Original Article

Effects of bilateral lesion of the locus coeruleus on the sleep-wake cycle in the rat

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Abstract

Introduction: Noradrenergic cells in LC participate in the process of cortical activation and behavioral arousal. The evidence suggests that locus ceoruleus (LC) plays an important role in the sleep-wake cycle. The aim of this study was stereological estimation of cavity caused by lesion and assessment of sleep stages after bilateral lesion of the LC.

Materials and Methods: Male Wistar rats weighting 250-275 gr were divided into four groups (control: n=6, sham: n=6, lesion1: n=6 and lesion2: n=6). 6 hydroxydopamine (6 OHDA) (2μg/0.5μl and 4μg/1μl) was sterotaxically injected bilaterally into LC to produced lesion. For sleep recording 3 EEG and 2 EMG electrodes were implanted respectively in the skull and dorsal neck muscle. Recordings were taken before and 7, 21 and 42 days after lesion. After 7 weeks, Rats first were anesthetized and then their brains were removed and cut in 7 μm serial sections and stained with cresyl violet. The volume of LC and the lesion induced cavity were evaluated through the stereological technique.

Results: Lesion - induced cavity volume (0.5 μl) was restricted to LC, whereas Another group (1 μl), total LC and structures adjacent to the LC were also damaged. A significant decrease was seen in non-rapid eye movement (NREM) and paradoxical sleep (PS) stages and a significant increase was seen in duration of wake and paradoxical sleep without atonia (PS-A) in lesion group in comparison with control and sham groups.

Conclusion: The results of this study demonstrate 2μg/0.5μl 6-OHDA is suitable dose for LC lesion and bilateral lesion of LC causing disrupt wake, NREM ,PS and also produce the PS-A.

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Introduction

Sleep has a critical issue in medical sleep disorder research, basic neuroscience research, and industrial and transportation safety. In humans, a sleep cycle involves the time from one rapid eye movement (REM) sleep episode to another one (REMS-REMS cycle). Sleep cycle in mammals has been included slow-wave sleep (SWS), also known as non-REM sleep, and

REM sleep. In rodent, sleep is typically divided into wakefulness, REM and non REM (NREM) (Low *et al.*, 2008, Zoubek and Chapotot, 2011), which in rats, proceeds in cycles of SWS to wake without REMS cycles (Simasko and Mukherjee, 2009). A dense collection of noradrenaline (NA)-containing neurons in the dorsolateral pontine tegmentum named locus coeruleus (LC) is involved in the mediation of several behavioural and physiological functions, such as the sleep-wake cycle (Gonzalez and Aston-Jones, 2006). Noradrenergic-LC (NA-LC) system modulates structures involved in sleep regulation (Osaka and Matsumura, 1994, Aston-Jones *et al.*, 2000, Berridge and Waterhouse, 2003, Sakai and Crochet, 2003). NA-LC neurons display state-dependent neuronal activity, discharging at highest rates during wakefulness, at lower rates during SWS, and exhibiting complete cessation of discharge during paradoxical sleep (PS), or REM sleep (Jacobs, 1986, Sara, 2009). In 1965, Jouvet and Delorme demonstrated that bilateral electrolytic lesions of the locus ceruleus, cause to eliminate the atonia of PS (Jouvet and Delorme, 1965). PS exhibits a pattern resembling that of wakefulness while the body musculature is tonically paralysed due to the inhibition of a-motoneurons (Mallick *et al.*, 2002, Aston-Jones *et al.*, 2007). PS without muscular atonia (PS-A) is a common finding that follows bilateral lesions in LC and other pontine areas, while the usual EMG atonia is present during PS after unilateral pontine lesions (Morrison and Reiner, Morrison, 1988). Lesions and stimulation studies identified a critical role of the brain stem neural systems, including the NA-LC system, in the induction and maintenance of arousal (Sakai and Crochet, 2003). 6-hydroxydopamine (6-OHDA) as a destroying agent of dopaminergic and noradrenergic neurons in the brain is toxic at both a peripheral and central level. However, toxicity in the CNS is achieved just by injection into the brain because of the blood-brain barrier exist (Simola *et al.*, 2007). In this study, 6-OHDA was used to clarify further the effect of bilateral LC lesion on the amount of sleep-wakefulness states. Note that in none of the previous studies, a stereological study and assessment of all stages of the sleep cycle in rat bilateral lesion of the LC have been not investigated.

Materials and methods

Animals

Twenty-four adult male Wistar rats weighing between 250-275 g, obtained from Pasteur Institute of Tehran, were used as subjects. The rats were categorized in four groups (Control, Sham (cannula implantation), lesion1 [2μg/0.5μl 6-OHDA] and lesion2 [4μg/1μl 6-OHDA]) and housed individually each animal in one cage at 22 ± 2 °C with a 12h: 12h cycle and given free access to food and water. All studies were performed in accordance with the ethical guidelines set by the "Ethical Committee of Faculty of Medical Sciences, Tarbiat Modares University".

Surgery and electrode implantation

One week prior to any sleep recordings the rats were anesthetized with intra peritoneal (i.p.) injection of ketamine 10% (100 mg/kg) and xylazine 2% (10 mg/kg) and placed in the stereotaxic apparatus. In all animals skull was surgically exposed and 3 EEG electrodes were implanted, one in the left frontal (5 mm A and 2 mm L, from bregma), one over the right parietal (−5 mm A and 6 mm L, from bregma), and a ground electrode over the left occipital cortex (−11 and 4 mm L, from bregma). Two EMG electrodes were implanted into dorsal neck muscle (Simola *et al.*, 2007). In sham and lesion groups stainless-steel guide cannula (23 gauge needle) was bilaterally implantated 1mm above the injection site in the LC to minimize cellular damage at the injection site. The coordinates for LC region were −9.8mm caudal to bregma, right & left ± 1.3mm from the medial suture, and−7.2mm below the skull surface (Paxinos and Watson, 2009). After surgery, the rats were allowed to recover for the next 7 days.

Optimal dosage of 6-OHDA for LC lesion

To obtain the proper dosage of 6-OHDA (Sigma, USA) for lesion of the LC, 12 rats were categorized in two groups (lesion1, lesion2), One group of animals received 2μg/0.5μl 6-OHDA and another group received 4μg/1μl 6-OHDA.

Procedure of bilateral LC lesion

Seven days after electrode implantation, Rats in lesion groups received 6-OHDA (4µg/1µl in 0.1%ascorbic acid and 0.9% saline solution) lesion of the LC by 30 gauge needle that extended 1 mm beyond the implanted guide cannula tip (Boyeson *et al.*, 1993). In the lesion groups (lesion 1 & 2) respectively 0.5 & 1 µl of 6-OHDA was injected slowly at 5 min. The injector cannula was connected to a 1µl Hamilton microsyringe by a polyethylene-20(PE-20) tubing.

Sleep recordings

All the recordings were performed after the rat had been transferred from the home cage to a recording box (nutrient cage into faraday cage, 40 × 40 ×40 cm). The head-stage of the rat was connected to a flexible, shielded cable. The signals were amplified (×100) by polygraph amplifier (SienceBeam, Tehran, Iran) and low-passed filtered at 100 Hz for EEG and 1 kHz for EMG recordings. The EEG/EMG recordings were analyzed with 10-sec epochs and each epoch was assigned to a particular vigilance state included wake, SWS, PS or REM by standard criteria embedded in the software program. If the wake episode exceeds 300 sec the wake episode is assigned to long duration wake (LDW), if the episode is less than 300 sec it is assigned to brief wake (BW). We then used LDW episodes to separate one sleep episode from another vigilance cycling (Simasko and Mukherjee, 2009).

6 hour polygraphic recordings (EEG, EMG) [from 9.00 to 15.00 h] were taken from animals housed in sound attenuated cages with controlled temperature and humidity with food and water provided at libitum. Each recording was preceded by at least 24 h of adaptation to the recording cage. Recordings were taken prior to lesion (7 days after electrode implantation), then 7, 21and 42 days following the lesions.

Sleep analysis

Active wakefulness was defined by low-amplitude, high-frequency waves (beta waves, 15–30 Hz) in the EEG accompanying sustained EMG activity (Fig. 1A). NREM was defined by slow EEG waves with low-frequency (less than 13 Hz), high-amplitude activity and lowered EMG activity (Fig. 1B- D). Fig. 1E indicates the transition from high-amplitude and low-frequency (NREM) to low-amplitude and high-frequency (wake) EEG activity and EMG activity. PS was defined by high-frequency, low-amplitude activity similar to wakefulness waves in the EEG and the absence of EMG activity (Takahashi *et al.*, 2010). All scoring was visually inspected to ensure correct assignment of vigilance states and to eliminate any epochs with artifact noise.

Histology and estimation of the LC volume and extent of damage

After completing all records, intra cardiac perfusion was performed under deep anesthesia with 0.9% sodium chloride and 10% formalin solutions. After paraffin embedding, the brains were cut coronally with rotary microtome into parallel sections with 7µm thickness. Then sections were stained with cresyl violet. 15 sections were used to estimate volumes. In stereological studies, LC and cavity volumes (Left & Right) were determined by Cavalieri method that were estimated by the following formula (Monsefi, 2008):

$$v = \frac{\sum p.ap.t}{m2}$$

V=volume; p=total number of points hitting the LC section, ap=area associated with one test point, t=Interval of between sections.

Statistical Analysis

Data have been presented as the mean ± SE and one-way analyses of variance (ANOVA) used to analyze group differences of the resultant data. The difference between groups was considered as statistically reliable if (P≤0.05). OLYSIA Bio Report software was used for estimation of the LC volume and extent of damage.

Results

All animals except one, which died after the 10 days postlesion (lesion2: n=6-1), achieved a stable recovery following the lesions. Also the LC-lesioned rats lost approximately 50-100 g of weight during the seconds post lesion week.

The LC volume and extent of lesion

In this study, morphology of the LC in control and sham groups was normal and without damage (Fig. 2A, B). LC volume in these two groups was not significantly different from each another. Cavity volume caused by lesion in lesion group with 1 µl 6-OHDA was more significantly in comparison with lesion group with

0.5 µl 6-OHDA [(P≤0.05)] (Table 1). In the group of rats that received 2µg/0.5µl 6-OHDA, lesion was restricted to LC (Fig. 2C), Whereas Another group that received 4µg/1µl 6-OHDA, total LC and structures adjacent to the LC were also damaged (Fig. 2D) According to the obtained results for the cavity and LC volume, 2µg/0.5µl 6-OHDA is Suitable dose for LC

Table 1. The mean volumes of the LC and cavity caused by lesion (Left & Right).
*: Significant differences with lesion2 group (P≤0.05).

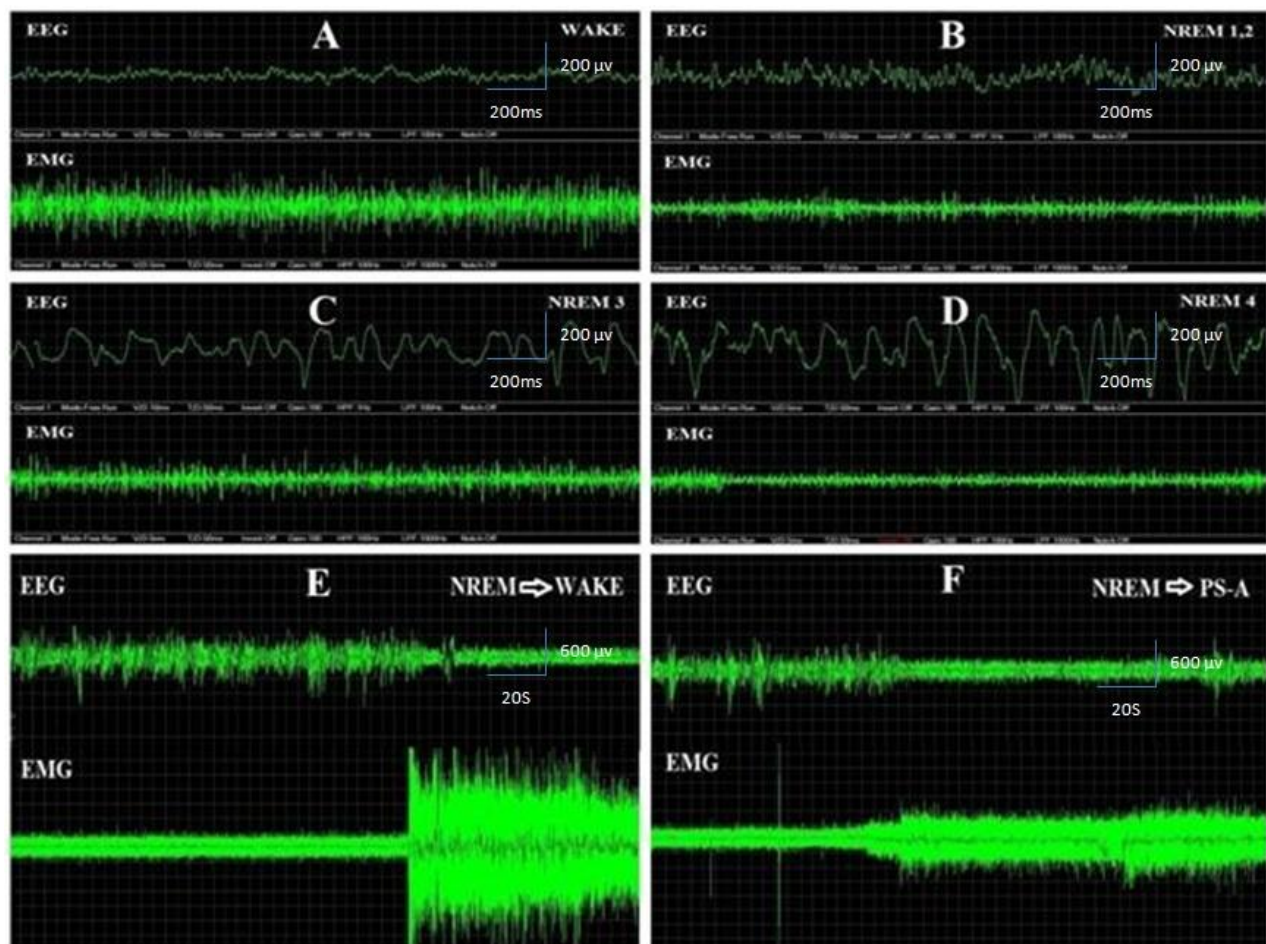| Groups	Number	LC of volume (μm^3)	cavity of volume (μm^3)
Control	6	$(174\pm5.9)\times10^6$	
Sham	6	$(172\pm5.2)\times10^6$	
Lesion 1; 2µg/0.5µl 6-OHDA	6		*$(152\pm6.7)\times10^6$
Lesion 2; 4µg/1µl 6-OHDA	5		$(219\pm10)\times10^6$

Fig 1: Continuous record of the EEG and EMG. The records in A is Related to the wakefulness, in B-D are episodes of NREM sleep and in E is Waking up from the NREM stage. In fig. F transition from high-amplitude to low-amplitude EEG activity and the gradual increase in the EMG, Indicates the beginning of an episode of paradoxical sleep without atonia.

lesion. The amount of LC lesion was directly related to dose of 6-OHDA. So, the mean of the Cavity volume were lesser than mean LC volume in control and sham group (Table 1, P≤0.05).

Sleep patterns in rats

In fig. 3, Hypnograms of sleep recordings are shown. In the 6 hour polygraphic recordings the rats have many brief periods of sleep activity (NREMS with some PS or PS-A) separated by long periods of wake. There were many brief wakening during sleep periods. The long and brief periods of wake between periods of sleep activity are as LDW and BW and also the periods of sleep activity separated by LDW episodes are as vigilance cycling (VC). In all groups VC number is 2 or 3. There was not any PS-A in control and sham groups (Fig. 3A, B). The PS-A appearing in lesion group and amount of PS the Descending is reduced at 7 (Fig. 3C), 21 (Fig. 3D) and 42 (Fig. 3E) days after the lesions.

Bilateral lesion of LC and PS-A

The result indicates bilateral lesion of LC produced the PS-A, The EEG activity was consisted of high-frequency, low-voltage waves in both wakefulness and PS episodes.

EMG show high activity during wakefulness. In the PS muscles activity gradually diminished, whereas in the PS-A, EMG activity gradually increased (Fig. 1F). After the LC lesioning, characteristic atonia of paradoxical sleep was lacking (Fig. 1F). Each PS-A episode followed a period of NREM. The transition in fig. 1F from high-amplitude to low-amplitude EEG activity and the gradual increase in the EMG, indicates the beginning of an episode of paradoxical sleep without atonia.

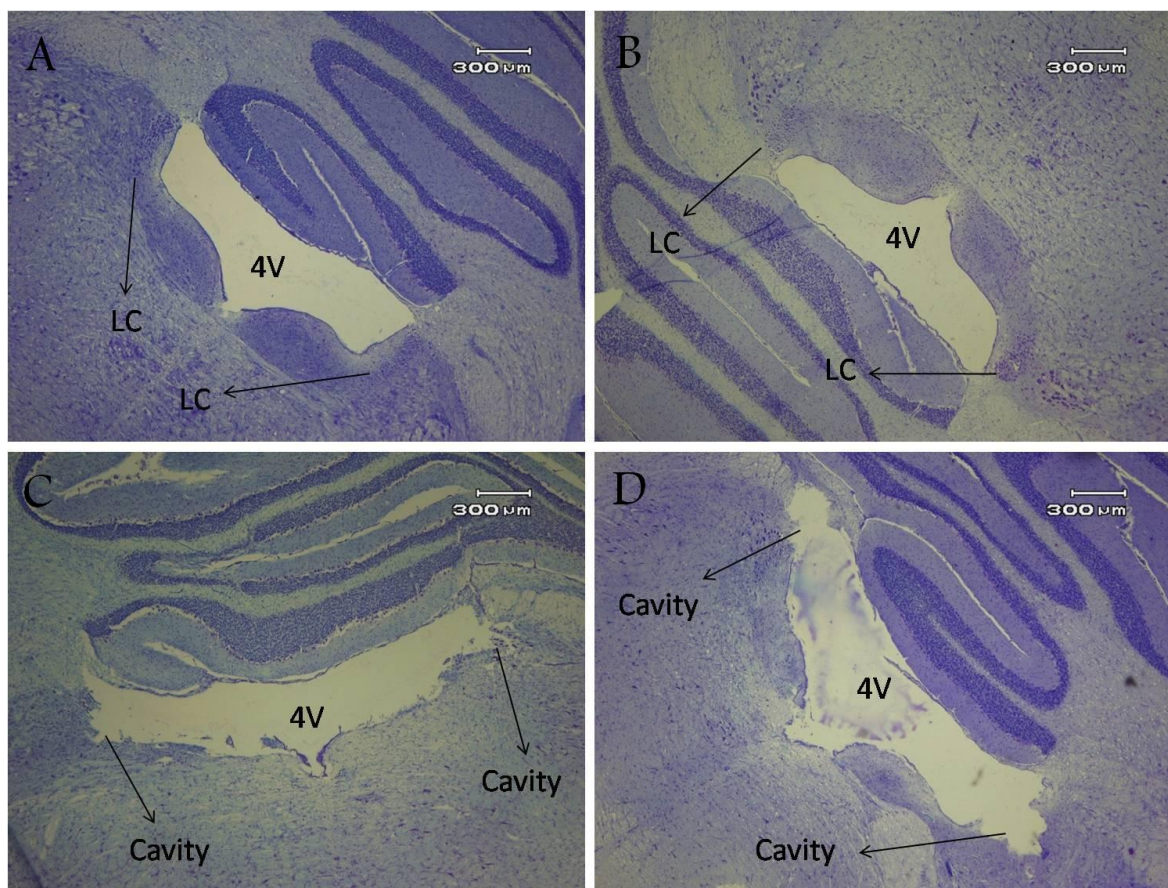## Effects of lesions on the amounts of sleep-waking states

Fig 2: Coronal sections of the brain stem. In control (A) and sham (B) groups, the LC was without damage. The lesion groups that received respectively 2μg/0.5μl 6-OHDA (C) and 4μg/1μl 6-OHDA (D).
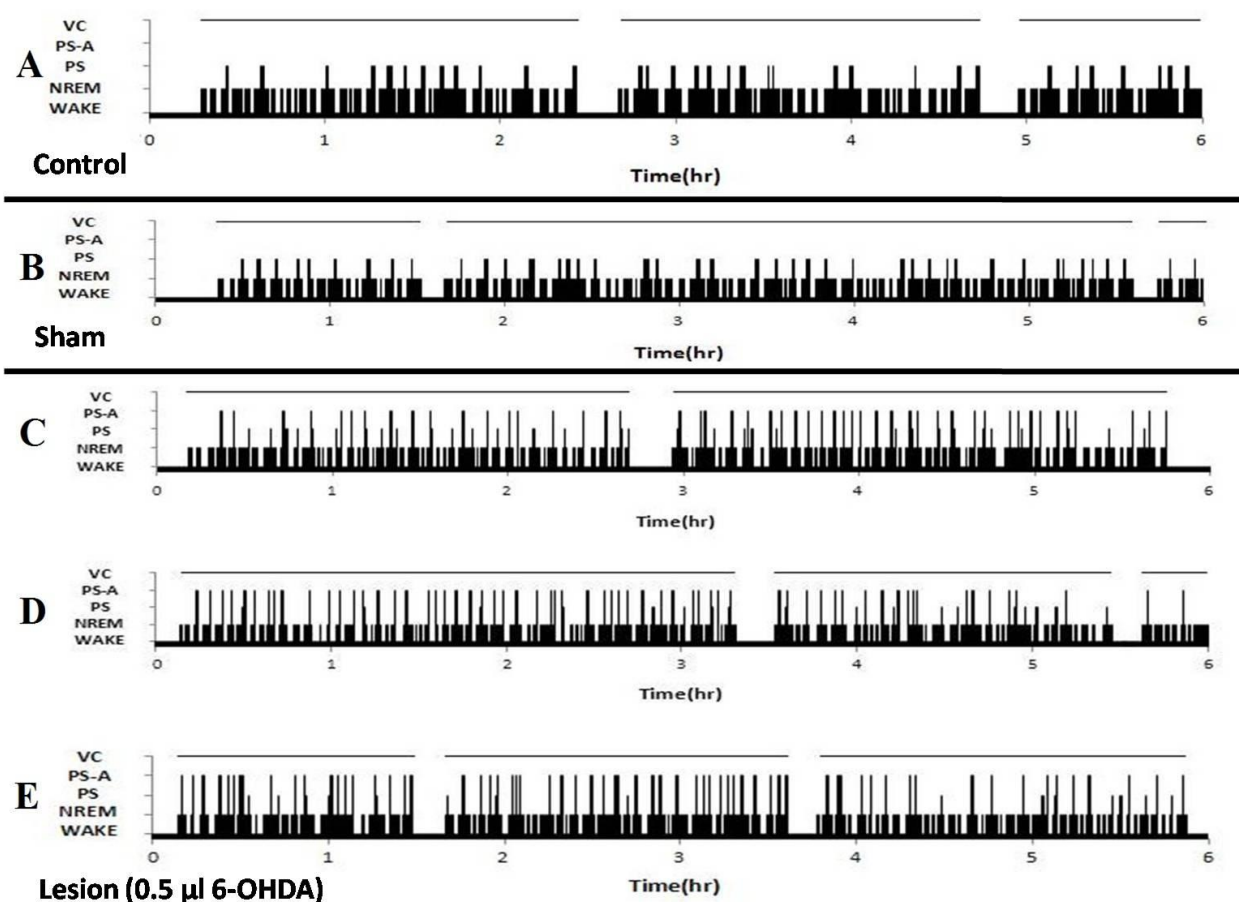
Fig 3: Hypnograms from five representatives sleep recording. Control (A), Sham (B) and lesion: 7 (C), 21(D) and 42 (E) days after the lesions.

The fragmentation of the sleep-waking cycle observed following LC lesions. The number of episodes of each state excluding PS increased after lesioning. The neural damage caused by implanted guide tubes in sham group did not produce significant alterations in sleep-waking states in comparison with control group. The amount of NREM and PS in lesion group were significantly reduced at 7, 21 and 42 days after the lesions in comparison with control and sham groups (Fig. 4B, C) and also amount of wakefulness and PS-A in this group were significantly increased at 7, 21 and 42 days after the lesions (Fig. 4A, D).

Discussion

In this study we observed that 2μg/0.5μl dose of 6-OHDA is suitable dose for LC lesion, whereas using the 4μg/1μl dose of 6-OHDA, In addition to total LC, structures adjacent to the LC were also damaged.

Different lessoning results may be due to not only the different concentration of 6-OHDA but also different volume of solution. There are some studies on lesion of LC by electrolytic radiofrequency (Mallick *et al.*, 2005) chemical and 6-OHDA (Arvin *et al.*, 1992, Fulceri *et al.*, 2007, Wang *et al.*, 2010) But none of these studies have not been used as a Model for study Sleep-Wake Cycle. The present study has demonstrated that cells and/or fibers of passage in the region of the nucleus locus coeruleus are essential to the integrity of sleep.

Our study shows that bilateral lesion of LC produce specific changes in the amount of sleep-waking cycle. Mean percentages of NREM and PS in the bilateral lesion of LC were significantly decreased. On the other hand, mean percentages of wakefulness and PS-A were significantly increased. Previous studies on the unilateral lesion of LC in cat, have confirmed that inactivation of the caudal part of LC enhanced the

generation of PS. In these studies, results suggest an inhibitory role of the LC caudal part for the appearance of PS (Jones *et al.*, 1977, Caballero and De Andres, 1986). An inhibitory role of the LC caudal area in PS generation has not been detected in other studies involving bilateral permanent destruction of this region produced by electrolytic radiofrequency or chemical lesions (Jones *et al.*, 1977).

In line with other studies involving LC unilateral lesions, we observed PS-A in our bilaterally operated rats. Similar to our study bilateral lesions of LC in cat (Henley and Morrison, 1974) and rabbit (Braun and Pivik, 1981) produced PS-A. Additionally, we found that abolition of PS atonia is the main effect of bilateral lesion of LC.

Studies involving LC lesions have not exceeded post lesion survival periods beyond 21 days with exception of one study which maintained 6 cats for periods of at least two months post lesion, and one cat up to six months (Henley and Morrison, 1974). In another study on 6 rabbits, post lesion survival periods were 30 days (Braun and Pivik, 1981). Moreover, sleep states were not quantified so that it was difficult to judge whether the PS-A phenomenon described was a permanent effect. In the present study, quantified analyses of recordings obtained in 6 LC-lesioned rats maintained 42 days post lesion. The results indicated persistence of all LC lesion associated behaviors, including the presence of PS-A, the decrease in the amount of time spent in PS, and the waking behavioral effects.
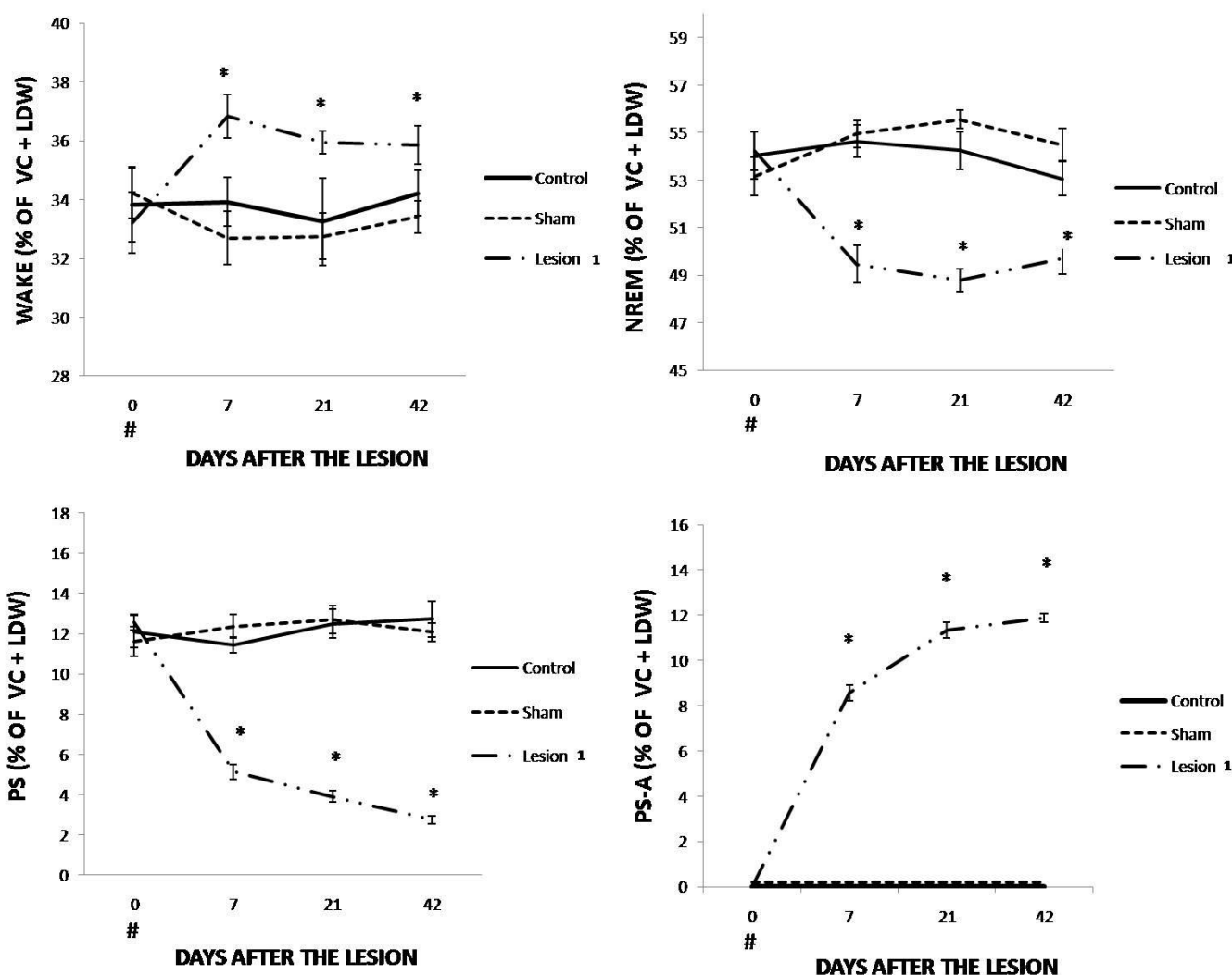

Fig 4: Percentages of wakefulness (W), non rapid eye movement (NREM), paradoxical sleep (PS), and PS without atonia (PS-A) reached in the baseline records of all rats. A: Wake, B: NREM, C: PS, D: PS-A.
#: Time of lesion, 7 days after electrode implantation. *: Significant differences with other groups (P≤0.05).

Lastly, and in agreement with previous data (Henley and Morrison, 1974, Braun and Pivik, 1981) our results showed that PS-A is observed after bilateral lesion LC. Collectively, our study suggests that the dose of 2µg (6 –OHDA) is more suitable than dose of 4µg for LC lesion and cavity volume caused by lesion is restricted to LC. In other words, the amount of LC lesion was directly related to dose of 6-OHDA. Also, we showed that in lesion group amount of PS and NREM were significantly reduced and amount of wake and PS-A were significantly increased in comparison with control and sham groups.

Acknowledgments

Conflict of interest

The authors declare that they have no conflict of interest.

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