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Late Posttraining Memory Processing by Entorhinal Cortex: Involvement of NMDA and GABAergic Receptors

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FERREIRA, M. B. C., R. C. DA SILVA, J. H. MEDINA AND I. IZQUIERDO. *Late posttraining memory processing by entorhinal cortex: Involvement of NMDA and GABAergic receptors.* PHARMACOL BIOCHEM BEHAV **41** (4) 767–771, 1992.—The NMDA receptor antagonist, D-2-amino-5-phosphonopentanoic acid (AP5) (5 μg) and the $GABA_A$ receptor agonist, muscimol (0.03 μg) were infused bilaterally into the entorhinal cortex of rats 0, 90, 180, or 360 min after training in habituation to a novel environment or in step-down inhibitory avoidance. Animals were tested for retention 22 h after training in each task. AP5 and muscimol were amnestic for both tasks when given 90 or 180 min after training, but had no effect when given 0 or 360 min after training. In contrast, intraamygdala injections or AP5 or muscimol were amnestic when given 0 but not 90 min after inhibitory avoidance training. The results indicate that the entorhinal cortex plays a late but important role in posttraining memory processing; this role involves glutamatergic NMDA receptors and is inhibited by $GABA_A$ receptors. The intervention of the entorhinal cortex in posttraining memory processing is subsequent, and could be secondary, to that of the amygdala and other limbic structures.

Posttraining memory processing Memory consolidation Entorhinal cortex NMDA receptors $GABA_A$ receptors

THE immediate posttraining infusion of agonists or antagonists of glutamatergic N-methyl-D-aspartate (NMDA), cholinergic muscarinic, beta-noradrenergic, or gamma-amino butyric type A ($GABA_A$) receptors into the amygdala, medial septum, and dorsal hippocampus alters memory in rats (7,16). This suggested that similar, parallel mechanisms in the three structures play a role in memory consolidation (6,7). Possibly, each of these regions handles different aspects or types of memory since inhibitory avoidance was affected by treatments administered into the amygdala, septum, and hippocampus, whereas habituation to a novel environment was altered only by the intrahippocampal treatments (6,7,16). Inhibitory avoidance is a highly alerting, aversive task that uses working memory and spatial and olfactory cues; the habituation of exploration of a novel environment, on the other hand, is to a large extent a purely spatial/olfactory task in the rat (6,7,16). The amygdala is involved in the processing of alerting and aversive memories (2); the medial septum and hippocampus play a role in working memory and in the processing of spatial and olfactory information (1,9). Due to their different input/output connections, it is likely that the specific role played by the medial septum and hippocampus in these type of memory may be different. Indeed, exploration enhances hippocampal unit excitatory postsynaptic potentials (EPSP's) to perforant path stimulation, but this is unhindered by temporary or permanent inactivation of the medial septum (1).

The parahippocampal region, particularly the entorhinal cortex, is directly interconnected with the amygdala and hippocampus and indirectly with the medial septum (4,15). Thus, it is strategically located to integrate memory-relevant information previously processed by these brain regions (7). Small, restricted lesions of the parahippocampal cortex in primates cause profound memory deficits, larger, in fact, than those produced by isolated or combined amygdala and hippocampal lesions (17). In a recent study, Sif et al. (12) measured brain regional metabolic activity by the incorporation of 2-deoxyglucose at 15 or 215 min from nonaversive training in a Skinner box. The septum and hippocampus showed an increased 2-deoxyglucose uptake 15 min after training, whereas the entorhinal cortex did so only at 215 min. This suggested that

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the septum and hippocampus are activated earlier than the entorhinal cortex following the training experience. The amygdala showed no changes in this study, possibly because this was not a highly alerting or aversive task [see (2)].

Here, we examine the effect of D-2-amino-5-phosphonopentanoic acid (AP5) and muscimol infused into the entorhinal cortex at various times after training on the retention test performance of inhibitory avoidance and habituation to a novel environment in rats. AP5 is an antagonist at glutamatergic NMDA receptors and muscimol is an agonist at $GABA_A$ receptors. These are respectively the most abundant excitatory and inhibitory receptors in the brain [see (6,7)] so both AP5 and muscimol should be expected to seriously hinder the activity of the area into which they are infused.

METHOD

Subjects and Surgical and Injection Procedures

For the study on the entorhinal cortex, 142 Wistar rats (median weight, 250 g) were bilaterally implanted under thionembutal anesthesia (30 mg/kg, IP.) with 27-ga guide cannulae aimed 2.0 mm above the surface of the entorhinal cortex at coordinates A 6.8 from bregma, L 5.0 from midline, and V 6.8 of the atlas by Paxinos and Watson (10). Four animals were used to study the spread of methylene blue from the injection site and 138 were used to study behavior.

For the study on intraamygdala injections, 40 male animals (median weight, 255 g) were implanted with cannulae aimed 1.0 mm above the central amygdala nucleus at coordinates A −2.3, L 4.2, and V 7.4 of the atlas by Paxinos and Watson (10).

For injection (see below), a 30-ga cannula connected by a polyethene tube to a 5-μl microsyringe was fitted into the guide cannula. The tip of the injection cannula protruded 1.0 mm beyond that of the guide. The spread of injections given into the entorhinal cortex was determined in four rats given microinjections of 0.5 μl 0.2% methylene blue and sacrificed by decapitation 30 min later. As shown in Fig. 1, the dye spread in a cone-line fashion from the injection site; it reached the entorhinal cortex but did not invade the perirhinal cortex, amygdala, or hippocampus. Injection placements were verified by histology as described elsewhere (7,16). Placements were found to be correct in 132 of the 138 animals used for behavioral studies and in the 4 animals used for methylene blue injection (Fig. 1). Only data from the animals with correct cannula placements were analyzed.

The cannulae aimed at the amygdala were found to be correctly placed [i.e., within the central, lateral, or basolateral nuclei, (7,16)] in 37 of 40 animals. These will not be illustrated here for the sake of brevity.

Behavioral Procedures

Three to 18 days after surgery, animals with cannulae in the entorhinal cortex were trained and tested over 3 consecutive days in two different tasks. The same apparatus was used for both tasks: a 50 × 25 × 25 cm acrylic box whose floor

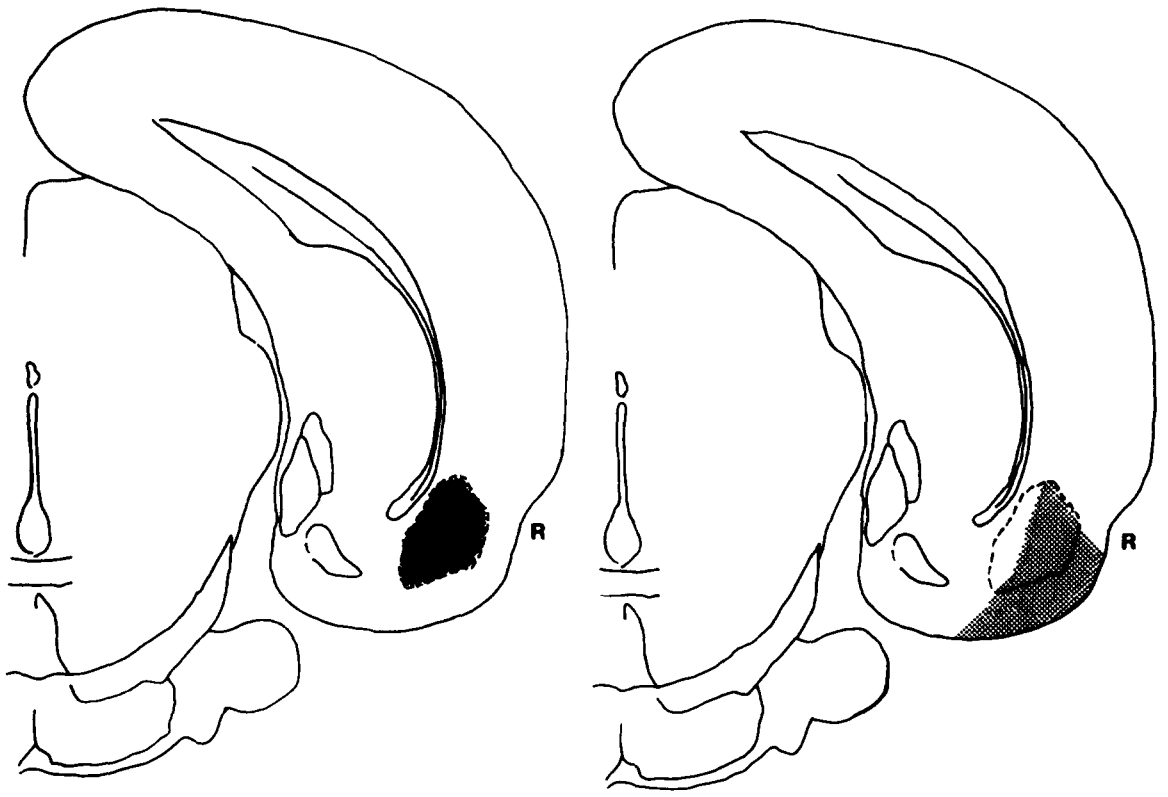

FIG. 1. Plane A −6.8 of the rat brain atlas of Paxinos and Watson (12). R indicates the rhinal fissure. The broken lines in both drawings encircle the area in which the tips of the cannulae aimed above the entorhinal cortex were placed in 136 of 142 animals. In the left drawing, this area is filled with a dark stipple; in the right drawing, it is superimposed upon a light stippled triangle-like area that represents the region dyed by methylene blue infused through the injection cannulae in four of the animals.

was a grid of 1.0-mm caliber steel bars; a 5-cm high, 8-cm wide formica platform covered the left end of the grid. The tasks were: habituation to the apparatus and step-down inhibitory avoidance (7,16). Training–test interval was 22 h in both tasks. Avoidance training was carried out within 2 h of habituation testing. In animals implanted in the amygdala, only the avoidance task was studied.

In the two tasks, both sessions started in the same way: Animals were gently placed on the platform facing the rear left corner and their latency to step down placing their four paws on the grid was measured. Following this, in the habituation task, the number of rearings and number of crossings of two imaginary lines on the grid, at 14 and 28 cm from the border of the platform, were counted in the training and test sessions (duration of each session = 1 min) (7,16). Stepping down from or back onto the platform occurred in all animals and were counted as crossings. All animals in all groups made more crossings in the first than in the second half of the training session (data not shown; differences within each group, $p < 0.001$ in sign tests). Thus, there was evidence of habituation during the training session in all animals. The retention of habituation was measured by the test minus training difference in performance of crossings and rearings.

In the training session of the inhibitory avoidance task, as soon as animals stepped down from the platform placing their four paws on the grid they received a 0.5-mA, 6-s scrambled foot-shock. In the test session, no foot-shock was given. The test minus training step-down latency difference, to a ceiling of 180 s, was taken as a measure of retention (7,16).

Animals implanted in the entorhinal cortex received 0, 90, 180, or 360 min after training in each task a bilateral microinjection of vehicle (0.1 M sodium phosphate buffer in saline to a pH of 7.4), of the NMDA receptor antagonist, (AP5) (5.0 μg), or of the $GABA_A$ agonist, muscimol (0.03 μg) dissolved in vehicle. Injection volume was 0.5 μl in all cases. The doses of AP5 and muscimol used here had been found to block consolidation completely when given immediately posttraining into the amygdala, medial septum, or hippocampus (7). Animals implanted in the amygdala received vehicle, AP5, or muscimol infusions only at 0 and 90 min from training.

RESULTS

There were no significant group effects on acquisition parameters. In the habituation task, differences among groups in training step-down latency (overall mean, 7.0 s) or in the number of crossings (overall mean, 13.8) or rearings (overall mean, 10.7) in the training session were not significant [$F(11,120) = 0.7$, 0.9, and 0.8, respectively, $p > 0.1$, in all cases]. In the avoidance task, differences among groups in training step-down latency were also not significant [for entorhinal cortex, overall mean, 6.2 s, $F(11,120) = 1.9$, $p > 0.1$; for amygdala, overall mean, 6.5 s, $F(5,31) = 1.2$, $p > 0.1$].

Effects of the treatments given into the entorhinal cortex on retention are shown in Tables 1 (habituation) and 2 (inhibitory avoidance). In the two tasks, AP5 and muscimol were amnestic when given 90 or 180 min, but not 0 or 360 min, after training. In the habituation task, amnesia was expressed by the reduction of the test minus training difference in crossings and rearings. In this task, muscimol was slightly more amnestic at 90 than at 180 min (Table 1). As is usual in this habituation task (7,16), test minus training step-down latency differences were not significant.

In the avoidance task, amnesia was expressed by the reduction of test minus training step-down latency difference (7,16). In the avoidance task, both the effect of AP5 and muscimol were slightly larger at 90 than at 180 min (Table 2).

TABLE 1

EFFECT OF BILATERAL INFUSION INTO THE ENTORHINAL CORTEX, 0, 90, 180, AND 360 MIN AFTER TRAINING OF VEHICLE, MUSCIMOL (0.03 μg), OR AP5 (5.0 μg) ON THE RETENTION OF HABITUATION TO A NOVEL ENVIRONMENT IN RATS

			Mean ± SEM Test Minus Training Difference		
Time (min)	Treatment	n	Step-Down Latency (s)	Number of Crossings	Number of Rearings
0	Vehicle	12	3.0 ± 1.2	3.4 ± 0.8	4.1 ± 0.8
	Muscimol	11	1.3 ± 1.6	5.2 ± 1.0	4.7 ± 0.8
	AP5	12	1.1 ± 1.8	3.5 ± 1.1	5.2 ± 1.1
			(F = 1.4, NS)	(F = 2.3, NS)	(F = 0.8, NS)
90	Vehicle	12	2.8 ± 1.8	4.0 ± 0.7	3.6 ± 0.6
	Muscimol	12	3.3 ± 1.2	−0.3 ± 0.4*	−0.6 ± 0.5*
	AP5	12	2.4 ± 1.7	0.6 ± 0.6*	−0.5 ± 0.5*
			(F = 0.1, NS)	(F = 14.9, $p < 0.01$)	(F = 19.6, $p < 0.01$)
180	Vehicle	11	1.7 ± 2.0	4.4 ± 0.5	3.6 ± 0.5
	Muscimol	10	−1.7 ± 1.8	2.1 ± 0.7†	0.9 ± 0.9*
	AP5	11	−0.2 ± 1.6	1.2 ± 0.7*	0.2 ± 0.6*
			(F = 0.9, NS)	(F = 6.2, $p < 0.01$)	(F = 7.3, $p < 0.01$)
360	Vehicle	9	2.4 ± 1.5	3.3 ± 0.8	3.8 ± 1.3
	Muscimol	10	0.5 ± 1.3	4.1 ± 1.0	3.7 ± 0.9
	AP5	10	2.4 ± 1.7	3.5 ± 1.1	4.5 ± 0.8
			(F = 0.8, NS)	(F = 0.4, NS)	(F = 0.3, NS)

F values and significance levels given below the figures for each group. (NS), not significant at $p < 0.1$ level. *Significant difference from vehicle group at $p < 0.01$ level in Newman-Keuls test; †same at $p < 0.05$ level. The effect of muscimol on both crossings and rearings was significantly higher at 90 min than at 180 min ($p < 0.05$ level in Newman-Keuls test).

TABLE 2

EFFECT OF BILATERAL INFUSION INTO THE ENTORHINAL CORTEX, 0, 90, 180, AND 360 MIN AFTER TRAINING OF VEHICLE, MUSCIMOL (0.03 μg), OR AP5 (5.0 μg) ON RETENTION OF STEP-DOWN INHIBITORY AVOIDANCE IN RATS

Time (min)	Treatment	n	Median (Interquartile Range) Test Minus Training Difference in Step-Down Latency (s)
0	Vehicle	11	47.9 (11.2/55.0)
	Muscimol	11	60.0 (42.4/104.5)
	AP5	10	63.4 (5.1/0.0)
90	Vehicle	12	52.1 (16.2/76.9)
	Muscimol	12	3.5 (2.7/4.9)*
	AP5	12	0.0 (−1.2/3.0)*
180	Vehicle	12	48.7 (36.1/79.0)
	Muscimol	12	12.5 (6.8/19.7)†
	AP5	12	16.5 (9.0/22.5)†
360	Vehicle	10	46.5 (10.8/53.8)
	Muscimol	10	42.7 (15.9/60.7)
	AP5	9	32.9 (9.3/75.1)

All differences in training latencies not significant at $p < 0.1$ level (F values, 1.3, 1.1, 1.9, and 1.7, respectively). *Significant difference from vehicle group at $p < 0.002$ level in Mann-Whitney U-test, two-tailed; †same and in addition significantly higher than 90-min group treated with the same drug at $p < 0.02$ level.

The effect of intraamygdala injections of AP5 and muscimol on retention of the avoidance task is shown in Table 3. The treatments were amnestic when given 0 but not 90 min after training.

DISCUSSION

AP5 and muscimol were amnestic for the two tasks when given into the entorhinal cortex at 90 or 180 min from training. The effect was more intense at 90 min and appeared to wane slightly at 180 min. Therefore, NMDA synapses in this structure appear to be necessary for, and $GABA_A$ synapses to inhibit, memory processing of the two tasks 90–180 min after acquisition.

TABLE 3

EFFECT OF BILATERAL INFUSION INTO THE AMYGDALOID NUCLEUS 0, AND 90 MIN AFTER TRAINING OF VEHICLE, MUSCIMOL (0.03 μg), OR AP5 (5.0 μg) ON RETENTION OF STEP-DOWN INHIBITORY AVOIDANCE IN RATS

Time (min)	Treatment	n	Median (Interquartile Range) Test Minus Training Difference in Step-Down Latency (seconds)
0	Vehicle	7	38.0 (16.8/63.3)
	Muscimol	6	1.1 (−2.4/6.0)*
	AP5	6	2.0 (−5.1/7.3)*
90	Vehicle	6	44.1 (16.9/54.0)
	Muscimol	6	35.0 (13.2/48.0)
	AP5	6	47.9 (13.0/64.0)

All differences in training latencies not significant at $p < 0.1$ level (F values, 0.9, and 2.4, respectively). *Significant difference from vehicle group at $p < 0.002$ level in Mann-Whitney U-test, two-tailed

This time course of the amnestic effect of AP5 or muscimol given into the entorhinal cortex was different from that observed with the same drugs given into the amygdala, medial septum, and hippocampus (7). Like other treatments that alter memory consolidation (6,8), intraamygdala, intraseptal, and intrahippocampal AP5 and muscimol were effective when given immediately after training (7). In the experiment shown in Table 3, intraamygdala AP5 and muscimol were effective when given 0 but not 90 min after training. The data indicate that the entorhinal cortex intervenes in memory processing *after* the amygdala, medial septum, or hippocampus. The results are consistent with those of Sif et al. (12), who reported that entorhinal cortex 2-deoxyglucose uptake increases *after* that of the septum or hippocampus following training in an alimentary task.

The results are also consistent with our previous suggestion (7) that the entorhinal cortex may participate in memory processing *after* the amygdala, medial septum, and hippocampus had each processed a given aspect or component of memory [alertness and aversiveness, working memory, spatial/olfactory information; see (1,3,6,9)]. The entorhinal cortex is directly interconnected with most nuclei of the amygdala and with the hippocampus (4,15) and is therefore indirectly connected with the medial septum as well (7). Lesions of the parahippocampal area including the entorhinal cortex produce severe memory impairment in monkeys (17). Lesions of the entorhinal cortex and neighboring areas are rather typical of Alzheimer's disease (4). The lesion of patient H.M., who developed a pronounced anterograde amnesia following surgery of the temporal lobes, probably includes the entorhinal cortex on both sides [see Fig. 35 of (14)].

There are several possible mechanisms that may substantiate this role of the entorhinal cortex. The entorhinal cortex projects to and receives fibers from the amygdala, hippocampus, parahippocampal cortex, and other regions of the cortex (3,15). Thus, a variety of feedforward loops could build up activity in the entorhinal cortex lasting for several hours. For example, the perforant path could generate hippocampal long-term potentiation [see (1)] and this, in turn, feeds back into the entorhinal cortex. AP5 and muscimol given into the entorhinal cortex would be expected to block both locally generated and reflex activity.

Whatever the mechanism, the present findings suggest a late role of the entorhinal cortex in posttraining memory processing. In light of the literature, it may be presumed that this consists of the integration of memory-relevant information previously processed by the amygdala, hippocampus, and medial septum. Whether this should be called a consolidation or a postconsolidation role depends on what we choose to call consolidation. If usage of this term is restricted to designate the rapid postacquisitional decrease of memory lability (8), a function possibly associated with the amygdala, medial septum, and hippocampus (6,7,16), then the entorhinal cortex would play a postconsolidation role. If one widens the meaning of the term consolidation to include the postacquisitional addition of information (5,13) or other processes that may gradually shape memories over long periods (14), then the delayed posttraining intervention of the entorhinal cortex may be considered part of the consolidation process.

Finally, the late but apparently crucial intervention of the entorhinal cortex in memory processing suggested by the present experiments is indeed remindful of the also late and crucial role of the lobus paraolfactorious in the storage of inhibitory avoidance in the chick brain described by Rose and associates (4,11). As happens in the chick, the indispensability of brain

structures for memory processing in the rat moves from one area to another within a matter of minutes. In the rat, the amygdala, medial septum, and hippocampus are necessary immediately after training (7,16) and the entorhinal cortex becomes necessary later. Whether in the rat the biochemical mechanisms involved in this "transfer" are similar to those that have been suggested for the chick (11) is not known. Interestingly, in all brain regions studied so far—amygdala, medial septum, hippocampus (6,7,16), and now entorhinal cortex,—NMDA receptors appear to be necessary for, and $GABA_A$ receptors to inhibit, the role of those regions in post-training memory processing.

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