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In Vivo Microdialysis Measures of Extracellular Norepinephrine in the Rat Amygdala during Sleep-wakefulness

Norepinephrine (NE)-containing locus ceruleus (LC) has been known to participate in the regulation of the sleep-wake cycle according to the differential firing rate. The aim of this study was to know the change of extracellular NE level in the rat amygdala, which are reciprocally connected with LC, during sleep-wakefulness. Extracellular NE levels in the rat amygdala were inrestigated during different stages of the sleep-waking cycle using in vivo microdialysis and polygraphic recording. Dialysates were collected every 5 min and correlated with the results of polygraphic recording. The content of NE was measured by high-performance liquid chromatography with electrochemical detection. NE level was the highest in active waking (AW) and, when compared to AW, NE level was progressively lower in quiet waking (QW; 86%), quiet sleep (QS; 72%), and active sleep (AS or REM sleep; 61%). This result suggests that the rat amygdala also participates in the regulation of the sleep-wake cycle according to the differential NE release.

Key Words : *Sleep; Norepinephrine; Amygdala; Rats; Microdialysis*

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INTRODUCTION

Norepinephrine (NE) is synthesized by the neurons of brainstem locus ceruleus (LC) nuclei, and NE-containing LC neurons are reciprocally connected with forebrain structures, including the amygdala (1, 2). NE has been implicated in the regulation of the sleep-wake cycle on the basis of numerous investigations (3-6). The firing rate of LC with extracellular electrophysiologic recordings during the sleep-wake cycle has been described to be the highest during wakefulness (W), lower during slow wave sleep (SWS), and virtually absent during paradoxical sleep (rapid eye movement sleep, REM sleep) in animals (5, 6). Given these observations, fluctuations of NE neuronal activity in the LC might be expected to correlate with NE release in the amygdala that receives projections from these nuclei.

Microdialysis is a sampling method that is used to determine the extracellular concentration of neurotransmitters in the brain. The method can be applied to conscious and unrestrained animals and is very suitable for the study of chemistry of endogenous behavior (7). During the last 12 yr a number of in vivo microdialysis studies have demonstrated that monoamines regulate the sleep-wake cycle in animals. Especially, the central serotonergic system is closely linked to the sleep-wake cycle (8-12). In cat studies, extracellular serotonin (5-HT) level was higher during W, lower during SWS, and even lower or absent during REM sleep in the dorsal raphe nucleus (DRN) (8), the striatum and hypothalamus (9). A rat study, which measured extracellular 5-HT levels in DRN and frontal cortex, demonstrated similar results to those shown in cat studies (12). The same changes of 5-HT level in rat hippocampus was also proved (13). While the microdialysis studies of 5-HT level during the sleep-wake cycle are abundant, those of NE level are relatively few (10, 11). In the cat study, the extracellular level of NE in LC and amygdala declined progressively from W to SWS and then to REM sleep (10). A rat study, which measured extracellular level of NE in the hippocampus without scoring the sleep stages, showed similar results (11). However, there have been no microdialysis studies to measure NE level in the rat amygdala.

This experiment used in vivo microdialysis designed to determine whether NE release in the rat amygdala is correlated with differential firing rate of LC across the sleep-wake cycle.

MATERIALS AND METHODS

Experiments were performed in five adult male Sprague-Dawley rats weighing 300-580 g. The rats were kept on a 12 hr light/dark cycle with lights on at 07:00 a.m.. Surgery was done under anesthesia with a combination of ketamine HCl (100 mg/kg, intra-muscular, i.m.), xylazine (4 mg/kg, i.m.), and acepromazine (0.75 mg/kg, i.m.), and preoperatively treat-

ed with atropine (0.02 mg, subcutaneous s.c.). Buprenorphrine (0.01 mg, s.c.) was used for additional operative and postoperative analgesia. Animals were implanted with stainless steel screw electrodes for lateral (bregma coordinates AP=-1.0 mm, L=3.0 mm) and medial (AP=midpoint between bregma and lambda, L= 1.0 mm) epidural electroencephalogram (EEG) recording. Bipolar depth electrodes were used for dorsal hippocampal recording (AP=-3.6 mm, L=2.0 mm, H=-4.0 mm) (14). Teflon-coated stainless steel wires, stripped of the insulation in the last 3 mm, were placed in the neck muscle for electromyogram (EMG) recording. An intracerebral 20-gauge stainless steel guide cannula was implanted 2 mm above the target (AP=-3.6 mm, L=5.0 mm, H=-6.0 mm) to guide and secure the probe in the amygdala. Guide cannulae were implanted in the left amygdala (3 rats) or right amygdala (2 rats).

One week was allowed for recovery from surgery. Rats were connected to a polygraph (Grass Instruments, Model 8-16, U.S.A.) with a flexible recording cable linked to a combined fluid swivel and electrical commutator. Ambient temperature in the chamber was approximately 28℃. Rats had free access to water and food pellets. Probes were inserted 24 hr before starting sampling, in order to allow for equilibration of the extracellular environment surrounding the microdialysis membrane.

Microdialysis probes (CMA 11, 2 mm membrane, CMA /Microdialysis) were perfused with artificial cerebrospinal fluid (aCSF=125 mM NaCl, 2.5 mM KCl, 0.9 mM NaH_2PO_4-H_2O, 5 mM Na_2HPO_4, 1 mM $MgCl_2$-$6H_2O$, 1.2 mM $CaCl_2$-$2H_2O$, and pH 7.4-7.6) at a flow rate of 2 μL/min. Samples were collected at 5 min intervals during the light period for 3 hr (from 10 a.m. to 1 p.m.). Dialysate samples were collected for three or four consecutive days for each amygdala site. Samples were stored at -70℃.

High performance liquid chromatography with electro-

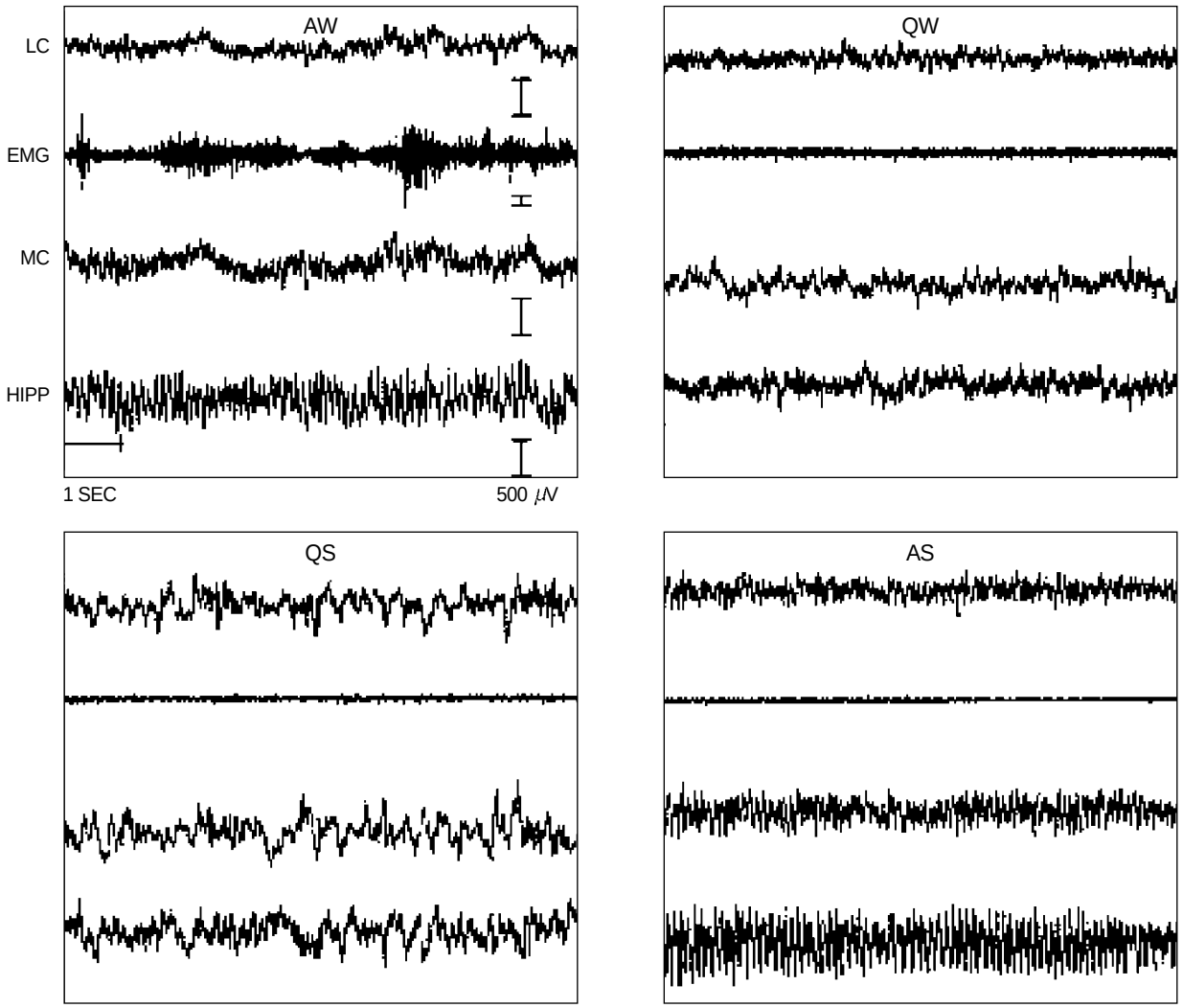

Fig. 1. Examples of polygraphically defined states. Abbreviations: LC-lateral cortex; EMG, Electromyogram; MC, medial cortex; HIPP, dorsal hippocampus; AW, active waking; QW-quiet waking; QS quiet sleep; AS active sleep.

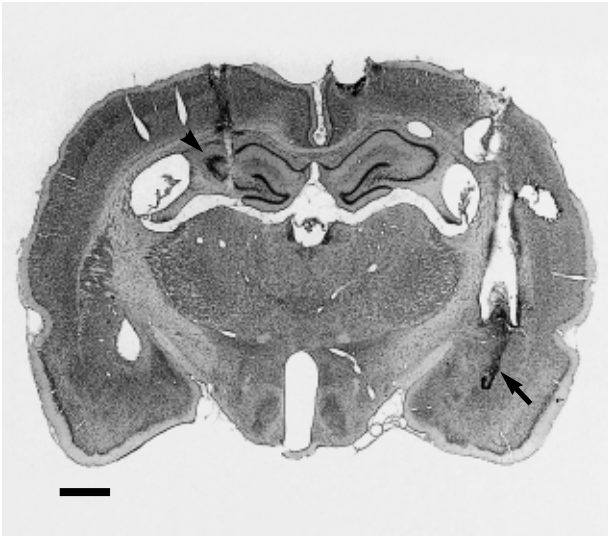

Fig. 2. Composite photomicrograph of a rat brain section which shows the location of the microdialysis probe (arrow) and dorsal hippocampal depth electrode (arrowhead). The tip of the guide cannula was located over the striatum, so the membrane was entirely positioned within the amygdala. Calibration bar: 1 mm.

chemical detection HPLC-EC, Antec) was used for determination of NE in dialysates using a mobile phase consisting of sodium acetate (75 mM), sodium dodecane sulphonate (0.75 mM), EDTA (10 μM), triethylamine (0.01%), acetonitrile (12%), methanol (12%), and tetrahydrofuran (1%), pH 5.5, pumped at a rate of 200 μL/min (Shimadzu model LC-10AD) through a 100 × 2 mm column (3 μm, Hypersil C18 resin). Under these conditions NE retention time was around 4 min. The system was calibrated at regular intervals and provided a limit of detection of 20 pM for a 5 μL injection.

EEG and EMG were recorded using a Grass polygraph, Model 8-16, and digitized at 500 Hz/channel on a Pentium PC using R-C Electronic software. Behavioral states were scored in epochs of 16 sec, into 4 stages based on modified criteria given by Marrosu et al. (15): (1) Active waking (AW): gross body movements, cortical desynchronization, medium-high EMG amplitude, and sustained hippocampal theta activity. (2) Quiet waking (QW): EEG desynchronization and low amplitude EMG potentials. (3) Quiet sleep (QS) or slow wave sleep (SWS): cortical and hippocampal high voltage slow waves and low amplitude EMG potentials. (4) Active sleep (AS) or REM sleep: desynchronized cortical EEG, remark-

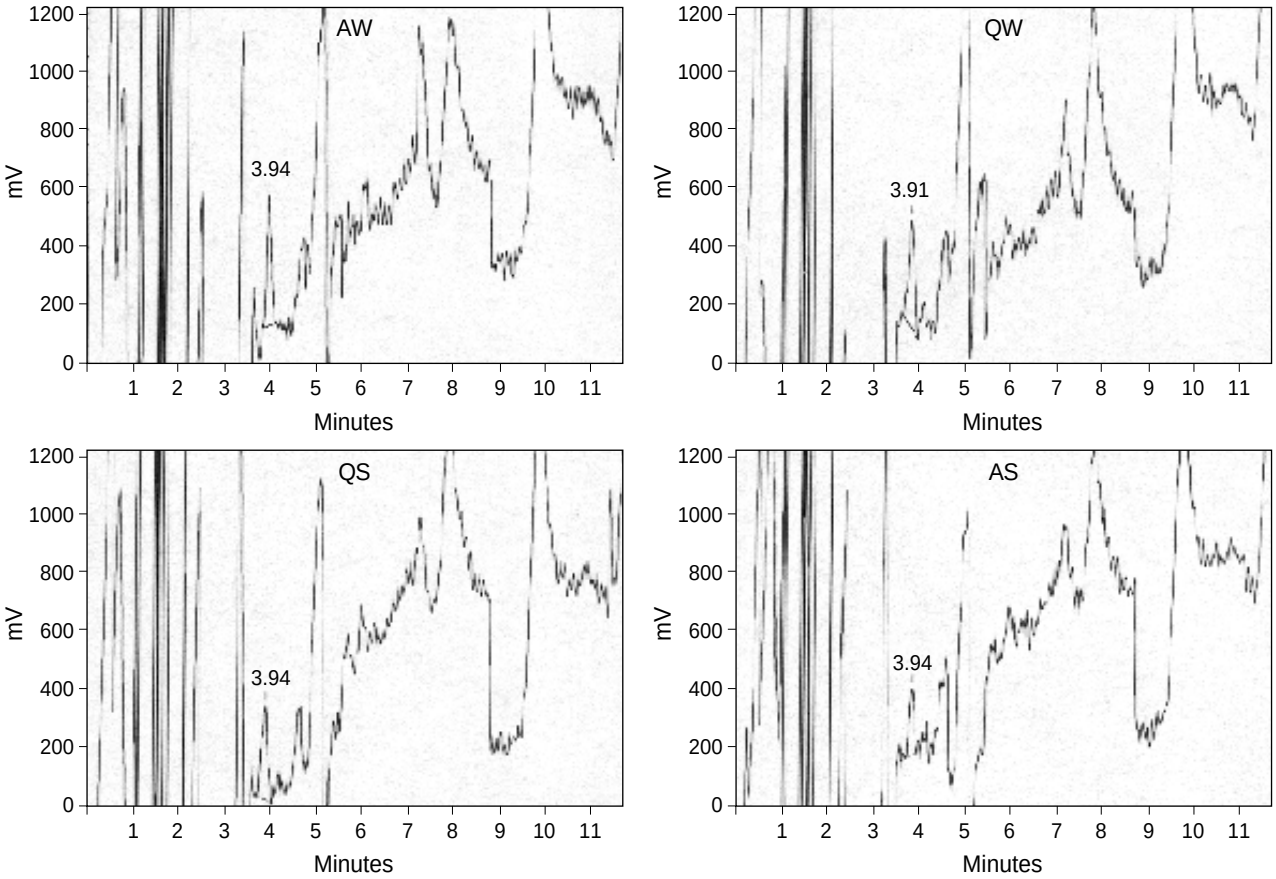

Fig. 3. Examples of chromatograms of norepinephrine (NE) peak from amygdala. NE peaks are indicated with their retention times in minutes. Note the progressive reduction in NE levels from active waking to active sleep. In this particular example, NE peak during AW was equivalent to 411 pM. Abbreviations: AW active waking; QW-quiet waking; QS quiet sleep; AS active sleep.

ably regular pattern of hippocampal theta activity, and very low amplitude EMG (Fig. 1). Sixteen-second epochs were scored according to the dominant state in that particular epoch. Samples categorized as AW, QW, or QS were chosen from those containing AW, QW, or QS for more than 4 of the 5 min in the sampling period. Because AS durations are generally shorter than those of the other stages, samples that included periods of AS lasting more than a half of the 5 min were categorized as AS samples.

Following completion of the experiments, the rats were euthenized with pentobarbital overdose and perfused with saline and 4% paraformaldehyde, prior to cresyl violet staining to verify the placement of probes within the amygdala.

Statistical analysis was performed using repeated measures one-way analysis of variance (ANOVA) to compare dialysate NE concentrations measured from AW, QW, QS, and AS samples. Neuman-Keuls test was used for post-hoc comparisons. For graphing purposes, the values for QW, QS, and AS were converted into percent of each animal's AW mean value in each amygdala sample.

RESULTS

In histological examinations, the probes were all successfully implanted in the amygdala. An example of a probe placement in the amygdala is shown (Fig. 2).

Data were available from 5 amygdala in 5 rats. A distinct peak was observed in 620 samples. Among these 620 samples, 320 samples met the criteria described above with a single state occupying at least 80% (for AW, QW, and QS) or 50% (for AS) of the 5 min sample period. Thus, 68 samples met the criterion for AW, 73 for QW, and 126 for QS. The less stringent '50% of 5 min period' criterion for AS was met in 53 samples. In these samples categorized as AS, the second most predominant state in 40 of the 53 samples was QS (75% of the AS samples).

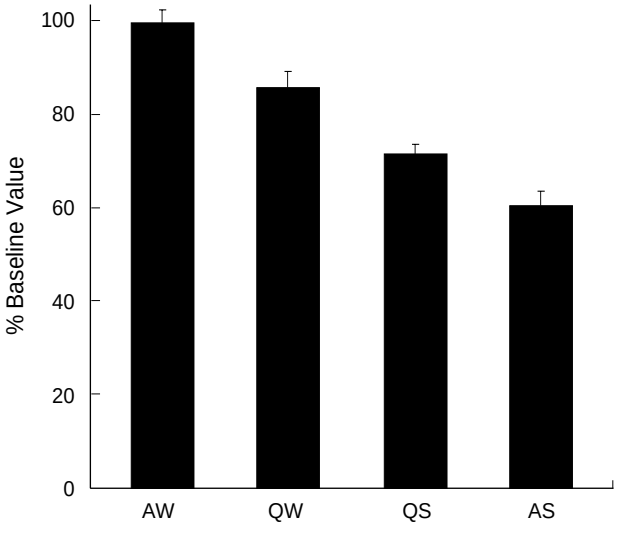

Fig. 4. Bar histogram depicting mean percentage changes of norepinephrine (NE) compared to baseline in each behavioral state. Baseline is calculated as the average of NE in active waking (AW) in each amygdala. Error bars represent S.E.M. Note the reduction of NE from AW to quiet waking (QW), to quiet sleep (QS), and to active sleep (AS). All these changes are statistically significant ($p<0.001$). Baseline value for AW was 223 ± 6 pM.

Dialysate NE concentration was highest in AW (223 ± 6 pM), decreased to 192 ± 8 pM in QW, to 160 ± 5 pM in QS, and to 136 ± 7 pM in AS ($p<0.001$). Neuman-Keuls post-hoc analysis showed that the differences between each of the states' mean NE concentrations were also statistically significant ($p<0.01$ in all pair-wise comparisons). Illustrative chromatograms of NE peak from the amygdala are shown according to the sleep-wakefulness (Fig. 3). Converted to percentage of AW, the mean NE level decreased significantly from AW to QW (86%), to QS (72%), and to AS (61%) (Fig. 4).

DISCUSSION

To the best of my knowledge, the present study is the first to monitor extracellular NE levels in the rat amygdala during the sleep-wake cycle using in vivo microdialysis with simultaneous polygraphic staging of sleep and waking. The analysis of behavioral state with polygraphic recordings allows accurate scoring of sleep-wake cycle states. Recently, there was a microdialysis study comparing the concentrations of NE derived from feline LC and amygdala (10). The NE change in this study is similar to that of the present study. Therefore, the present study demonstrates that the sleep-related changes in NE release as described in cats are also present in rats.

Several related hypotheses propose that the LC is involved in initiating or maintaining stages of the sleep-wake cycle (3, 4). Extracellular electrophysiological recordings in freely moving rats and cats proved this view since LC neurons fired tonically at a rate of 2.2 Hz during W, decreased their activity during SWS to an average rate inferior to 1 Hz, and were nearly quiescent during REM sleep (5, 6). Intracellular recordings in slices and in culture evidenced the spontaneous tonic firing of LC neurons during W mainly due to their intrinsic pacemaker properties (16, 17). In addition, stimulation of the LC promoted W and localized cooling of the LC promoted REM sleep (18, 19). Thus, the noradrenergic nuclei of the LC and synthesized NE appear to inhibit REM sleep production and promote W. The present study demonstrated that the level of extracellular NE in the amygdala was highest in AW and progressively decreased in QW, QS, and AS. Although the 5 min samples collected during AS also included other behavioral states, most of these 5 min periods were a combination of AS and QS. Because the mean NE level during QS was higher than that collected during

periods consisting of more than 50% of AS, my assumption is that the behavioral state with the least NE release is AS. This pattern of sleep-related NE change is consistent with that of firing rate of LC with extracellular electrophysiological recordings (5, 6). The parallel between noradrenergic activity measured by microdialysis and LC firing rates supports the conclusion that changes in NE levels in the amygdala measured by microdialysis reflect changes in synaptic release during the different sleep-wake states and their participation in the modulation of the sleep-wake cycle.

The level of extracellular 5-HT in the rat DRN, frontal cortex and hippocampus was the highest in AW and progressively decreased in QW, QS, and AS (12, 13). This pattern is similar to that of the present amygdala study. Therefore, a plausible assumption would be that NE might complement the action of 5-HT in promoting W and participating in the inhibition of REM sleep (20).

In conclusion, the rat amygdala participates in the regulation of the sleep-wake cycle according to the differential NE release. From a practical point of view, the state-dependent changes in NE levels indicate that sleep states must be controlled during any experiment of the behavioral correlates of NE release.

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