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Improvement of Two-Way Active Avoidance Memory Requires Protein Kinase A Activation and Brain-Derived Neurotrophic Factor Expression in the Dorsal Hippocampus

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Abstract

Previous studies have shown that two-way active avoidance (TWAA) memory processing involves a functional interaction between the pontine wave (P wave) generator and the CA3 region of the dorsal hippocampus (DH-CA3). The present experiments examined whether the interaction between P wave generator activity and the DH-CA3 involves the intracellular protein kinase A (PKA) signaling system. In the first series of experiments, rats were subjected to a session of TWAA training followed immediately by bilateral microinjection of either the PKA activation inhibitor (KT-5720) or vehicle control into the DH-CA3 and tested for TWAA memory 24 h later. The results indicated that immediate KT-5720 infusion impaired improvement of TWAA performance. Additional experiments showed that KT-5720 infusion also blocked TWAA training-induced BDNF expression in the DH-CA3. Together, these findings suggest that the PKA activation and BDNF expression in the DH-CA3 is essential for the improvement of TWAA memory.

Keywords

BDNF; Learning; Pontine wave; PKA; Rat; Rapid eye movement sleep

Introduction

Over the last four decades, an impressive number of studies have shown that sleep confers a beneficial effect on learning and memory (Smith 1995; Gottesmann 1999; Maquet et al. 2003; Peigneux et al. 2003; Datta 2006; Stickgold and Walker 2007). To understand the neurobiological mechanisms involved in the beneficial effects of sleep in cognitive functions, recent physiological and behavioral studies have demonstrated that rapid eye movement (REM) sleep-dependent memory processing depends on pontine wave (P wave) generator activation-mediated interaction with the dorsal hippocampal CA3 region (DH-CA3) (Mavanji et al. 2004; Datta et al. 2005). The P wave generator contains a group of glutamatergic cells in the pons, which project directly to the dorsal hippocampus (DH), amygdala, cerebral cortex, and many other regions of the brain that are directly involved in learning and memory processing functions (Datta et al. 1998; Datta 2006). These P wave-generating cells remain silent during wakefulness and slow-wave sleep (SWS), but during the transition from SWS to REM sleep and throughout REM sleep, these cells fire in high-frequency bursts (Datta and Hobson 1994; Datta 1997; Gottesmann et al. 1998). Activation of the P wave generator increases

glutamate release and frequency of theta waves in the DH; both of these conditions have a positive influence on memory processing (Booth and Poe 2006; Datta 2006).

Recent studies have demonstrated that chemical activation of the P wave generator and/or two-way active avoidance (TWAA) learning training increases the phosphorylation of the cAMP response element-binding protein (CREB) and expression of the immediate early genes, activity-regulated cytoskeletal-associated protein (Arc), brain-derived nerve growth factor (BDNF), and early growth response 1 (Egr-1) in the DH, amygdala, and cerebral cortex (Saha and Datta 2005; Ulloor and Datta 2005; Datta et al. 2008). Neuronal activation-dependent expression of Arc, BDNF, and Egr-1 in the hippocampus and amygdala has also been shown to be critical to the formation of long-term memories (Guzowski et al. 2000; Hall et al. 2000, 2001; Ribeiro et al. 2002). Some other studies have shown that activation of the intracellular signaling molecule protein kinase A (PKA) in the DH is involved in memory processing (Abel et al. 1997; Bernabeu et al. 1997; Bourchouladze et al. 1998; Schafe et al. 1999; Ouyang et al. 2008). However, it remains to be determined whether the intracellular PKA system in the DH is involved in the processing of TWAA memory. The results of the present study demonstrate that the localized inhibition of intracellular PKA activation blocks TWAA training trials-induced BDNF gene expression in the DH-CA3 and improvement of TWAA performance in the test session.

Materials and Methods

Subjects and housing

Experiments were performed on 35 Sprague–Dawley rats (Charles River, Wilmington, MA, USA) weighing between 250 and 300 g. Rats were housed individually at 24°C with free access to food and water. Lights were on from 7:00 A.M. to 7:00 P.M. (light phase) and off from 7:00 P.M. to 7:00 A.M. (dark phase). Animals were cared for in accordance with National Institutes of Health guidelines for laboratory animal welfare. All experiments were approved by the Boston University Institutional Animal Care and Use Committee (Protocol no: AN-14085). To minimize stress that might be imposed by the experimental protocol, animals were handled daily for 15–20 min between 09:00 A.M. and 10:00 P.M. This habituation handling began 2 weeks prior to surgery and continued for the duration of the experiment.

Surgical procedures for the implantation of electrodes and guide tubes

All surgical procedures were performed under pentobarbital anesthesia (40 mg/kg, i.p; Abbott Laboratories, Chicago, IL, USA) under aseptic conditions. Stereotaxic coordinates for electrode placement were taken from the atlas of Paxinos and Watson (1997). To record behavioral states of vigilance, cortical electroencephalogram (EEG), dorsal neck muscle electromyogram (EMG), hippocampal EEG (to record theta wave), and pontine EEG (to record P wave) recording electrodes were chronically implanted as described in our earlier publication (Datta et al. 2008). In addition to these electrodes, bilateral 26 gauge stainless steel guide tubes containing equal length stylets were stereotaxically implanted for microinjection of saline (as control) or KT-5720 (membrane permeable inhibitor of PKA; Calbiochem, EMD Biosciences, La Jolla, CA, USA) into the DH-CA3 (stereotaxic coordinates, anterior–posterior, −3.80 mm; lateral, 3.8 mm; horizontal/dorso-ventral, 3.2 mm) of the freely moving rat (for details of guide tube implantation and microinjection, see Datta et al. 2005). All electrodes and guide tubes were secured to the skull with dental acrylic. Electrodes were crimped to mini-connector pins and brought together in a plastic connector. Following completion of the surgical procedure, animals were administered saline (5 cc, s.c.), to prevent dehydration, and ampicillin (50 mg/rat, s.c.; Bristol-Myers Squibb Company, Princeton, NJ, USA) to control any potential postsurgical infection. Potential postoperative pain was controlled with buprenorphine (0.05 mg/kg, s.c; Abbott Laboratories, Chicago, IL, USA).

Habituation and baseline recordings

After a postsurgical recovery period of 7–10 days, rats were habituated to the experimenter and a sound-attenuated recording cage under free moving recording conditions for 7 days as described in previous publications (Datta et al. 2005, 2008). During their recovery, habituation, and free-moving recording conditions, all rats experienced the same 12-h light/dark cycle with free access to food and water. These habituation sessions were considered to be the adaptation recording sessions for this study. After the adaptation recording sessions, all rats underwent two sessions of baseline recording for electrode testing and additional habituation with the recording setup.

Two-way active avoidance learning paradigm

The test apparatus is an automated two-way shuttle scan shock-avoidance box (45.7×20.3×30.5 cm) made of high-grade acrylic (Shuttle-flex test chamber; Model: SF II, AccuScan Instruments, Columbus, OH, USA). The floor is made of stainless steel bars suitable for application of electrical shock. The box is bisected by a vertical partition with an opening in the middle (near the bottom). This opening permits the animal to travel freely from one side of the shuttle box to the other. The box contains a front and a rear sensor containing eight infrared light beams. These light beams determine positively which side the animal is on. Located on the lid of the shuttle box are three light bulbs (one in each compartment and one in the center) that provide light stimuli (adjustable intensity, 6 W at 115 V AC) and three beepers (3,600 Hz; adjustable 0.00–85 dB at 30 cm) that produce sound stimuli. The interface unit permits interconnections between the computer and the shuttle box. A personal computer using remote monitoring system software controls experimental protocols and data collections (Datta 2000; Datta et al. 2004).

The procedure involved first placing the rat in one compartment of the apparatus at 9:05 A.M.. Training trials began following 15 min of acclimatization. During acclimatization and the training trials, the rats could move freely from one compartment to the other within the shuttle box. Rats were trained on a massed 30-trial shuttle box TWAA task beginning at 9:20 A.M.. The procedures for the conditioned stimulus (CS) and unconditioned stimulus (UCS) are detailed in an earlier publication (Datta et al. 2004). In brief, a tone (3,600 Hz, 65 db) and a pulsatile light (2.5 Hz) were presented as the CS in the compartment with the animal, paired 5 s later with a 0.3-mA scrambled foot shock (UCS) delivered through the floor grid (steel rods 0.5 cm in diameter, spaced 1.5 cm between center). To avoid receiving a foot shock, the rat had 5 s to move to the opposite compartment. If the animal did not move to the other compartment, the UCS was delivered for a maximum of 5 s and the CS ended with the UCS. While receiving the UCS, if the animal moved to the other compartment, both the CS and the UCS ended immediately. The intertrial interval was variable with a mean of 60 s. To test for improvement in TWAA memory, some of these rats were again subjected to a session of (between 9:05 A.M. and 9:50 A.M.) TWAA trials 24 h later (test trials). The number of trials, the procedure for CS and UCS, and intertrial interval during the test trial session were identical to those employed in the training trial session.

Experimental design and tissue collection

After the postsurgical recovery period and adaptation recording sessions, 35 rats were randomly divided into five groups as follows: (1) SC-TT, saline control—training and test; (2) SC-T, saline control—training; (3) DD-TT, drug—training and test; (4) DD-T, drug—training; (5) SC-CC, Saline control—cage control. On the experimental day (the day after the final adaptation recording session), rats in groups 1–4 were subjected to a session of TWAA training trials. The rats of group 5 (SC-CC) remained in the shuttle box for a period of 45 min without receiving any CS-UCS trials and were designated the shuttle box control group. Immediately after the training trial, DD-TT and DD-T groups of rats received bilateral DH-CA3

microinjections of KT-5720 (2 mM solution; 200 nl/site; a total of 0.2 µg/site). SC-TT and SC-T groups of rats received bilateral DH-CA3 microinjections of control saline (200 nl/site). Similarly, immediately after the shuttle box (without CS-UCS trials) period, the SC-CC group of rats received bilateral DH-CA3 microinjections of control saline (200 nl/site). The dosage of KT-5720 was chosen based on previous studies indicating that this dose of KT-5720 effectively blocks PKA activity (Bernabeu et al. 1997; Izquierdo et al. 2000; Fu et al. 2008). After microinjection, rats were transferred to the sleep-recording cage for a 3-h recording session (10:00 A.M. to 1:00 P.M.) of undisturbed sleep–wakefulness. At the end of the 3-h recordings, all animals were awakened for 1 min by shaking their cages. Rats from groups SC-TT and DD-TT were returned to the housing room, and rats of groups SC-T, DD-T, and SC-CC were euthanized with CO₂. Following euthanasia, rat brains were rapidly removed and the DH-CA3 was removed under a dissecting microscope to an ice-chilled Petri dish as described earlier (Datta et al. 2008). The amount of BDNF in the DH-CA3 tissue was measured using the ELISA technique (for the details of BDNF measuring method, see Ulloor and Datta 2005). In this study, we have selected a 3-h interval between TWAA training and tissue collection because, in our earlier studies, we showed that P wave generator activation induced by TWAA training peaks at the 3-h interval (Datta 2000; Datta et al. 2005). Additionally, we have shown that TWAA training trials-induced BDNF expression in the DH peaks at the 3-h interval (Ulloor and Datta 2005). Twenty-four hours after the TWAA training trials session, the SC-TT and DD-TT rats were subjected to a session of TWAA test trials to evaluate their memory. After this, rats were deeply anesthetized with pentobarbital (60 mg/kg, i.p.) and then perfused transcardially with phosphate buffer (0.1 M; pH 7.4) followed by 10% buffered formalin. The brains were then removed and processed for staining and histological localization of injection sites.

Data analysis for sleep–wake states, P wave density

To determine the possible effects of TWAA training trials on sleep and wakefulness, polygraphic data were digitized using the “Gamma” software (Grass product group, Astro-Med, West Warwick, RI, USA). From this data, three behavioral states were distinguished and scored visually using “Rodent Sleep Stager” software (Grass product group, Astro-Med, West Warwick, RI, USA), as described earlier (Datta et al. 2004). Wakefulness (W), slow-wave sleep (SWS), and REM sleep were scored in successive 5-s epochs. The polygraphic measures provided the following dependent variables which were quantified for each recording session: (1) percentage of recording time spent in W, (2) percentage of recording time spent in SWS, (3) percentage of recording time spent in REM sleep, and (4) REM sleep P wave density. To calculate P wave density, P wave spikes were visually identified and isolated from the background EEG activities as described earlier (Datta et al. 1998). These spikes are monophasic and always of the same polarity. Their amplitudes are between 100 and 150 µv with durations between 75 and 150 ms. P waves from one side of the brain were counted during all REM sleep periods and expressed as the number of waves per minute (REM sleep P wave density). For statistical analyses, analysis of variance (ANOVA) procedures and post hoc Scheffe *F* tests were performed using StatView® statistical software (Abacus Concepts, Berkeley, CA, USA). Specific statistics for specific comparisons are described in the results section.

Results

Effect of KT-5720 in the DH-CA3 on the TWAA training trials-induced expression of BDNF and improvement of TWAA memory

The effects of DH-CA3 PKA activation suppression on TWAA training-induced BDNF synthesis and on TWAA memory were measured as follows: four groups of rats were subjected to TWAA training; immediately afterwards, two groups (SC-T and SC-TT) received bilateral saline control microinjections in the DH-CA3, and the other two groups (DD-T and DD-TT)

received bilateral DH-CA3 microinjections of KT-5720. Twenty-four hours later, SC-TT and DD-TT groups of animals were subjected to a session of TWAA test trials. To determine possible group differences in the avoidance learning performances in the training session, the numbers of avoidances of those four different groups of rats were subjected to one-factor ANOVA test and post hoc tests (Scheffe *F* test). The one-factor ANOVA revealed no significant group effect ($DF=3$; $F=1.013$; $p=0.404$) in the number of avoidances. The individual post hoc tests also failed to reveal any significant differences between the four different groups (Fig. 1a; group of bars in the left). Similarly, during the learning training session, the total foot shock time in the four different groups of rats (Fig. 1b) were not significantly different (one-factor ANOVA; $DF=3$; $F=0.025$; $p=0.994$). Thus, these results indicate that, before KT-5720 microinjections, the groups were equal in terms of TWAA performance. Since the number of avoidances and total foot shock times were similar in the four different groups, these results exclude the possibility that any differences observed in the magnitude of BDNF changes between groups may be due to differences in their performance during the TWAA training trials. These results also exclude the possibility that differences in TWAA test performance between groups might be due to differences in nonspecific effects of foot-shocks in the TWAA training trials.

To assess the effects of KT-5720 microinjections into the DH-CA3 on TWAA memory, in the SC-TT and DD-TT groups, the number of avoidances were compared (Scheffe *F* test) between training and test sessions. This comparison revealed that the number of avoidances in the SC-TT group was significantly higher in the test session ($F=19.143$; $p<0.001$) than they were in the training, but in the DD-TT group, the number of avoidances in the test session was not significantly different ($F=2.88$; NS) from the training session (Fig. 1a). Comparison of test data also shows that the number of avoidances in the SC-TT group was significantly higher ($F=12.9$; $p<0.001$) than in the DD-TT group (Fig. 1a). This demonstrates that following TWAA training, bilateral microinjections of KT-5720, but not saline control, into the DH-CA3 blocked improvement in TWAA test performance.

Having demonstrated deficits in the improvement of TWAA memory following inhibition of PKA activation in the DH-CA3, we next determined the impact of this inhibition on TWAA training-induced BDNF expression. The results show that the amount of BDNF in the DH-CA3 was significantly higher (Scheffe *F* test; $F=44.915$; $p<0.001$) in the SC-T group than in the SC-CC group; however, there were no significant differences ($F=0.727$; NS) between SC-CC group and DD-T group (Fig. 1c). A comparison between SC-T and DD-T groups also showed that the amount of BDNF in the DH-CA3 was significantly higher ($F=57.071$; $p<0.001$) in the SC-T group. Thus, these results demonstrate that bilateral microinjections of KT-5720 into the DH-CA3 suppressed TWAA training-induced BDNF expression in the DH-CA3.

Changes in wake–sleep stages and P wave density

Figure 2 shows the total percentages of time spent in W (2A), SWS (2B), and REM sleep (2C) and REM sleep P wave density (2D) in the five different groups of rats. Although these five groups received three different treatments immediately before they were recorded for the sleep–wake activity, one-factor ANOVAs revealed no significant group effects on the total percentages of time spent in W ($DF: 4$; $F=1.47$; $p=0.24$) or SWS ($DF: 4$; $F=0.35$; $p=0.84$). These results suggest that the TWAA training and/or microinjection of KT-5720 into the DH-CA3 did not change the total percentages of time spent in W and SWS (Fig. 2a and b). However, ANOVA revealed a significant group effect in the total percentage of time spent in REM sleep ($DF=4$; $F=14.14$; $p<0.001$). Subsequent post hoc Scheffe *F* tests revealed that the SC-T, SC-TT, DD-T, and DD-TT groups of rats spent significantly more time in REM sleep than the SC-CC group (Fig. 2c). Similar post hoc tests on the total percentages of REM sleep did not show any significant differences when compared between any other groups. Collectively, these

results suggest that the TWAA training increased REM sleep in both saline and KT-5720-treated rats. These results also suggest that the bilateral microinjections of KT-5720 into the DH-CA3 did not alter the TWAA training-induced REM sleep increases we observed. One-factor ANOVA analysis revealed that like the total amount of REM sleep, there was a significant group effect in the P wave density ($DF=4$; $F=38.10$; $p<0.001$). Post hoc comparisons (Scheffe F tests) revealed that the REM sleep P wave densities in the SC-T, SC-TT, DD-T, and DD-TT groups were significantly higher than that in the SC-CC group (Fig. 2d). Like the percentages of REM sleep, the P wave densities were not significantly different (Scheffe F tests) between any other groups (Fig. 2d). In summary, TWAA training trials increased both the total percentage of time spent in REM sleep and P wave density. These results suggest that the homeostatic demand of increased REM sleep and increased P wave density for TWAA memory improvement remained intact even after the inhibition of PKA activity in the DH-CA3.

A photomicrograph of a representative bilateral microinjection site in the DH-CA3 is shown in Fig. 3a. The microinjection sites in the DH-CA3 of all animals of SC-TT and SC-DD groups are shown in Fig. 3b. The dark circles represent the loci of KT-5720, and the white circles represent control injection sites. Placement of drug and control injections was comparable, and there were no instances of excessive mechanical damage induced by the guide and injector tubes. While effort was made to center the microinjections on the DH-CA3, we acknowledge that some diffusion of injectate into neighboring areas is possible.

Discussion

The principal findings of this study are as follows: localized application of the PKA activation inhibitor, KT5720, into the DH-CA3 blocked improvement of TWAA memory; the inhibitor also suppressed TWAA training trials-induced BDNF expression in the DH-CA3; and application of KT5720 into the DH-CA3 did not change TWAA training-induced increases in REM sleep or P wave density. The results suggest that TWAA memory processing involves both PKA activation and BDNF expression in the DH-CA3. In addition, these results provide further support to the hypothesis that the P wave generator activation-dependent TWAA memory processing involves a functional interaction between the P wave generator and the DH-CA3.

In this study, we used a PKA inhibitor, rather than an activator, because we sought to determine the role of endogenously activated PKA. KT5720 is a widely used selective PKA inhibitor (at nanomolar to low micromolar concentrations) that binds to the catalytic subunits of PKA, causing the displacement of the regulatory subunit and thereby inhibiting the phosphorylating activity of the kinase (Kase et al. 1987; Bernabeu et al. 1997; Huang et al. 2000; Fu et al. 2008). Since the application of KT5720 inhibits PKA activation, the results of this study demonstrate that inhibition of PKA activity in the DH-CA3 blocked memory processing of TWAA training. The notion that PKA activity in the DH is involved in long-term memory processing is also supported by Bernabeu et al. (1997) who demonstrated that infusion of KT 5720 into the DH blocks memory consolidation of step down inhibitory avoidance learning in rats. Other studies have used another PKA inhibitor, Rp-cAMPS, and have also shown that the inhibition of DH PKA activity disrupts consolidation of fear memory in mice (Bourtchouladze et al. 1998; Schafe et al. 1999; Ouyang et al. 2008). Furthermore, fear memory consolidation is impaired in transgenic mice that overexpress an inhibitory isoform of PKA (Abel et al. 1997; Bourtchouladze et al. 1998). Although it appears that PKA in the DH is a common signaling pathway, processing more than one type of memory, the present observations are critical because this is the first time that activation of a specific area of the REM sleep generating network has been linked to the PKA system in the DH. Any other signaling pathways in the DH that are more specific to TWAA memory, is an area for future investigations. The

results of our study demonstrate that the inhibition of PKA activity within the DH-CA3 blocks P wave generator activation-dependent TWAA memory processing. Indeed, our earlier studies have shown that the P wave activity positively influences TWAA performance (Datta 2000; Datta et al. 2004; Mavanji et al. 2004).

Several other studies have indicated that BDNF in the DH is involved in the modulation of contextual and spatial memory processing (Kesslak et al. 1998; Mu et al. 1999; Hall et al. 2000; Mizuno et al. 2003; Rattiner et al. 2004). The results of the present study also demonstrate that the inhibition of PKA activity not only blocked improvement of TWAA memory but also suppressed TWAA training trials-induced BDNF expression in the DH-CA3. An earlier study has shown that the level of improvement in TWAA memory depends on the level of BDNF expression in the DH during posttraining sleep period (Ulloor and Datta 2005). Thus, it is reasonable to suggest that the deficit in TWAA memory improvement following PKA activity inhibition was caused, at least in part, by suppressing BDNF expression in the DH-CA3. Another possibility is that reduction in activation of NMDA receptors caused by reduced PKA activity might have caused this TWAA memory-improvement deficit. This suggestion is based on the fact that, in the DH, phosphorylation of CREB and the expression of different genes and proteins that are known to be involved in memory processing depend on the activation of postsynaptic NMDA receptors (Link et al. 1995; Lyford et al. 1995; Cammarota et al. 2000; Vianna et al. 2000; Athos et al. 2002). It is also known that PKA phosphorylates NMDA receptors, and this enhances ion flow through the receptor and also accelerates the kinetics of the ion channel (Raman et al. 1996; Tingley et al. 1997; Westphal et al. 1999; Chen et al. 2006; Fu et al. 2008).

In conclusion, the present study reveals that the activation of PKA in the DH-CA3 is an important molecular step in the improvement of TWAA memory that may involve the regulation of BDNF gene expression in the DH-CA3. These new findings significantly enhance our understanding of the cellular and molecular links between the P wave generator and the DH-CA3 involved in the formation of long-term memory of TWAA task.

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Abbreviations

Arc, activity-regulated cytoskeletal-associated protein
 BDNF, brain-derived neurotrophic factor
 CREB, cAMP response element-binding protein
 DD-T, drug—training
 DD-TT, drug—training and test
 DH-CA3, CA3 region of the dorsal hippocampus
 P wave, pontine wave
 PKA, protein kinase A
 REM, rapid eye movement
 SC-CC, saline control—cage control
 SC-T, saline control—training
 SC-TT, saline control—training and test
 SWS, slow-wave sleep
 TWAA, two-way active avoidance
 W, wakefulness

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Figure 1.

Improvement of two-way active avoidance memory and learning training trials-induced BDNF expression in the DH-CA3 depends on the activation of DH-CA3 PKA. **a** Number of avoidances (mean and SEM) in the training (*bars in the left*) and test (*bars in the right*) sessions. Note that the learning performances (expressed as the number of avoidances), in the training session, before application of KT-5720 into the DH-CA3 are not significantly different between the four different groups. Furthermore following application of vehicle control (SC-TT), the number of avoidances is significantly higher ($*p < 0.001$) in the test session, but not in those animals receiving KT-5720 (DD-TT; 0.2 $\mu\text{g}/\text{site}$). This shows that the application of KT-5720 into the DH-CA3 blocked improvement of avoidance memory. **b** Total shock time (mean and

SEM) in the training session. Note that the total shock times between four different groups are not significantly different. **c** Amount of BDNF protein (mean and SEM) in the DH-CA3. Note that 3-h after training session, BDNF protein increased in the vehicle control microinjected group of animals but not in the KT-5720 microinjected group. Indicating that the application of KT-5720 in the DH-CA-3 blocked learning training trials-induced BDNF expression. *SC-TT* saline control—training and test, *SC-T* saline control—training, *DD-TT* drug (KT-5720)—training and test, *DD-T* drug (KT-5720)—training, *SC-CC* saline control—cage control

Figure 2.

Effects of TWAA learning training trials and infusion of KT-5720 into the DH-CA3 on the total percentages of W (a), SWS (b), REM sleep (c), and REM sleep P wave density (d). Bars represent the total percentages (mean and SEM) time spent in W, SWS, REM sleep, and REM sleep P wave density of the five different groups. Note that the total percentages of W and SWS are not significantly changed after training trials, but the total percentages of REM sleep and REM sleep P wave densities in all four groups of rats were significantly ($*p<0.001$) increased. Also note that the total percentages of W, SWS, REM sleep, and REM sleep P wave density were not significantly different between groups of rats that received a session of learning training and infusion of KT-5720 into the DH-CA3 (DD-T and DD-TT groups) and rats that

received learning training and vehicle control (VC-T and VC-TT groups). These results indicate that the application of KT-5720 into the DH-CA3 did not change learning training trials-induced increases in REM sleep and P wave density. *SC-TT* saline control—training and test, *SC-T* saline control—training, *DD-TT* drug (KT-5720)—training and test, *DD-T* drug (KT-5720)—training, *SC-CC* saline control—cage control

Figure 3. Location of microinjection sites in DH. **a** Photomicrograph of a representative histological section showing bilateral microinjection sites in the DH-CA3. **b** Microinjection sites from rats infused with vehicle control (*open circles*) and KT-5720 (*filled circles*). Note that both vehicle control and KT-5720 microinjection sites were in the DH-CA3