# The Effects of Lidocaine Reversible Inactivation of the Dorsal Raphe Nucleus on Passive Avoidance Learning in Rats

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## ABSTRACT

**Introduction:** The role of serotonergic fibers in avoidance learning is controversial. Involvement of the dorsal raphe nucleus (DRN), the main source of hippocampal projecting serotonergic fibers in acquisition, consolidation and retrieval of passive avoidance (PA) learning, was investigated by functional suppression of this area.

**Materials and Methods:** DRN functional inactivation was done by lidocaine (0.5µl, 2%) injection into the DRN, 5 min before training (n=10); and 5 (n=9), 90 (n=10) and 360 min (n=9) after acquisition trial. In the last experiment, lidocaine was injected into the DRN 5 min before the retrieval test , which was 48 h after the training (n=10).

**Results:** Our results showed that PA learning was not impaired by DRN inactivation 5 min before training nor 5 and 360 min after training. Lidocaine injected 90 min after the acquisition trial significantly reduced avoidance of the dark compartment ($P<0.001$). Intra-DRN injection of lidocaine before retrieval significantly increased PA retention ($P<0.001$). Therefore, it seems that DRN has opposite effects on consolidation and retrieval of passive avoidance learning, but it has no effect on PA acquisition.

**Discussion:** It is suggested that functional ablation of DRN may disrupt integrity of subcortical circuits participating in PA consolidation, but DRN inactivation by increasing brain awareness may affect PA retrieval in rats.

## 1. Introduction

The ascending pathways (cholinergic, serotonergic, noradrenergic, dopaminergic and histaminergic), arising from the brainstem and basal forebrain, have important state dependent effect on the information networks of the forebrain underlying higher cerebral functions such as learning and memory (Erb et al., 1997; Meneses, Terron, & Hong, 1998). The cell bodies of neurons that provide 5-HT innervations to the forebrain are mainly located in the dorsal raphe (DRN) and median raphe nuclei (Takagi, Shiosaka , Tohyama , Semba , & Sakanaka, 1980). Ascending projections of these two nuclei travel through the medial forebrain bundle (Takagi, Shiosaka, Tohyama, Semba, & Sakanaka, 1980). Projections from

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the DRN terminate on several neural structures such as hypothalamic nuclei, thalamic nuclei, basal ganglia, spinal cord, septal area, cerebral cortex and hippocampus (Steckler & Sahgal, 1995). Animals with serotonergic system lesions exhibit alterations in tasks aimed at measuring cognitive behavior (Altman, Nordy, & Ogren, 1984; Altman, Normile, Galloway, Ramirez, & Azmitia, 1990; Lorens, Sorensen, & Yunger, 1971; Santucci, Knott, & Haroutunian, 1996). However, the literature concerning the role of serotonin (5-HT) in cognition is controversial. Electrical stimulation of the raphe nuclei disrupted consolidation of passive avoidance learning (Fibiger, Lepiane, & Phillips, 1978). The effect was reversed by pretreatment with p-chlorophenylalanine (PCPA), a 5-HT depleting compound (Fibiger, Lepiane, & Phillips, 1978). In the same way, other studies have shown that treatment with p-chloroamphetamine (PCA), a drug that releases 5-HT, impairs active and passive avoidance (PA) learning (Ogren & Johansson, 1985). In contrast, interference with serotonergic activity has been shown in most instances to enhance both retention and acquisition in similar procedures (Brody, 1970; Wetzel, Getsova, Jork, & Matthies, 1980).

Some studies have shown that permanent lesions of the midbrain raphe nuclei enhance acquisition and retention of avoidance task (Plaznic, Kostowski, Bidzinski, & Hauptmann, 1980), whereas others have shown disruption of acquisition in one way avoidance (Lorens, Kohler, & Guldberg, 1975) and in latent inhibition tasks (Cassaday, Hodges, & Gray, 1991; Asin,Wirtshafer, & Fibiger, 1985). We have shown that the lesion of DRN has no effect on PA retention (Sarihi, Motamedi, Naghdi and Rashidy-Pour, 2000). There is no direct evidence concerning the role of DRN in different phase of PA learning. In the present study, the role of DRN in passive avoidance (PA) learning and memory was investigated by reversible functional inactivation of DRN with lidocaine hydrochloride. This technique has made it possible to define the phase or phases during which a given neural structure is functionally involved in memory processing (Brody, 1970; Asin,Wirtshafer, & Fibiger, 1985). Neuronal blockade with lidocaine has been used to study a variety of behaviors such as avoidance of noxious stimuli (Steckler & Sahgal, 1995) and memory (Plaznic, Kostowski, Bidzinski, & Hauptmann, 1980; Rowan, Cullen, & Moulton, 1990; Sarihi, Motamedi, Naghdi, & Rashidy-Pour, 2000). Therefore, the aim of the present study is to examine the role of DRN in acquisition, consolidation and retrieval of passive avoidance learning by reversible inactivation of DRN with lidocaine in rats.

## 2. Materials and Methods

### 2. 1. Subjects

One hundred two adult male albino rats (220-250 g), 3-months old were obtained from the breeding colony of the Hamadan University of Medical Sciences. Rats were housed three per cage and maintained on a standard 12:12h light-dark cycle with lights on at 7:00 a.m. Food and water were available ad lib while the animals adapted to the laboratory and for up to one week following surgery. All Experiments were done in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals (NIH Publication No. 80-23, revised 1996) and were also approved by the local ethical committee of Hamadan University of Medical Sciences.

### 2. 2. Surgery

Approximately 10 days prior to initiation of the behavioral experiments, the rats were anesthetized with sodium pentobarbital (50 mg/kg i.p.) and were implanted with a cannula (15 mm, 23 gauge) aimed at a site 0.5 mm above the DRN (AP: -7.8 mm from bregma; ML: 0.0 mm and DV: 5.8 mm below the dura mater) according to the atlas of Paxinos and Watson (1986). The cannula and two anchoring screws were fixed to the skull with dental cement. Rats were given Penicillin (300,000 units/ml, 0.2 ml i.m.) immediately after the surgery to prevent postoperative infection. The cannula was sealed with occluding stylet over recovery period (7 days), during which rats were handled daily to decrease stress and to become habituated to the microinjection procedure on the test day.

### 2. 3. Microinjection Procedure

Before injection, the animal was held in hand, the stylet was removed and replaced with the injection needle (30 gauge) connected with a short piece of polyethylene tubing to a Hamilton syringe. The needle was inserted 0.5 mm beyond the tip of the cannula. A volume of 0.5 µl saline or 2% lidocaine hydrochloride (Bayer) was injected into the DRN over 60 sec. The injection cannula remained in place for an additional 60 seconds and then stylet was replaced to minimize backflow of the drug.

### 2. 4. Step-Through Passive Avoidance Apparatus

The apparatus used for PA training consisted of a two-compartment box. The larger illuminated chamber (35×20×15 cm) made from transparent plastic was con-

nected by an 8×8 cm guillotine door to the smaller dark compartment (25×15×15 cm) with black opaque walls and ceiling. The floor of the dark compartment was constructed out of stainless steel rods (3 mm in diameter, 10 mm apart) through which foot-shock could be delivered from a constant current source.

## 2. 5. Training Procedure

First, all the rats in the experimental groups became habituated to the apparatus. The rat was placed in the illuminated compartment and 5 sec later the guillotine door was raised. Upon entering the dark compartment the door was closed and the rat was taken from the dark compartment into the home cage. The habituation trial was repeated after 30 min and followed after the same interval by the acquisition trial during which the guillotine door was closed and a 50 Hz, 1.2 mA constant current shock was applied for 1.5 sec immediately after the animal had entered the dark compartment (Shahidi, Motamedi, Bakeshloo, & Taleghani, 2004). In experiment 2 and 3, after 20 sec, the rat was removed from the dark compartment and received appropriate treatments if necessary and placed into the home cage. In experiment 1, the rat was retained in the apparatus and received a foot-shock each time it reentered the dark compartment. Training was terminated when the rat remained in the light compartment for 120 consecutive seconds. The number of trials to acquisition (entries into the dark chamber) was recorded. The non-shocked control animals were handled in the same way, but received no electrical shock upon entering the dark compartment and were not given the second test.

## 2. 6. Retention Test

Two days after PA training, the rat was placed in the illuminated chamber and 5 sec later the guillotine door was raised and the latency of entering the dark compartment (step- through latency) and the time spent there during 10 min were recorded.

## 2. 7. Histological Verification

At the end of each experiment, the animals were deeply anesthetized with sodium pentobarbital and 1 µl neutral red was injected via the injection cannula. The perfusion-fixation was performed intracardially with saline followed by 10% formalin/ phosphate buffer solution. The brains then were removed and post-fixed in the same fixative. Then, the paraffin-sections (20µ thick) were stained (H & E) for histological examination. The location of cannula was verified by examining enlarged projections of the slides (Fig. 1). The volume of lidocaine injected into the DRN in this experiment has been reported to spread from 0.5 to 1.5 mm from the site of injection (Lorens, Kohler, & Guldberg, 1975; Randich & Aicher, 1988). Therefore a cannula positioned more than 0.5 mm from the intended site of injection was not considered in statistical analysis. In 6 rats the cannula position was not within acceptable area and their data was removed from the statistical analysis.

## 2. 8. Experimental Design

**2.8.1. Experiment 1.** The aim of this experiment was to determine the effect of pre-training reversible inactivation of DRN on PA acquisition and retention. Eighteen rats were divided into two experimental groups. SS, shocked-saline control group (n =8) receiving intra DRN injection of saline 5 min before acquisition trial, and ST, shocked test group (n =10) receiving intra-DRN injection of lidocaine. Number of trials to PA acquisition, step-through latency and time spent in dark compartment during the retrieval test were recorded.

**2.8.2. Experiment 2.** The aim of this experiment was to determine the effect of post-training reversible inactivation of DRN on PA consolidation. Forty four rats were divided into five experimental groups: NS, non-shocked control group (n=8); SS5, shocked-saline groups receiving intra-DRN injection of saline at 5 min after foot-shock (n=8), and shocked test groups ST5 (n=9), ST90 (n=10), and ST360 (n=9) receiving intra-DRN lidocaine at 5, 90 and 360 min after foot-shock, respectively. Step-through latency and time spent in the dark compartment during the retrieval test were recorded.

**2.8.3. Experiment 3.** The aim of this experiment was to determine the effect of pre-retrieval reversible inactivation of DRN on PA retention. Thirty four rats were divided into four experimental groups. NS, non-shocked control group (n =8); NST, non-shocked test group (n =8); RS, shocked-saline group (n = 8) receiving intra DRN injection of saline 5 min before retention test, and RT (n = 10), shocked test groups receiving intra DRN lidocaine 5 min before retention test. Step-through latency and time spent in dark compartment during the retrieval test were recorded.

## 2.9. Statistical Analysis

Data showed a normal distribution. Evaluating the differences in number of trials in experiment 1 was done by student t-test. Step-through latency and time spent in dark chamber in experiment 1 were compared using un-

paired-student t-test. In experiments 2 and 3 differences between the groups were tested by one-way ANOVA followed by Tukey's test for multiple comparisons. All results were shown as mean ± S.E.M. The level of $P<0.05$ was considered significant.

## 3. Results

### 3.1. Effect of pre-training reversible inactivation of DRN on PA acquisition and retrieval

Comparing the number of trials to acquisition showed no significant difference between SS (2 ± 0.79) and ST (1.1 ± 0.37) groups. Result indicates that DRN inactivation has no effect on PA acquisition (Fig. 2). Effect of pre-training reversible inactivation of DRN on PA retention is summarized in Fig. 3. Unpaired-student t-test showed absence of significant difference between the step-through latency and the time spent in dark compartment of two groups, indicating that pre-training inactivation of DRN has no effect on PA retention.

### 3.2. Effect of post-training reversible inactivation of the DRN on PA consolidation

One-way ANOVA indicated that there was no significant difference in step-through latency before the acquisition trial among the five experimental groups [$F_{4,43} = 0.68$, n.s.]. Fig. 4 shows the results of the retrieval test performed two days after training. One-way ANOVA indicated that there were significant differences in step-through latency [$F_{4,43} = 21.702$, $P<0.0001$] between groups in the retrieval test. Tukey's multiple comparison test showed that step-through latency in the group which received saline 5min after training (SS5) was significantly different from NS ($P<0.001$), ST90 ($P<0.001$), and ST360 ($P<0.001$) but not from ST5. Additionally, One-way ANOVA indicated that there were significant differences in time spent in the dark compartment between groups in the retrieval test [$F_{4,43} = 60.33$, $P<0.0001$; Fig. 4]. Tukey's test for multiple comparisons showed that time in the dark compartment of SS5 group was significantly different from NS ($P<0.001$), ST90 ($P<0.001$), and ST360 ($P<0.001$), but there was no significant difference compare with ST5 group.

### 3.3. Effect of pre-retrieval reversible inactivation of DRN on PA retrieval

One way ANOVA indicated no overall significant differences in step-through latency before acquisition trial between groups [$F_{3,33} = 0.57$, n.s.). This indicated the

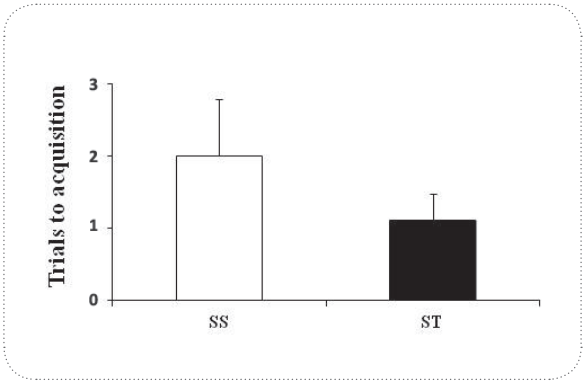


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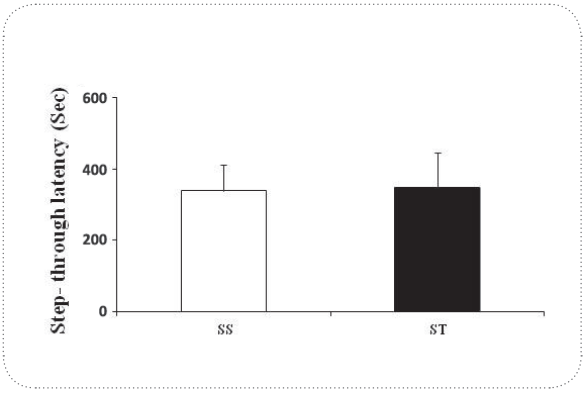
**Figure 1.** Schematic drawing of coronal plane through the DRN has been adapted from the atlas of Paxinos and Watson (1986). Solid dots reveal the location of the cannula tips in the multiple experimental cases having the acceptable placements.



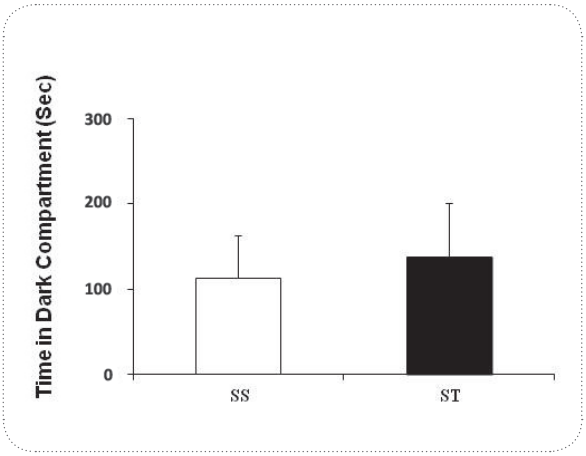




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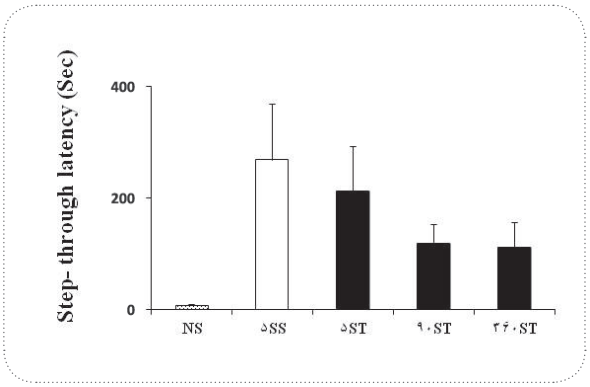
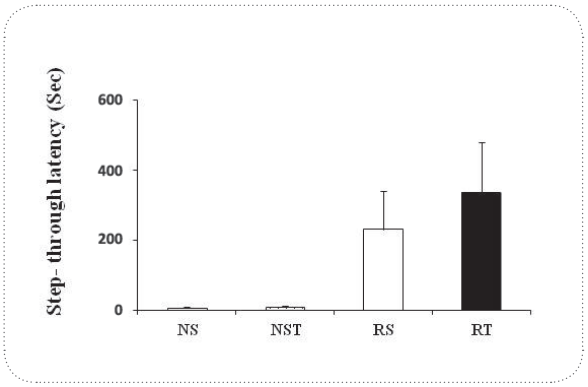
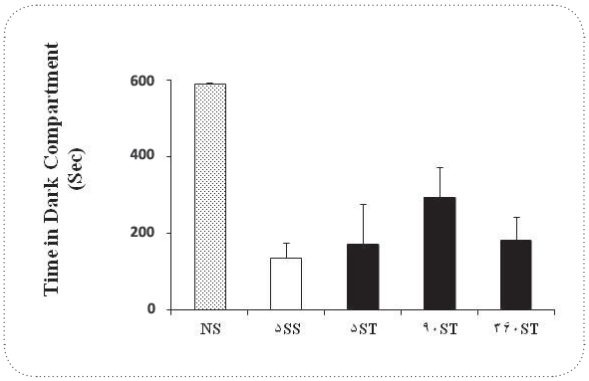
**Figure 2.** The effect of pre-training reversible inactivation of DRN on PA acquisition. Ordinate: mean (±S.E.M.) of trials to acquisition during training procedure. SS, control group receiving saline 5 min before acquisition trials. ST, test group receiving lidocaine 5 min before acquisition trials.



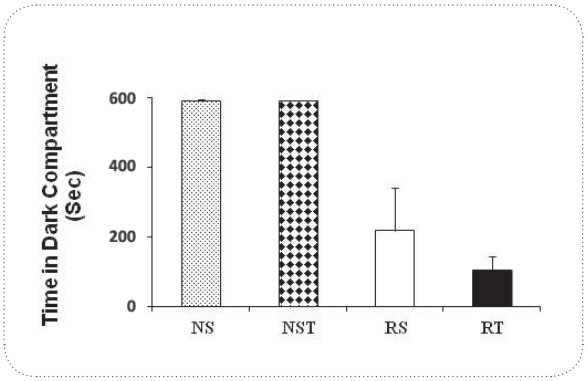




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**Figure 3.** The effect of pre-training reversible inactivation of DRN on PA retention. Ordinate: mean (±S.E.M.) step-through latency (A), and time spent in dark compartment (B) during the retrieval test performed 2 days after PA acquisition. SS, control group receiving saline 5 min before acquisition trials. ST, test group receiving lidocaine 5 min before acquisition trials.

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**Figure 4.** The effect of post-training reversible inactivation of DRN on PA consolidation. Ordinate: mean (±S.E.M.) step-through latency (A), and time spent in dark compartment (B) during the retrieval test performed 2 days after PA acquisition. NS, non-shocked control group; SS5, shocked control group receiving intra-DRN injection of saline 5 min after the acquisition trial; ST5, ST90, and ST360, shocked animals receiving intra-DRN injection of lidocaine 5, 90, and 360 min after the acquisition trial respectively.

uniformity between groups. Fig. 5 shows the results of the retrieval test performed 2 days after training. The step-through latency was different between groups [$F_{3,33} = 26.371$; $P<0.0001$]. Tukey's multiple comparison test revealed that step-through latency of the RS group was significantly higher than the NS ($P< 0.001$) and NST ($P< 0.001$) and less than RT ($P< 0.01$). The step-through latency was significantly higher in RT group which received lidocaine 5 min before retention test with respect to saline treated group (RS) ($P< 0.01$). There was no significant difference between NS and NST groups (Fig. 5). On the other hand, ANOVA of the time spent in the dark compartment shown in Fig. 5 showed a significant difference between groups [$F_{3,33} = 140.43$; $P< 0.0001$]. Tukey's test for multiple comparisons showed that the RS was significantly different from groups NS ($P<0.001$), NST ($P<0.001$) and RT ($P<0.01$). There was no significant difference between NS and NST groups. This result indicated that pre-retention test inactivation of DRN enhances passive avoidance retention.

## 4. Discussion

### 4. 1. Dorsal raphe inactivation does not interfere with motor activity of rats

Lidocaine induces reversible block of impulse conduction by changing the membrane permeability to sodium ions ( Ritchie, 1975 ; Sandkuhler & Gebhart, 1984) . The spatial extent of 0.5µl lidocaine (2%) blockade (Martin, 1991; Sandkuhler & Gebhart, 1984), suggested that inactivation was effective within 1 mm, which is restricted to the DRN. The blocking effect last for a period

of up to 30 min and DRN returned to the pre-injection level in less than 1 h (Randich & Aicher, 1988; Petkov, 1980).

The similar step-through latencies of rats before the acquisition trial are proof of the behavioral homogeneity of the animals as indicated in other reports (Shahidi, Motamedi, Bakeshloo, & Taleghani, 2004). Moreover, during lidocaine inactivation of the DRN, the animals did not exhibit alterations in their spontaneous exploratory and locomotor behavior. In fact, the data shows that there was neither significant difference in step-through latency and time spent in the dark compartment in the first acquisition trial between SS and ST groups (Expt. 1), nor in the retrieval test between NS and NST (Expt. 3). This indicates that neither the control treatment nor lidocaine inactivation was followed by significant modifications in either of the rat's normal explorative behavior or of spontaneous preference for dark compartment. This is in agreement with the results reported by Albinsson et al., showing that movement in DRN lesioned rats was unchanged (Albinsson, Andersson, Andersson, Vega-Matuszczyk, & Larsson, 1996). In addition, it has been reported that neurotoxin lesion of the dorsal raphe-serotonergic fibers does not interfere with motor activity of rats (Ruotsalainen Miettinen, MacDonald, Koivisto, & Sirviö, 2000). Therefore, the result of the present study is due to changes in memory processes of PAL but not motor activity.

### 4. 2. Pre-training inactivation of DRN has no effect on PAL acquisition

There is little evidence supporting the role of DRN neurons on passive avoidance response. Lorens and colleagues (1975) found that electrolytic lesions of midbrain raphe nuclei deteriorate acquisition in one way avoidance task. In the present study, reversible inactivation of DRN had no effect on PA acquisition. This discrepancy might be due to different experimental procedure. In addition, serotonergic lesions of fimbria fornix by 5,7-DHT have been reported to disrupt acquisition in a latent inhibition task (Brody, 1970; Rowan, Cullen, & Moulton, 1990). This difference may be due to inactivation of all DRN neurons including non serotonergic ones (Sarihi, Motamedi, Rashidy-Pour, Naghdi, & Behzadi, 1999; Schneider, Wilkins, Firestone, Everbach, & Naylor, 2003) and difference in learning task.

### 4. 3. Pre-training inactivation of DRN has no effect on PAL retention

Previous studies have shown that pre-training administration of serotonin agonists impaired PAL retention (Lorens, Sorensen, & Yunger, 1971; Lorens, Kohler, & Guldberg, 1975) while Altman et al. reported that administration of serotonin antagonist impaired PAL retention (Altman, Nordy, & Ogren, 1984). Therefore, the role of serotonergic fibers on PAL retention is controversial. In this study, all types of the DRN fibers (serotonergic and non serotonergic) were inactivated. It is possible that serotonergic and non serotonergic fibers of DRN have different role in PAL acquisition; therefore inactivation of both types of neuronal fibers had no net effect on PAL retention in this study.

### Inactivation of DRN, 90 and 360 min (but not 5 min) after training significantly impaired consolidation of PAL

Igaz, Vianna, Medina and Izquierdo (2002) found that hippocampal gene expression is critical in two time windows: around the time of training and 3-6 hr after training. Also, there is another report concerning the consolidation of memory in the hippocampus. The results suggest that hippocampal protein synthesis is critical in two periods, around the time of, and 3 hr after training (Quevedo et al., 1999).

The present findings indicate that post-training inactivation of DRN impaired PA retention. It has been shown that post-training administration of serotonergic antagonists ( ketanserin, pirenperone and mianserin) improved PA retention (Santucci, Knott, & Haroutunian, 1996). Most studies show that enhancement of retrieval is specific to post-training drug administration which shows the role of serotonergic system on consolidation and retrieval (Altman, Normile, Galloway, Ramirez, & Azmitia, 1990; Lorens, Sorensen, & Yunger, 1971; Martin, 1991; Ruotsalainen Miettinen, MacDonald, Koivisto, & Sirviö, 2000 ), but our results are in contrast with previous findings.

### Pre-Retrieval Functional Ablation of DRN Enhanced PAL Retention

The present work showed enhancement of PA retention when lidocaine was injected before retrieval test but not before acquisition trial. Santucci, Knott and Haroutunian (1996) have shown that excessive serotonin release, not depletion, leads to memory impairments in rats. Our finding is in agreement with their results.

The performance of a step-down passive avoidance response is disrupted by a range of compounds known to be 5HT agonists (Sanger, & Joly, 1989). Misane and Ogren (2000) found that administration of multiple 5-HT receptor agonists before PA training produced a

dose-related impairment of the 24-hour retention. The crucial involvement of the postsynaptic 5-HT1A receptors was confirmed. In contrast, the 5-HT2A and 5-HT2C receptors were of negligible importance in the 24-hour retention deficit induced by PCA. However, the ability of the 5-HT2C receptor antagonist Ro 60-0491 to block the inhibitory effects of mCPP indicated an important regulatory role of the 5-HT2C receptor in PA. The non-selective 5-HT receptor antagonist methiothepin attenuated the PA deficit by PCA but lacked activity versus 8-OH-DPAT. In conclusion, multiple 5-HT receptors are involved in PA with roles that probably differ at various stages of information processing.

Previous studies have shown that disruption of hippocampal serotonergic activity increases acetylcholine release via removal of inhibitory input to hippocampal cholinergic neurons (Fibiger, Lepiane, & Phillips, 1978; Ruotsalainen Miettinen, MacDonald, Koivisto, & Sirviö, 2000; Carli, Tranchina, & Samanin, 1992), which mainly originates from medial septal area (MSA). Therefore, the facilitatory effect of DRN inactivation on PA retention can be explained on the following way: inactivation of DRN may increase the activity of MSA cholinergic neurons and this in turn, enhances retention of PA. In fact, it has been previously demonstrated that reversible inactivation of MSA, and therefore decreasing of cholinergic neuronal activity in the hippocampus impairs PA consolidation 5 and 90 min after acquisition trials (Rashidy-pour, Motaghed-Lerijani & Bures, 1996).

### Interaction between serotonergic system and other neurotransmitters on PA learning and memory

In addition, involvement of other neurotransmitter systems which participate more closely in PA learning and memory must be considered. Aznar, Qian, and Knudsen (1986) found a significant non-serotonergic projection from dorsal raphe nucleus onto calbindin- and parvalbumin-containing interneurons in septum and hippocampus, with a preference in hippocampus for projecting onto calbindin-positive neurons. These results indicate that the raphe nuclei may exert their control on hippocampal and septal activity not only through a serotonergic projection, but also through a significant non-serotonergic pathway (Aznar, Qian, & Knudsen,1986). It is possible that DRN could affect PAL by influencing the hippocampus and septum areas, structures that have important role in PAL.

Meneses, Terron and Hong (1998) believe that the role of 5-HT receptors in learning seem to require an interaction with glutamatergic, GABAergic and cholinergic neurotransmission systems. For example, Carli, Tranchina, and Samanin(1992), reported that blockade of postsynaptic hippocampal 5-HT1A receptors antagonized the effect of intrahippocampal scopolamine in the two-platform spatial discrimination task, the results suggest that drugs with presynaptic stimulatory and postsynaptic blocking actions on 5-HT1A receptors, such as partial agonists at these receptors, may be useful in the symptomatic treatment of human memory disturbances associated with loss of cholinergic innervation to the hippocampus (Carli, Bonalumi, & Samanin, 1998). The electrical stimulation of the midbrain raphe increases extracellular noradrenaline in the hippocampus, however, experiments in 5-HT-lesioned animals suggest that this response is not mediated by 5-HT (Shahidi, Motamedi, Bakeshloo, & Taleghani, 2004; Hajós-Korcsok, & Sharp, 2002).

Ruotsalainen, Miettinen, MacDonald, Riekkinen and Sirviö (1998) studied the role of the dorsal raphe-serotonergic system and its interaction with muscarinic or nicotinic receptors in the modulation of performance of rat in working memory task.

Fibiger, Lepiane and Phillips (1978) reported that stimulation of the dorsal raphe nucleus caused disruption of memory. The administration of the YM992 (a selective serotonin (5-HT) reuptake inhibitor) [into the DRN] markedly increased the effectiveness of the electrical stimulation of ascending 5-HT fibers on firing activity of the postsynaptic hippocampus pyramidal neurons (Dong, De Montigny, & Blier, 1999). It is possible that inactivation of DRN serotonergic fibers after training prevents principle hippocampal neuronal activity and disturbs consolidation of the PAL.

According to our results it seems that DRN has a different role in learning and memory processes but it is not clear whether this structure only relays the information between subcortical structures and hippocampus or it directly participates in different phases of avoidance learning and memory.

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