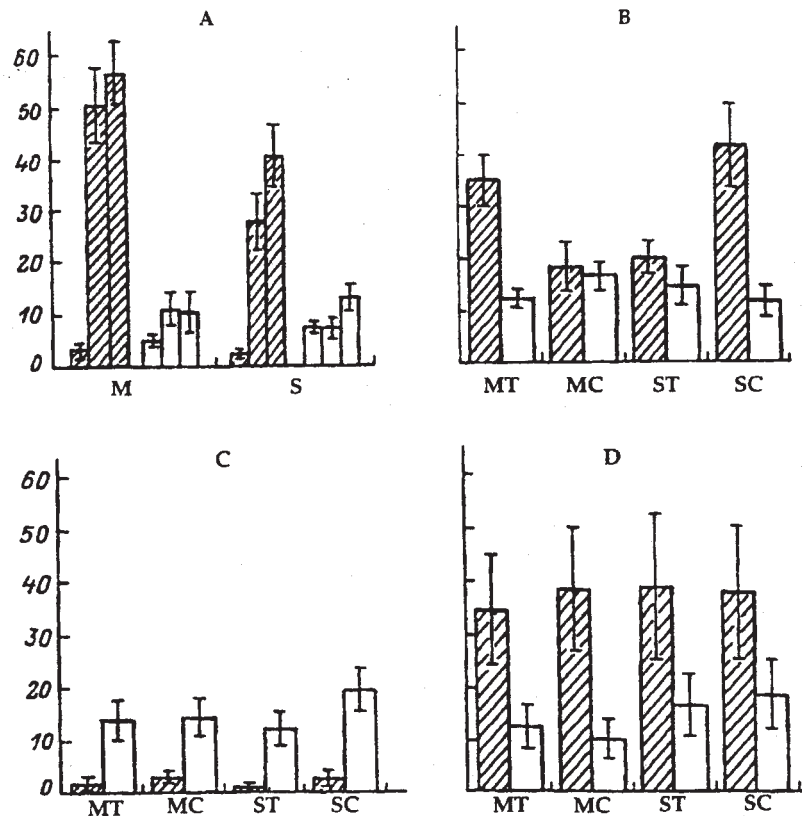


Fig. 2. Freezing (shaded columns) and washing (white columns) on training before presentation of the sound (A), testing to context (B), testing to the conditioned signal before its presentation (C), and during its presentation (D). Vertical axes show freezing or washing as a proportion of the total recording time, %. A) M indicates massed training, S indicates spaced training. Groups of three columns represent three sequential training sessions. B, C, D) MT and ST are massed and spaced training with drug (L-pyroglutamyl-D-alanine amide), MC and SC are massed and spaced training with isotonic saline.

During training and testing, the absolute durations (in sec) of freezing and washing were measured. Behavioral recording was carried out visually, with immediate input of data into a computer via the keyboard.

The significance of differences between groups was assessed using the nonparametric Wilcoxon-Mann-Whitney test.

## RESULTS AND DISCUSSION

The experimental results are shown in Fig. 2. This indicates that the initial levels of freezing in animals of the various groups were not significantly different ( $p < 0.75$ ). The duration of freezing increased from the first training session to the third training session in both the massed- and spaced-training groups (Fig. 2, A). The increase was slightly faster in

the group in which massed training was used than in the group with spaced training. In the second training session, the difference was significant at the level of  $p < 0.017$ , though in the third session the difference ceased to be statistically significant ( $p < 0.084$ ), suggesting that the two training protocols were essentially equieffective.

Results obtained from testing to context are shown in Fig. 2, B. The differences between the groups subjected to massed (MC) and spaced (SC) training and given isotonic saline were statistically significant ( $p < 0.034$ ). The spaced-training group showed much stronger freezing than the group subjected to massed training.

Administration of L-pyroglutamyl-D-alanine amide 24 h after massed training had a positive effect on long-term memory: the animals demonstrated an increase in freezing on testing to context ( $p_{MTMC} < 0.030$ ). In animals subjected to spaced training, the



same dose of the same substance had a negative effect on subsequent extraction from long-term memory, decreasing freezing in this experimental group as compared with context-testing results for the control group ( $p_{SCMC} < 0.034$ ).

Thus, the results of testing for contextual signals, 72 h after training, show that: 1) spaced training leads to a higher level of learning than massed training (this is in agreement with data published by other groups [15, 20]); 2) administration of i.p. L-pyroglutamyl-D-alanine amide (0.5 mg/kg) 24 h after training (48 h before testing) significantly modified the animals' behavior, this modification being in opposite directions for animals subjected to massed and spaced training—an increase in memory performance in massed training and a reduction in memory in spaced training.

The results obtained from testing to the conditioned signal are shown in Fig. 2, C (the animals' behavior before presentation of the conditioned signal) and Fig. 2, D (during presentation of the conditioned signal).

Figure 2, C shows that there were no significant differences in testing to the conditioned signal. This result is evidence that the effect of the nootrope used here is specific for contextual memory, and agrees with data obtained by Morris [22] on the selective efficacy of nootropes for hippocampus-dependent spatial memory in a water maze model.

The finding that L-pyroglutamyl-D-alanine amide is active when given at a time well separated from training and testing is an important result of this present study. This phenomenon shows that the time interval of about 24 h after training is a sensitive period for the action of some memory-affecting chemical compounds; this may be associated with continuing active processing of contextual memory traces and linked with actualization of the results-of-action acceptor. It is of note that dosage with L-pyroglutamyl-D-alanine amide at this time had no effect on the other component of memory extracted using a triggering conditioned signal.

In general, the results obtained here are in good agreement with the hypothesis that the nootropic agent tested here acts on the hippocampal component of memory. Previous investigations have demonstrated that lesioning of the hippocampus one day after training to a freezing reaction disturbs contextual but not conditioned-reflex memory in rats [19]. Our data demonstrate for the first time that these two forms of memory differ not only in terms of the neuroanatomical substrate supporting them, but also in terms of the chemical mechanisms of processing at late post-training time points.

The fact that L-pyroglutamyl-D-alanine amide has activity in opposite directions depending on the use of spaced or massed training sessions is evidence that even 24 h after completion of the training session, the memories formed by these two forms of training have different chemical mechanisms and different sensitivities to modulating substances. This fact adds to the picture of previously identified differences in the molecular mechanisms of these types of memory [17, 24, 25] and draws attention to the persistence of these differences long after training has finished.

The phenomenon of nootrope-induced worsening of contextual memory formed by spaced training is unusual. In general, this result does not contradict the widely held idea that peptides play regulatory roles [2, 3], though it is difficult to apply this interpretation at the molecular level to the present results.

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