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Nucleus incertus inactivation impairs spatial learning and memory in rats

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HIGHLIGHTS

- Nucleus incertus inactivation impairs retrieval of working memory.
- Nucleus incertus inactivation reduces hippocampal c-fos and pCREB levels.
- Nucleus incertus is involved in acquisition and retrieval of spatial reference memory.

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ABSTRACT

Nucleus incertus (NI) is a pontine nucleus which releases mainly GABA and relaxin-3 in rats. Its suggested functions include response to stress, arousal, and modulation of hippocampal theta rhythm. Since the role of NI in learning and memory has not been well characterized, therefore the involvement of this nucleus in spatial learning and memory and the aftermath hippocampal levels of c-fos and pCREB were evaluated. NI was targeted by implanting cannula in male rats. For reference memory, NI was inactivated by lidocaine (0.4 μl, 4%) at three stages of acquisition, consolidation and retrieval in Morris water maze paradigm. For working memory, NI was inactivated in acquisition and retrieval phases. Injection of lidocaine prior to the first training session of reference memory significantly increased the distance moved, suggesting that inactivation of NI delays acquisition in this spatial task. Inactivation also interfered with the retrieval phase of spatial reference memory, as the time in target quadrant for lidocaine group was less, and the escape latency was higher compared to the control group. However, no difference was observed in the consolidation phase. In the working memory task, with inter-trial intervals of 75 min, the escape latency was higher when NI was inactivated in the retrieval phase. In addition, c-fos and pCREB/CREB levels decreased in NI-inhibited rats. This study suggests that nucleus incertus might participate in acquisition of spatial reference, and retrieval of both spatial reference and working memory. Further studies should investigate possible roles of NI in the hippocampal plasticity.

1. Introduction

The hippocampal formation as a major structure in the processes of learning and memory is connected to a variety of higher and lower brain areas and nuclei. Brainstem inputs to the hippocampus have modulatory roles in memory processes, and some are thought to exert their effects by generating or affecting hippocampal theta rhythm which is suggested to be involved in learning and memory [1]. A pontine structure which is recently identified and implicated in modulation of theta rhythm is nucleus incertus (NI) [2–4].

NI which is located ventral and medial to the posterodorsal tegmental nucleus constitutes a medial part, pars compacta (NIc) and the lateral pars dissipata (NId) [5]. NI neurons are heterogeneous and secrete GABA and some neuropeptides as co-transmitter, the most prominent of which is relaxin-3 and is highly abundant in NI in the mammalian brain [6]. On the other hand, glutamatergic projections from NI to septohippocampal system have been recently identified [7]. Many of NI neurons are rich in corticotropin releasing hormone receptor type 1 (CRF1), suggestive of its role in response to stress [8,9]. Indeed studies have reported that behavioral stress [10–12] or direct administration of CRF [13] activates NI neurons. Some other receptors are expressed in NI neurons including RXFP3 itself which is the cognate receptor of the relaxin-3 [6,12,14,15].

NI projects to the hippocampal formation, the medial septal nucleus, nucleus of the diagonal band, the amygdala and many other areas. And its main inputs are from the medial septum and nucleus of the diagonal band, the medial part of the lateral habenula, the median raphé nucleus, and the contralateral nucleus incertus [5,16].

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Diverse projections along with neurochemical, electrophysiological and behavioral investigations have urged investigators to propose different functions for NI such as response to stress [8,10,12,17], feeding behavior [18], arousal [5,16,19–22], and effects on hippocampal theta rhythm [3,4,23,24]. Brain theta rhythm which can be recorded from many areas including hippocampus is involved in functions like active waking behavior and REM sleep, encoding and retrieval of memory, spatial navigation/exploration, and also sensorimotor integration [2, 25,26]. Septal complex, hypothalamus and many brainstem nuclei, like reticularis pontis oralis (RPO), are thought to be principal structures engaged in the generation of theta rhythm [4]. RPO-elicited theta rhythm in hippocampus depends on NI, and RXFP3 antagonist in septal area reduces theta rhythm [3,24]. This functional connectivity was later approved by tracing studies [27]. These observations have led to the notion that NI is a relay point between RPO and the medial septum. Moreover, theta-like oscillations have been shown in NI under experimental conditions which is synchronous with hippocampal theta rhythm [23]. Lately, NI was shown to have relaxin-3 positive and negative neurons, with the former shown to have phase-lock activity with hippocampal theta when stimulated by CRF [28]. The contribution of NI to hippocampal theta rhythm raises the possibility that this nucleus in involved in cognitive tasks such as learning and memory. In a spontaneous alternation task, RXFP3 antagonist reduced alternation score, indicative of disrupted spatial working memory [24]. In another study, electrolytic lesion of NI in rats delayed extinction of the conditioned fear [29]. More recently NI ablation with CRF-saporin conjugate increased freezing behavior in cued fear conditioning [30].

Behavioral studies evaluating the role of NI in learning and memory are limited and long term hippocampus-dependent memory has not been investigated. The present study aimed to identify the role of NI in different phases of spatial reference and working memory formation in rats. This was achieved by using the Morris water maze (MWM) paradigm and injection of lidocaine into the NI. Reversible inhibition by lidocaine has been extensively used in our laboratory and elsewhere to inactivate neurons so that their functions can be eliminated for a limited time period [31–34]. And in the case of NI, it inhibits all types of neurons, leaving no NI-driven response. Since the MWM is a hippocampal dependent task, NI effects on learning and memory were assessed by measuring hippocampal c-fos and pCREB levels.

2. Materials and methods

2.1. Animals

Adult male Wistar rats (230–280 g) were obtained from our own breeding colony. They were housed four per cage in a temperature and humidity controlled animal house facility where a 12:12 h light/dark cycle, with lights on at 7:00 am, was applied. Rats had free access to food and water during the study. They were handled by the experimenter from two weeks before the surgery. The behavioral testing was done during the light phase. Experiments were carried out in accordance with recommendations from the declaration of Helsinki and the internationally accepted principles for the experimental use of rats.

2.2. Surgery

Rats were anesthetized by ketamine and xylazine (100 and 2.5 mg/kg respectively, i.p). Under aseptic conditions, scalp and fascia were removed and the skull was exposed. After drilling the trephine hole, a 15 mm cannula was inserted into the nucleus incertus (AP: −9.8, ML: 0, DV: 7–7.5). To avoid excessive hemorrhage, the cannula was inserted with a 16° angle, almost 2 mm caudal to the vertical insertion point. Two jewelers' screws were inserted into the skull, and dental cement was applied to fix the cannula. A stylet was inserted into the cannula to prevent its blockade. Rats were returned to their cages and after one week behavioral tests were started.

2.3. Microinjection procedure

Before injection, rats were gently restrained by hand and the stylet of cannula was removed. The injection needle (30-gauge) was connected to a Hamilton syringe through a 20-cm piece of polyethylene tubing. The needle was inserted 0.5 mm beyond the tip of the cannula to reach the NI; so the cannula itself was in the vicinity of NI and did not cause damage to it. Then 0.4 μl of sterile saline or 4% lidocaine hydrochloride (Sigma, USA) in saline was injected slowly over a 2-min period. The injection needle was left in the guide cannula for an additional 60 s to facilitate diffusion of the drug, before it was slowly withdrawn.

2.4. Behavioral testing

2.4.1. Morris water maze apparatus

The water maze used in our study is a dark circular pool (150 cm in diameter and 60 cm high) filled with water to a depth of 45 cm. A Plexiglas platform (11 cm diameter) covered with black rubber was located 2 cm below the water surface in the center of one of the arbitrarily designed north-east (NE), south-east (SE), south-west (SW) or north-west (NW) orthogonal quadrants. The platform provided the only escape from the water. Extra-maze cues included racks, curtains, a door, and pictures on the walls around the room where the water maze was housed. The cues were kept fixed in positions during the study so that all the rats could use the same visual cues. Swimming was recorded by a CCD camera (Panasonic Inc., Japan) hanging from the ceiling above the MWM apparatus and locomotion tracking was measured by a video tracking system for automated analyzing of animal's behavior using the Ethovision software (version XT7, Netherlands).

2.4.2. Habituation

To promote animal adaptation with the test environment and reduce the level of stress, one day before starting the hidden-platform training sessions, rats were given a 60 s swim in the tank where there was no platform.

2.4.3. Reference memory

The training sessions consisted of two blocks of 4 trials per day (block interval: 10 min, trial interval: 1 min), for three consecutive days as reported previously in our lab [35]. The platform was located in the center of the third quadrant near the southern labeled direction. Other seven directions, from southwest to southeast, were engaged to release the rats in a quasi-random manner, always facing the wall of the tank. Rats had 60 s to swim and find the platform, where after 2 s of being stationary on it, recording was stopped by the software. If the animal failed to reach the platform within 60 s, it was directed by the experimenter to the platform and left there for 10 s. Twenty-four hours after the third session the probe test was given, during which the platform was removed from the tank and rats swam for the whole 60 s. For the probe test, rats were released opposite to where the platform was placed. For the recorded tracks, escape latency, distance moved, velocity and time spent in target quadrant were calculated for subsequent analyses.

2.4.4. Working memory

The protocol for assessment of working memory started like the reference memory with two blocks of four trials each day, for three days while the platform was located in a same position [31,36]. Then with one day break, rats were given two trials per day (trial interval: 75 min) for four consecutive days with the platform placed in a different position each day. On the fifth day, rats did one training trial, and 75 min later the probe trial was performed in which the platform was removed.

2.4.5. Visuo-motor activity

In order to rule out the effect of NI inhibition on visuo-motor coordination, 5 min after the injection of lidocaine (n = 8) or saline (n = 8), the rats were allowed to swim in the MWM where the platform was in a new position, 1 cm above the water surface and made visible by a piece of bright latex. Latency to reach the platform and swimming speed were used as a measure of rats' visuo-motor ability to find the platform.

2.5. Western blot analysis

After rinsing extracted hippocampi with phosphate buffer saline, they were frozen in liquid nitrogen and then stored at −80 °C. Tissues were homogenized in ice-cold lysis buffer containing complete protease inhibitor cocktail. After determination of concentration of proteins (Bradford, 1976), 60 mg protein of samples was loaded into a 12% SDS-PAGE gel and electrophoresed at 120 V. Next, proteins were transferred to polyvinylidene difluoride membrane which was then incubated at room temperature in a 2% blocking solution (Amersham Biosciences, Piscataway, New Jersey, USA) for 75 min. Membranes were then incubated overnight at 4 °C with primary antibodies for c-Fos and pCREB (1:1000; Cell Signaling Technology, Beverly, Massachusetts, USA), and after washing with TBST, subjected to a secondary antibody (1:3000; Cell Signaling Technology, Beverly, Massachusetts, USA) for 90 min at room temperature. Following three washes, chemiluminescent reagent (Amersham Biosciences) was used to visualize protein bands on a radiographic film. Then, blots were stripped and re-probed with β-actin and CREB antibodies (1: 1000; Cell Signaling Technology). The density of protein bands was quantified using ImageJ software (NIH ImageJ, Bethesda, Maryland, USA). Individual pCREB values were divided by their respective CREB values to obtain the pCREB/CREB ratio for each sample, and c-Fos values were divided by their respective β-actin values to obtain the c-Fos/β-actin ratio.

2.6. Immunohistochemistry

To further identify levels of hippocampal c-fos, immunohistochemistry was performed. Following induction of deep anesthesia the rats were transcardially perfused by phosphate buffer saline and then 4% formaldehyde. Brains were rapidly removed and fixed in 4% buffered formalin, embedded with paraffin, cut into consecutive 5 μm coronal sections and loaded on poly-D-lysine-coated glass slides. Tissue sections were deparaffinized in xylene, rehydrated, and immersed in 3% hydrogen peroxide in order to block the endogenous peroxidase activity. Antigen retrieval was performed in a 0.01 M citrate buffer, pH 6.0 at 80 °C for 40 min, cooled in the same solution for 20 min, and rinsed three times in Tris solution. Non-specific binding was reduced by using blocking solution of 5% normal goat serum (NGS) for 30 min. Tissue sections were incubated with rabbit monoclonal anti c-fos primary antibody (Cell Signaling, USA, 1:100 dilution using distilled water), at 4 °C overnight. After rinsing three times with Tris, the sections were exposed with mouse and rabbit specific HRP/DAB (ABC) detection kit (abcam, ab64264) for 60 min at room temperature, followed by three washes in PBS. After three rinses in PBS, sections were reacted with diaminobenzidine tetrahydrochloride (DAB) for 10 min. Rinsing with Tris was done at the final stage. Sections were then dehydrated and cover slipped with Entellan.

2.7. Experimental design

The first experiment aimed to determine the effect of NI inactivation on spatial reference memory. Five minutes before the initiation of the training session each day, rats received 0.4 μl intra-NI injection of saline (n = 8) or lidocaine (n = 8). This inactivation thus targets acquisition phase of memory. On the fourth day, probe test was performed. For assessment of consolidation, NI was inactivated 5 min after completion of each training session in (n = 7), and another group received saline instead (n = 9). Thus each rat in acquisition and consolidation groups received one injection on each of the training days. Finally, to evaluate the effect of NI inactivation on retrieval, lidocaine (n = 8) or saline (n = 8) was injected into NI 5 min prior to the probe test. The second experiment aimed to assess the acquisition and retrieval of spatial working memory. On the fifth day of working memory training, rats received saline (n = 9) or lidocaine (n = 6) 5 min before the training trial; or 5 min before the probe trial (n = 8, saline and n = 6, lidocaine). The third experiment aimed to evaluate hippocampal neuronal activity in the NI-inactivated rats involved in learning and recall of memory. c-fos and phosphorylated cAMP response element binding protein (pCREB) levels were assessed by western blotting. Two groups (n = 4 each) were involved in acquisition and received saline (Acq/Sal: AS) or lidocaine (Acq/Lid: AL) 5 min before the single training session which included 8 trials and took about 25 min. Five minutes after the session rats were decapitated and the hippocampi were removed and rinsed with phosphate buffer saline (PBS) and kept in liquid nitrogen. Another two groups received saline (Non-Acq/Sal: NAS) and lidocaine (Non-Acq/Lid:NAL) but did not perform training trials and considered as naïve rats; they were decapitated with the same time gap from injections. For the retrieval phase which was performed 24 h later, two groups received saline (Ret/Sal: RS) or lidocaine (RL) before the probe test and another two groups were treated the same but did not perform the probe test (NRS and NRL). Hippocampi were then extracted 25–30 min after the injections and rinsed with PBS for western blotting [35]. For immunohistochemistry, two other rats for each of the AS, AL, RS and RL groups were transcardially perfused following completion of MWM tasks.

2.8. Verification of injection

After behavioral testing rats were killed in CO_2 chamber, then 0.2 μl of toluidine blue was injected into NI. Rats were decapitated and brains were extracted and kept in 4% paraformaldehyde solution. Four days later 200 μm coronal slices were taken, and the injection point trace of cannula was examined under a stereomicroscope. Rats whose injection spot was outside the area of NI were not included in the final analysis.

2.9. Statistics

Data were expressed as mean ± SEM (Standard Error of Mean). The Kolmogorov–Smirnov test was used to examine normal distribution of the data. Parameters of saline and lidocaine groups during training days were analyzed by 2-way analysis of variance (ANOVA) repeated measures followed by Bonferroni post-test. On the probe test the groups were compared by unpaired student's t-test. Groups assessed for western blotting were compared by one-way analysis of variance (ANOVA) followed by Tukey's test. A $P < 0.05$ was considered to be statistically significant.

3. Results

Fig. 1A shows brain coronal sections where a one-time injection point is marked with 0.2 μl injection of toluidine blue prior to decapitation. NI is easy to detect based on its location right under the fourth ventricle, and the paler color compared to the flanking posterodorsal tegmenti. In Fig. 1B the trace of cannula is evident in a rat that received NI injection three times during training days in MWM.

3.1. NI inhibition delays acquisition, but has no effect on consolidation of spatial reference memory

In order to assess whether the NI is involved in acquisition and/or consolidation of spatial reference memory, it was inactivated at the

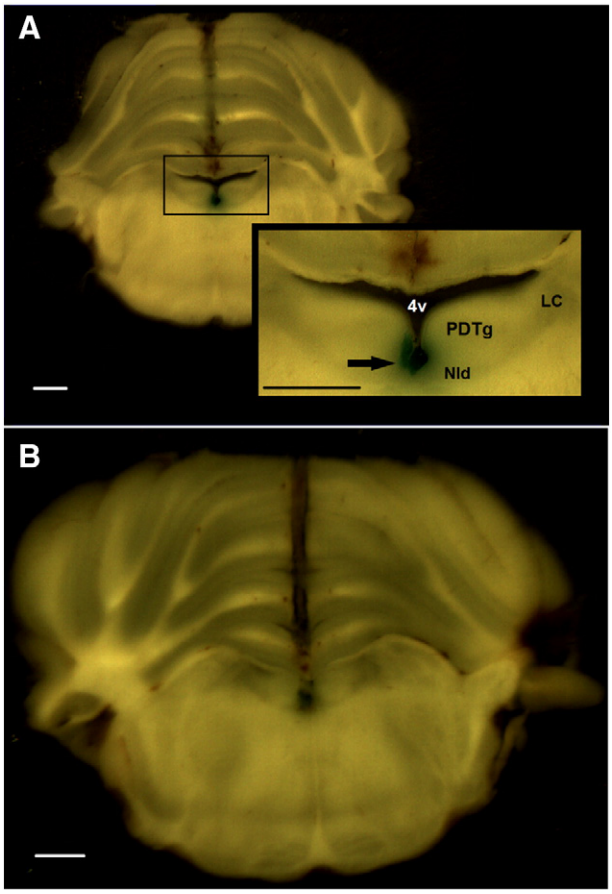

Fig. 1. Coronal sections showing the trace of the cannula in the brain. Sections of brain that received single (A) or three times injection (B) in NI. Lower magnified panel of (A) shows the injection spot of toluidine blue (arrow) in the NI. 4v: fourth ventricle, PDTg: posterodorsal tegmental nucleus, NId: nucleus incertus pars dissipata, LC: locus coeruleus. Scale bars: 1 mm.

beginning of, or just after each training session. Two-way ANOVA repeated measures showed that there was no interaction between the "training" and "inactivation". But the effect of training was significant in both acquisition and consolidation groups. This means that all rats learned the task during three days of training. As shown in Fig. 2, administration of lidocaine 5 min prior to the initiation of each training session (acquisition group) did not make a significant difference in the escape latency [$F(1,28) = 0.38$, $P = 0.54$] and distance moved [$F(1,28) = 4.44$, $P = 0.06$]. However, further analysis of trials of the first training day showed that distance moved for the lidocaine group was higher than the saline group [$F(1,98) = 13.07$, $P = 0.003$]; and post-test revealed that distance moved was significantly higher in lidocaine group by 1.9 and 2.6 folds in trials 3 and 4 respectively ($P < 0.05$). When the NI was inhibited 5 min after the completion of each training session (i.e. in consolidation phase), neither escape latency [$F(1,28) = 0.087$; $P = 0.77$] nor distance moved [$F(1,28) = 0.004$; $P = 0.95$] differed significantly compared to the saline group. In addition, time spent in target quadrant (on the probe test) showed no difference between the lidocaine and saline groups when NI was inactivated in acquisition ($t_{14} = 1.16$, $P = 0.26$) and consolidation ($t_{14} = 0.64$, $P = 0.529$) phases (Fig. 3A).

3.2. NI inhibition impairs retrieval of spatial reference memory

Rats in the retrieval groups, like acquisition and consolidation groups, performed three training days; but 24 h later, on the probe test, they received lidocaine or saline, 5 min before the probe trial. The lidocaine group, compared to the saline group, spent 0.72 folds less time in the quadrant where the platform was located ($t_{14} = 3.49$, $P = 0.0036$) (Fig. 3A). Within group analysis of quadrant occupancy using one-sample t-test also reveals significant difference of both saline ($t_7 = 7.51$, $P = 0.0001$) and lidocaine ($t_7 = 3.91$, $P = 0.006$) compared to the 25% of random occupancy. As shown in Fig. 3B, escape latency was also significantly higher in the lidocaine than the saline group ($t_{14} = 2.74$, $P = 0.016$). To make sure that the observed effect is related to inactivation of NI, and not adjacent areas, data of rats with missed injection points were compared between saline and lidocaine groups which showed no significant difference ($t_{11} = 0.89$, $P = 0.39$).

3.3. NI inhibition does not interfere with acquisition, but impairs retrieval of spatial working memory

To evaluate the effects of NI inactivation on acquisition of working memory, on the fifth day of working memory task in MWM rats received either lidocaine or saline 5 min before training (first trial), and 75 min later they performed the probe test (second trial) where there was no platform in the MWM. The escape latency was calculated and analyzed, but did not show a significantly higher value in the lidocaine group compared to the saline group ($t_{13} = 0.27$, $P = 0.790$) (Fig. 4A). To target the retrieval phase of working memory, on the fifth day lidocaine or saline was injected into the NI 5 min prior to the probe trial. The escape latency for rats of the lidocaine group was significantly higher than that of saline group by 2.38 folds ($t_{12} = 2.79$, $P = 0.016$) (Fig. 4B).

3.4. NI inhibition has no effect on visuo-motor activity

In order to make sure that inhibition of NI does not interfere with visuo-motor coordination of rats, the visible test was performed. Rats could find and rest on the platform, and the escape latency or swimming speed did not differ between the lidocaine- or saline-injected rats ($t_{14} = 1.23$, $P = 0.238$) (Fig. 4C,D).

3.5. Levels of hippocampal c-Fos and pCREB decreased in rats that microinjected with lidocaine in the acquisition phase of spatial reference memory

Hippocampal c-fos was assessed to evaluate effects of NI inactivation (Fig. 5). The levels of hippocampal c-Fos and pCREB significantly decreased in rats in which NI was inhibited in the acquisition phase [$F(3,12) = 63.48$; $P < 0.001$] and [$F(3,12) = 37.5$; $P < 0.001$] respectively. A decrease in c-Fos levels was observed in NAL and AL groups compared to the respective saline microinjected groups as shown in Fig. 5A,B. There was also a decrease in pCREB/CREB levels in the lidocaine-injected groups (NAL and AL) compared to the saline-injected groups (NAS and AS) respectively (Fig. 5C,D) ($P < 0.001$). Both c-fos and pCREB/CREB ratios increased in AS group compared to NAS group which were naïve rats and not involved in the learning task ($P < 0.001$). Learning even caused an increase in c-fos and pCREB levels in rats which received lidocaine (AL) compared to the naïve counterpart (NAL) ($P < 0.01$ and $P < 0.001$ respectively).

3.6. Hippocampal pCREB and c-fos levels decreased in rats that microinjected with lidocaine in the retrieval phase of spatial reference memory

In the retrieval phase, c-Fos decreased in lidocaine groups (NRL and RL) compared to the saline (NRS and RS) groups [$F(3,12) = 15.19$; $P < 0.001$] (Fig. 6A,B). Likewise, Hippocampal pCREB levels decreased in NI-inactivated rats involved in the probe test [$F(3,12) = 53.13$; $P < 0.001$]; but there was no difference in pCREB/CREB ratio between the naïve groups (NRL and NRS) (Fig. 6C,D).

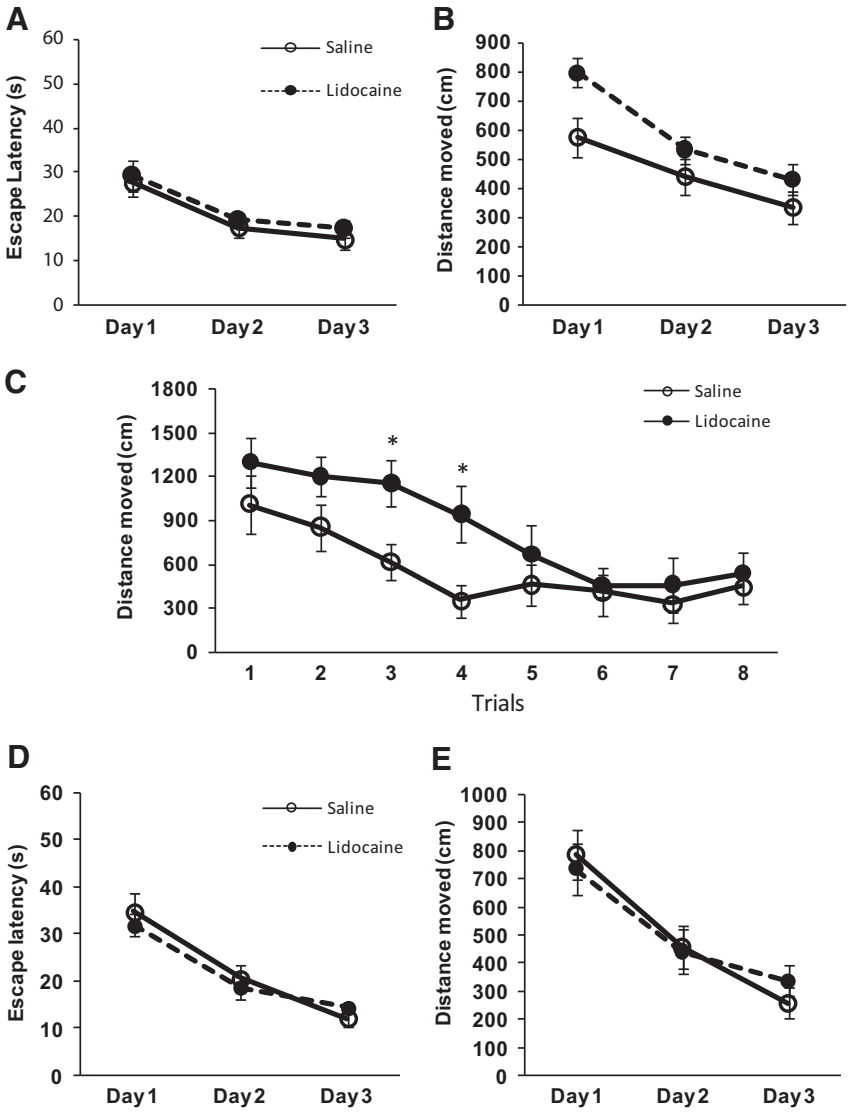

Fig. 2. Escape latency and distance moved in rats that NI was inhibited 5 min before each training session (acquisition: A, B, C) or after it (consolidation: D, E). Based on two-way analysis of variance (ANOVA) repeated measures, the differences between groups are not significant. However, within-session analysis of the first training day of acquisition group (C) showed that the distance moved is significantly higher in the lidocaine group; and post-test revealed significant difference in trials 3 and 4. Data is presented as mean ± SEM. *P < 0.05 compared to the saline group.

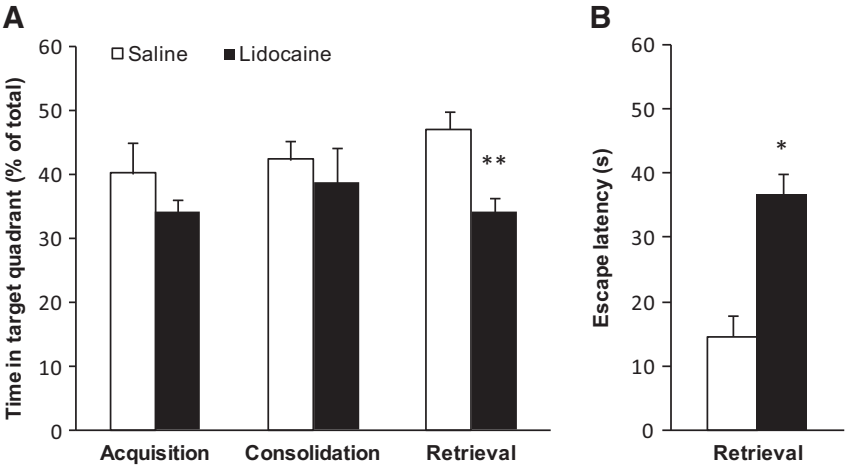

Fig. 3. (A) Time spent in target quadrant as percentage of total swimming time of the probe tests. In these groups, NI was inhibited in acquisition, consolidation or retrieval phases of spatial reference memory task. Acquisition and consolidation groups showed no difference on the probe test. However, NI inactivation before the probe test (retrieval phase) significantly reduced the time spent in the target quadrant, and increased the escape latency (B). Data is presented as mean ± SEM. *P < 0.05 and **P < 0.01 compared to the respective saline group.

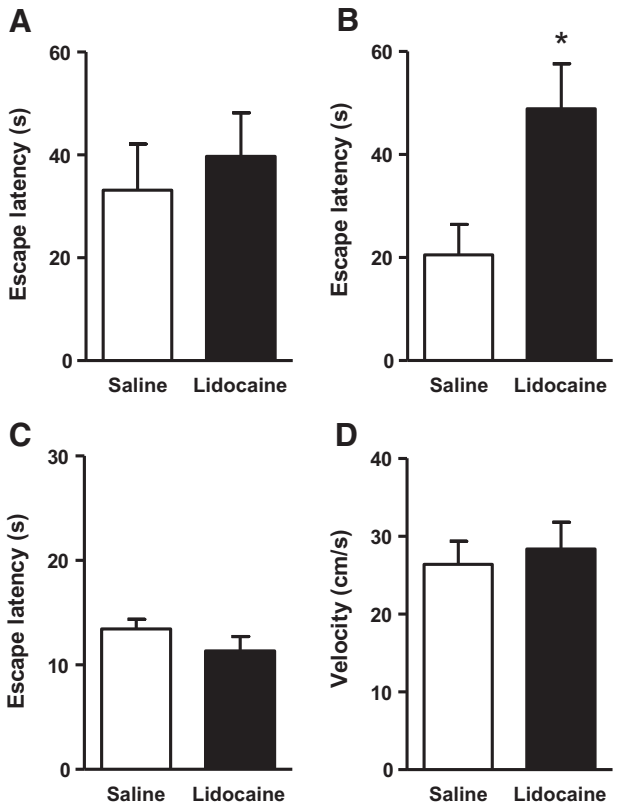

Fig. 4. Escape latency was not affected in acquisition phase of working memory (A), but significantly increased in the retrieval phase (B). Injection of lidocaine 5 min before the visible test did not make any difference in the escape latency (C) or swimming speed (D) between the two groups. Data is presented as mean ± SEM. *P < 0.05 compared to the saline group.

Rats involved in retrieval (RS) showed higher levels of phosphorylated form of CREB, compared to those not involved (NRS). The RL group did not reveal any increase in c-fos or pCREB/CREB levels compared to the NRL group.

3.7. C-fos immunoreactivity was decreased by NI inhibition

Immunohistochemistry against c-fos following completion of MWM task in rats injected with saline or lidocaine in NI revealed differences among the groups. Fig. 5E shows part of the CA1 of the dorsal hippocampus. C-fos positive neurons are evident in the pyramidal cell layer of CA1. Comparison of AS and AL groups suggests decreased immunoreactivity in lidocaine injected rats (Fig. 5 E1,2). Similarly, injection of lidocaine before the probe test reduced c-fos positive neurons in the CA1 area (Fig. 6E).

4. Discussion

The purpose of this study was to evaluate effects of NI inhibition on the spatial reference and working memory. Our findings showed that reversible inactivation of NI delays acquisition in the spatial reference memory and impairs retrieval of both spatial reference and working memory in rats. But the consolidation phase in the reference memory was unaffected. Hippocampal c-fos and pCREB levels increased as a result of training, but decreased in rats treated with lidocaine. Moreover, NI inhibition did not disrupt visuo-motor function. This suggests that the observed differences are not because of rats' physical ability, but rather related to their cognitive performance.

Reversible inactivation of a nucleus/group of neurons lends itself to study their function at different phases of a process [37,38]. The nucleus can be inactivated in one phase and become functional later on when the drug gradually disappears. In the present study lidocaine was used to reversibly inactivate NI at three stages of acquisition, consolidation and retrieval in learning a spatial task. NI contains a heterogeneous population of neurons [7,28], so lidocaine was used purposefully to inactivate all types of NI neurons not just a sub-population. The volume of the injected lidocaine was around 0.4 μl and it has been reported that in the rat's brain tissue this volume diffuses to about 500 μm away from the injection center [39]. However it should be taken into account that diffusion of lidocaine into adjacent areas cannot be ruled out. In interpretation of findings of this study, this should also be mentioned that the injection tip falls in NIc; however, NId is expected to be affected as well by diffusion of lidocaine.

Investigations on the functions of NI and relaxin-3/RXFP3 system are growing recently. Anatomic connections with the hippocampal formation and medial septum, along with electrophysiology findings are suggestive of NI roles in mnemonic processes [2,16]. In the present study NI inactivation in acquisition phase increased distance moved in the first training session. This difference in distance moved on the first training day, and the lack of it on the second and third days suggests that NI inactivation delays acquisition in MWM task. Although a single training session would also be possible and even effective in MWM learning [31,40], a 3-day training protocol gave the opportunity to observe NI inactivation effects on repeated training sessions.

The retrieval phase of reference memory was well influenced by NI inactivation as time in target quadrant reduced and escape latency increased in lidocaine-treated rats. The nature of MWM makes it difficult to exclude the role of stress in interpreting the results [41]. Especially NI has been shown to be an active element in response to stress [10,12,42]. However, the level of stress declines by repetition of swimming sessions [41], turning the thigmotaxic and splashy swimming pattern in the first trials to purposeful and secure in the final ones. The probe test, thus, takes place when rats are much less stressed than the beginning. Water temperature was also maintained around 25 °C to avoid excessive stress. Thus spending less time in the target quadrant seems to be more linked to mnemonic processes than a direct effect of stress. This observation in spatial memory may not be true in other types of memory as a recent study reported that electrolytic lesion of NI delayed extinction of conditioned fear, but had no effect on retrieval of extinguished fear [43].

Assessment of spatial working memory revealed that NI inactivation did not cause a failure in acquisition, but disrupted retention of working memory. In the working memory protocol used in our study rats should target the platform each day in a new position. The strategy to find the platform therefore differs from that in reference memory type: rats should search and scan the maze each new day. They actually should alternate between possible platform locations. Role of NI in working memory was first shown by Ma et al. in an alternation task, where administration of an RXFP3 antagonist into the medial septum decreased alternation behavior [24]. While NI neurotransmission system is basically inhibitory, blockade of its function disrupts some aspects of memory as reported by Ma et al. and here in our study.

The molecular part of this study was designed to reveal if hippocampal neurons' activity, as measured by levels of c-fos and pCREB, is in accordance with the observed effects in the behavioral memory task. It has been reported that c-fos expression increases following neural activity [44]. CREB on the other hand is an important factor in mammalian long term memory [45]. NI inactivation during the acquisition phase decreased c-fos and pCREB levels of the hippocampus compared to the group that received saline, which itself showed higher levels of expression compared to naïve rats not involved in learning (NAS, Fig. 5). This latter observation is in agreement with Porte's report that pCREB levels increase following MWM learning [46]. A similar pattern of expression was seen at the retrieval phase, thus suggesting that NI

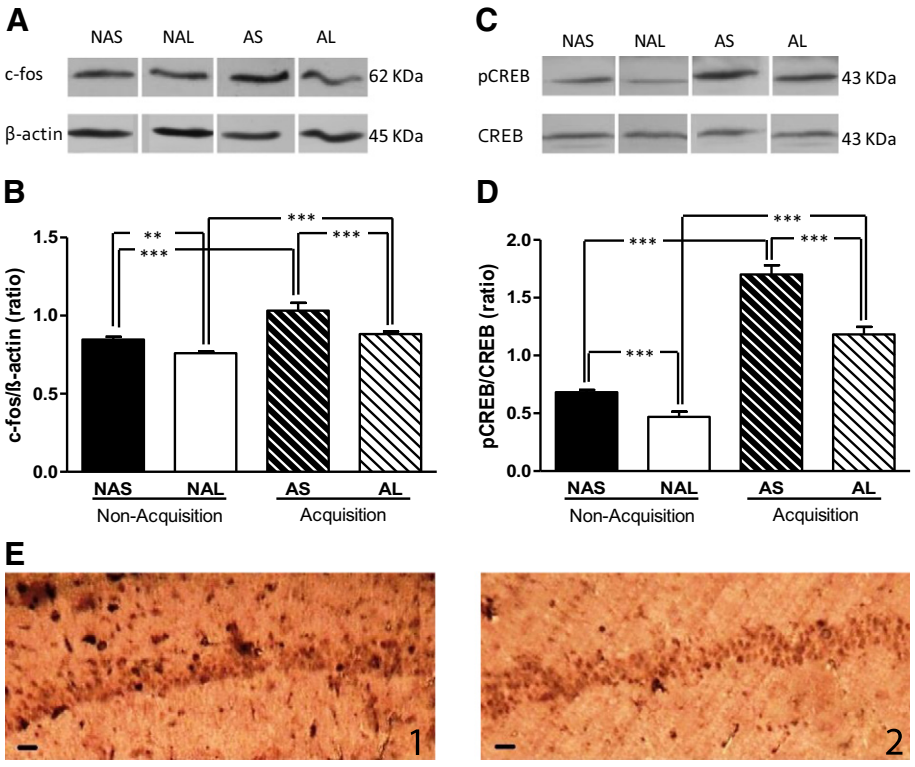

Fig. 5. C-Fos and pCREB levels in lidocaine and saline microinjected rats in the acquisition phase of spatial reference memory. A, B: The densities of c-Fos bands were measured and their ratios to β-actin were evaluated. One-way ANOVA revealed significant differences among the groups. C, D: Also the densities of pCREB bands were measured and their ratios to CREB were evaluated which revealed significant difference between the groups. E1: The CA1 area shows c-fos immunoreactivity in its principal neurons in a rat received saline in NI before training (AS group) in MWM. E2: Similar area in a rat that received lidocaine (AL group) shows much less immunoreactivity. Scale bar: 100 μm. NAL = non-acquisition/lidocaine, NAS = non-acquisition/saline, AL = acquisition/lidocaine, AS = acquisition/saline. Bars represent mean ± SEM. **P < 0.01 and ***P < 0.001.

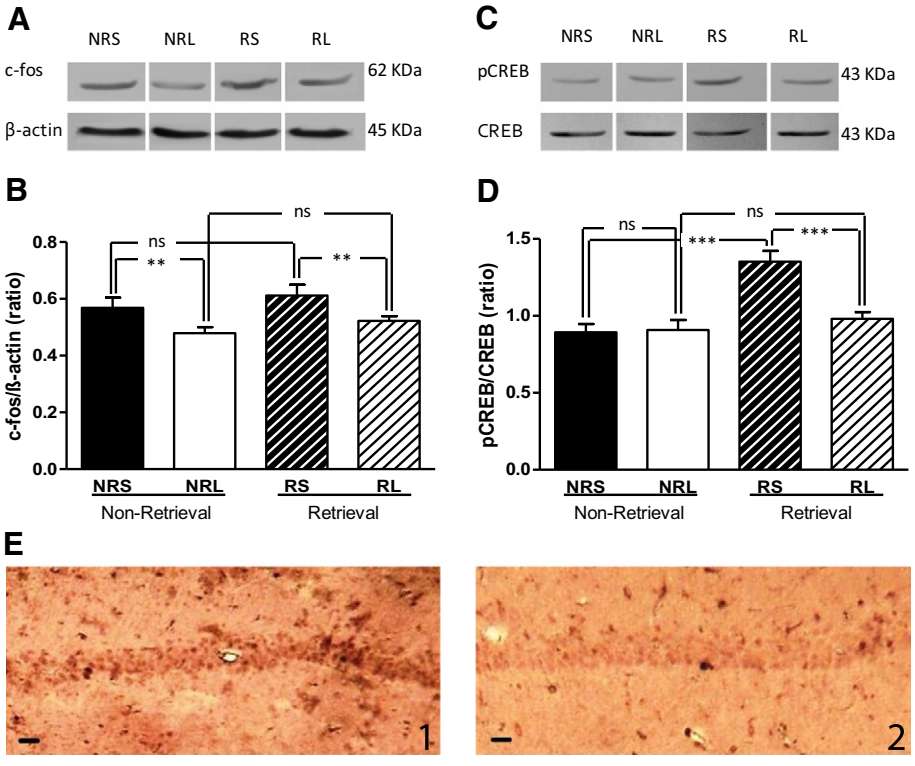

Fig. 6. A,B: c-Fos and pCREB levels in lidocaine and saline microinjected rats in the retrieval phase of spatial reference memory. The densities of c-Fos bands were measured and their ratios to β-actin were evaluated. One-way ANOVA revealed significant differences among the groups. C,D: Also the densities of pCREB bands were measured and their ratios to CREB were evaluated which revealed significant difference between the groups. E1: The CA1 area shows c-fos immunoreactivity in its principal neurons in a rat received saline in NI before the probe test (RS group) in MWM. E2: Similar area in a rat that received lidocaine (RL group) shows much less immunoreactivity. Scale bar: 100 μm. NRL = non-retrieval/lidocaine, NRS = non-retrieval/saline, RL = retrieval/lidocaine, RS = retrieval/saline. Bars represent mean ± SEM. **P < 0.01 and ***P < 0.001.

inactivation reduces hippocampal neural activity in rats involved in acquisition and retrieval of MWM task. Immunohistochemistry against c-fos in the dorsal hippocampus shows a similar pattern observed in western blotting. Differences in levels of positive staining of CA1 principal neurons, though not quantified here, suggest the influence of NI inactivation during learning and memory tasks.

The cognate receptor for relaxin-3 is relaxin family peptide 3 (RXFP3). It has been shown that relaxin-3 projections from NI overlap with RXFP3 mRNA/binding sites [6,12,15]. Hippocampal formation as a main component of spatial cognition receives direct input from the NI; and RXFP3 immunoreactivity in various areas of hippocampus suggests the functional connections [5,6,16,47,48]. The NI is also functionally and indirectly connected to hippocampus through its vigorous connections with the medial septum/diagonal band and RPO, being a relay point in modulation of theta rhythm in the septohippocampal system [3,4,23,24]. Theta oscillation is believed to be involved in spatial navigation through theta entrainment of the place cells [49]. Since theta rhythm is involved in many aspects of learning and memory [1,25,26], any theta-generating/modulating structure might be implicated in learning and memory. As discussed by Ma et al., NI favors theta rhythm by two possibilities [24]. The first is based on the assumption that NI projections to target structures like the medial septum and hippocampus end mainly on inhibitory neurons instead of primary neurons, exerting a net disinhibition effect. The other is that RXFP3 activation by relaxin-3 activates extracellular signal-regulated kinase (ERK)-1/2, and that this activation requires $G_{i/o}$ protein activation and receptor internalization which is protein kinase C-dependent [50]. With either of these mechanisms affecting theta rhythm, or by direct connections with the hippocampus, what has been found in both behavioral and molecular parts of the present study supports the memory-pro functions of NI. NI inactivation, possibly by disconnecting a main path from the brainstem reticular formation to the septal pacemaker and thus interrupting theta rhythm, can lead to disruption of encoding and retrieval. Yet, the direct inhibitory projections of NI have been reported to interfere with hippocampal–medial prefrontal cortex plasticity [51]. Thus, regarding diverse efferents of NI, any observed effect of NI manipulation should be perceived in light of its direct and indirect connections.

5. Conclusion

In conclusion, behavioral results of this study suggest that function of NI is required for acquisition and retrieval phases of spatial reference, and retrieval of spatial working memory. Molecular findings along with immunohistological observations revealed that NI inactivation reduces the enhanced hippocampal activity following learning of the spatial task. This supports behavioral results and suggests that NI is involved in the process of hippocampal learning and memory. The findings of this study, with the utilized NI inhibition method, pertain to spatial memory; and the role of NI in other aspects of memory needs further research. Future studies should also investigate possible role of NI in the hippocampal synaptic activity and plasticity.

Conflict of interest statement

The authors report no conflict of interest.

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