Behavioural Brain Research 250 (2013) 114–122

Contents lists available at SciVerse ScienceDirect

Behavioural Brain Research

journal homepage: www.elsevier.com/locate/bbr

Research report

Functional inactivation of orexin 1 receptors in the cerebellum disrupts trace eyeblink conditioning and local theta oscillations in guinea pigs

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HIGHLIGHTS

- We assessed the effects of endogenous orexins on cerebellum-dependent motor learning.
- Orexin 1 receptors located in the Purkinje cells and deep cerebellar nuclei neurons.
- SB-334867 disrupted both the onset and peak latencies of trace CRs.
- SB-334867 prevented the increase in peak amplitude of cerebellar theta oscillations.
- However, SB-334867 failed to reduce the acquisition rate of trace CRs.

ARTICLE INFO

Article history:
Received 4 April 2013
Received in revised form 1 May 2013
Accepted 4 May 2013
Available online 13 May 2013

Keywords:
Eyeblink
Trace conditioning
Motor learning
Orexin 1 receptor
Cerebellum
Guinea pig

ABSTRACT

The cerebellum plays an essential role in motor learning. Recently, orexins, the newfound lateral hypothalamic neuropeptides, have been found to excite Purkinje cells in the cerebellar cortex and neurons in the deep cerebellar nuclei (DCN). However, little is known about their roles in cerebellum-dependent motor learning. Therefore, the present study was designed to investigate the functional significance of hypothalamic orexinergic system during trace eyeblink conditioning, a tractable behavioral model system of cerebellum-dependent motor learning. It was revealed that the orexin 1 receptors (OXR1) were specifically localized on the soma of Purkinje cells and large DCN neurons. Furthermore, interfering with the endogenous orexins' effects on the cerebellum via the selective OXR1 antagonist SB-334867 disrupted the timing rather than the acquisition of trace conditioned eyeblink responses. In addition to the behavioral effects, the SB-334867 prevented the increase in peak amplitude of cerebellar theta oscillations with learning. These results suggest that the endogenous orexins may modulate motor learning via the activation of cerebellar OXR1.

1. Introduction

Classical eyeblink conditioning is a useful model system of motor learning which can be modified to tap into diverse aspects of the learning process [1,2]. Typically, the eyeblink conditioning experiment involves paired presentations of a behaviorally neutral conditioned stimulus (CS; e.g., a tone) and an aversive unconditioned stimulus (US; e.g., an airpuff in the eye). With repeated to the paired stimuli, the subject learns to close the eye before the onset of the US (called conditioned eyeblink response, CR). It has been demonstrated that the primary memory trace of CR is stored in the deep cerebellar nuclei (DCN) and/or the cerebellar cortex [3–8], although the forebrain regions are critically involved in the learning process when the cognitive demands are increased [9–13].

A growing body of evidence has led to the proposal that function of the cerebellum is under the influence of numerous neuropeptides [14,15]. Uniquely, orexins (orexin-A and orexin-B), the new reported neuropeptides with multiple physiological functions, are exclusively produced by one group of cells located in the lateral hypothalamus (LH) that diffusely project to various brain regions [16–20]. In addition to their fibers, both orexin 1 receptors (OXR1) and orexin 2 receptors (OXR2) are extensively distributed in central nervous system including the cerebellum [21–23], an area implicated in motor learning. Recently, it has been found that orexins

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http://dx.doi.org/10.1016/j.bbr.2013.05.009

can excite the Purkinje cells in the cerebellar cortex [24] and neurons in the DCN [25]. In spite of the excitatory effects on principle cells in the cerebellum, to our knowledge, no investigation has been made to address the roles of orexins in cerebellum-dependent motor learning.

Consequently, the present study aimed to determine the physiological and behavioral relevance of LH orexin system in trace paradigm of classical eyeblink conditioning, a cerebellum-dependent motor learning task [26–30]. Given that SB-334867 is a selective non-peptide OXR1 antagonist with high affinity [31], we microinjected SB-334867 into the cerebellum before daily conditioning training and assessed effects of the selective OXR1 antagonism on the acquisition and expression of trace CRs in guinea pigs. It was found that the SB-334867 microinjections disrupted the timing of trace CRs, and prevented the increase in amplitude of cerebellar theta oscillations with learning of the CS–US association.

2. Materials and methods

2.1. Animals

Adult male albino Dunkin-Hartley guinea pigs weighing between 450 and 550 g (3–4 months old) were used in this experiment. Before the experiment and between the conditioning sessions, the animals were individually housed and had free access to food and water ad libitum. All experimental procedures were approved by the Animal Care Committee of the Third Military Medical University and were performed in accordance with the principles outlined in the NIH Guide for the Care and Use of Laboratory Animals. All efforts were made to minimize the animals' suffering and the use of the animals.

2.2. Surgical and behavioral procedures

The detailed surgical procedures for the cannula implantation, local field potential (LFP) and eyeblink recording have been described previously [32–34]. In brief, each animal was implanted with a simultaneous bipolar electrode-microdialysis system (C315G-MS303/2/SP, PlasticOne, Roanoke, VA, USA), and the stainless steel bipolar electrodes were specified as needed (diameter: bare: 0.003 in., coated: 0.006 in.). This system allowed us to perform the drug microinjection and LFP recording at the same site simultaneously. Four stainless steel screws were implanted on the surface of the skull to serve as reference (+2.5 mm posterior to Bregma, ±5.0 mm from midline; +8.5 mm posterior to Bregma, ±5.0 mm from midline). Finally, the animals were fitted with a headstage and a loop attached to the apex of left upper eyelid. In this study, this loop was utilized to attach the left upper eyelid to a high-resolution movement-measuring device. After the surgery, the animals were allowed at least one week of recovery.

Following the postoperative recovery, the animals were adapted to the experimental environment for 2 days, 30 min/day. During the daily adaptation sessions, the animals were restrained in a Plexiglas containing box located in a sound- and light-attenuating chamber with no stimuli given. The adapted animals then were given trace conditioning training as described by Hu et al. [34,35]. The CS was a tone (2 kHz, 85 dB, 350 ms, and 5 ms rise/fall time), which was paired with a left corneal airpuff US (100 ms, 3.0 psi measured at the source). The CS offset was separated by 500 ms from the US onset. Conditioning training consisted of 5 blocks of 10 CS–US pairings trials for a total of 50 trials per session per day. The animals were consecutively trained for 8 days unless otherwise noted.

2.3. Microinjection

Drug microinjections were performed 10 min before the beginning of daily trace conditioning training. We microinjected 2.0 μg (5.0 mM, 1.25 μl) of the OXR1 antagonist SB-334867 (Tocris, Bioscience, UK) through a stainless steel needle that extended 0.5 mm below the base of the guide cannula. The SB-334867 was dissolved in 1.25 μl 0.9% saline and 5% DMSO (Sigma–Aldrich, St. Louis, MO, USA) prior to use. In order to functionally verify the effectiveness of each cannula implantation, we microinjected 1.25 μg (8.8 mM, 1.25 μl) of the $GABA_A$ receptor agonist muscimol (Sigma–Aldrich, USA) into the left intermediate cerebellum on day 9. The muscimol was dissolved in 1.25 μl sterilized aCSF prior to use, and the aCSF solution was prepared as described in our previous work [36]. The microinjections continued at a rate of 0.2 μl/min for 6 min. A constant injection rate was maintained using a syringe pump (CMA 402, CMA/Microdialysis, Solna, Sweden). The internal needle was kept in place for 4 min after each injection to allow the drug to diffuse from the needle tip. All the animals were allowed 24 h to recover between test sessions.

2.4. Electrophysiological recordings

The LFP signals were measured as the voltage difference between single electrode and the reference screws placed above the skull, as opposed to the voltage difference between two electrodes of the bipolar electrode to avoid the possibility that the LFP relevant for cortical communication would be subtracted-out [37]. The LFP signals, eyelid movement mechanogram and markers of the applied stimuli were digitized at a sample rate of 10 kHz by a data acquisition system (Powerlab/4sp, ADInstuments, Australia) and were acquired using the software Chart (v. 5.4.2). The LFP signals were amplified by 1000 times and bandpass filtered (0.5–30 Hz). A Windows PC was used to store the data.

2.5. Data analysis

2.5.1. Eyeblinks

Detailed description of eyeblink response analysis was previously described [34]. Briefly, for each conditioning trial, we calculated the average above-threshold activity for the baseline (1–350 ms before CS onset), startle eyeblink response (SR; 1–120 ms after the CS onset), conditioned eyeblink response (CR; 121–850 ms after the CS onset), and unconditioned eyeblink response (UR; 1–500 ms after the US onset). A significant eyelid movement was defined as an increase in the integrated activity that was greater than the mean baseline activity plus four times the standard deviation (SD) of the baseline activity. An eyelid movement also required to have a minimal duration of 15 ms and had to exceed the 1.0 mV baseline threshold (equaled 0.25 mm). Any significant eyelid movement during the periods mentioned above was counted as a SR, a CR or an UR, respectively. A total of 6 parameters were included to evaluate the effects of SB-334867 microinjections on the acquisition and expression of eyeblink responses: (1) CR incidence (2) CR peak amplitude, (3) CR peak latency, (4) CR onset latency, (5) UR peak amplitude, and (6) SR incidence.

2.5.2. Relative theta ratio

LFP signals from the electrode tip were re-sampled at 100 Hz using homemade software written in the Matlab (MathWorks, Natick, MA, USA). To determine the relative power of cerebellar theta activity during the 960 ms pre-CS period, Fast Fourier Transform (FFT) was run on the pre-CS period EEG with a resolution of 0.5 Hz, and was used to compute a power spectrum. To assess theta continuity, a sliding Hamming window continued to sample the incoming slow wave in 160-ms increments and re-calculated the ratio (480 ms previous data and 160 ms new data). Lastly, a spectral ratio of the proportion of theta (4.0–10.0 Hz) to non-theta (0.5–4.0 Hz and 10.0–30.0 Hz) was calculated (called relative theta ratio) [38,39].

2.5.3. CS-evoked change in theta amplitude

As the computational procedures described by Takehara-Nishiuchi et al. [40], the instantaneous peak amplitude of theta oscillations was averaged across trials in each animal. The average amplitude was subsequently normalized by subtracting the mean and dividing by the SD of the amplitude during a 960-ms pre-CS period. The normalized amplitude values then were averaged across training days and animals.

2.5.4. Statistical analysis

All data were expressed as the mean ± SEM unless otherwise noted. Behavioral statistical significance was determined by a two-way ANOVA with repeated measures using the SPSS software for Windows package (v. 13.0). A minimum of p value of <0.05 was an index of statistical significance for all tests.

2.6. Histology

After completion of the inactivation, recording and behavioral experiments, the animals were given a lethal dose of pentobarbital sodium (100 mg/kg) and perfused transcardially with 500 ml saline followed by 750 ml, 4% paraformaldehyde (PFA; prepared in 0.1 M phosphate buffered saline (PBS), pH = 7.4). The brain then was removed from the skull and fixed in formalin sucrose solution for 48 h. Frozen coronal sections (40 μm) were collected from the guide cannula site. The slices were mounted on the gelatinized slides and stained with toluidine blue (Sigma–Aldrich). The locations of the guide cannula and electrode in the cerebellum were histologically examined.

2.7. Immunofluorescence

To determine the cellular distribution of OXR1 in the cerebellum of guinea pigs, additional two animals were anesthetized with sodium pentobarbital (100 mg/kg) and perfused transcardially with 500 ml saline followed by 750 ml 4% PFA in 0.1 M PBS. Subsequently, the brain was removed, trimmed, and post-fixed in the same fixative for 24 h at 4 °C and then cryoprotected with 30% sucrose for 48 h. Frozen coronal sections (15 μm) containing the intermediate cerebellar tissue were obtained by using a microtome (CM1900, Leica, Germany). We rinsed the coronal slices in 0.01 M PBS containing 0.4% Triton X-100 (PBST) and incubated them in 1% normal bovine serum in PBST for 30 min at 37 °C. Sections then were incubated overnight at 4 °C with the primary antibody to OXR1, a rabbit anti-OXR1 serum diluted at 1:100 (AB3092, Millipore, USA). The adjacent slice was incubated with no primary OXR1 antibody-as a negative control, for 24 h at 4 °C in darkness. After a complete wash in 0.01 M PBS, the slices were incubated with tetramethylrhodamine isothiocyanate-labeled lectin (TRITC) goat anti-rabbit IgG (1:100, Millipore, USA) at room temperature for 2 h. Nucleic acids were stained with the 4,6-diamidino-2-phenylindoldihydrochloride (DAPI; Merck, Germany). Digital photographs of areas of interest were generated by an Olympus microscope (BX53, Tokyo, Japan) using a 10× and 20× lens coupled to an Olympus CCD camera (DP73) with *Stem cell* image