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Modulation of electrically evoked hippocampal epileptiform activity by exogenous orexins in the rat CA1 field

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In vivo electrophysiological experiments were conducted to evaluate the effects of intraventricular and cortical administration of orexins on electrically evoked epileptiform discharges (EEDs) in the hippocampal CA1 field. Bipolar electrodes were stereotaxically implanted into the CA1 region of anesthetized rats, and epileptiform activity was induced by high-frequency electrical stimulation (HFES). Orexin-A and orexin-B (10 µg/10 µl) were administered into the lateral ventricle or applied to the frontal cortex. Intracerebroventricular administration of orexin-A significantly reduced the duration of EEDs within 20 minutes and abolished the progressive, HFES-related prolongation of epileptiform discharges observed in control animals. In contrast, cortical administration of orexin-A selectively reduced the incidence of high-amplitude (7–10 mV) population spikes during EEDs without affecting the duration of epileptiform activity. Neither cortical nor intraventricular administration of orexin-B produced significant changes in evoked epileptiform activity in the CA1 field, suggesting a possible involvement of OX1 receptor signaling in mediating the effects of orexin-A. The differential effects observed after cortical versus intraventricular administration of orexin-A appear to reflect site-specific mechanisms of action. The effects of intraventricular administration of orexin-A were predominantly induced *via* direct activation of hippocampal orexinergic receptors, whereas cortical application of orexin-A induced its effects *via* modulation of cortico-hippocampal mechanisms.

Key words: epilepsy, orexins, hippocampus, *in vivo* electrophysiology

INTRODUCTION

Epilepsy is a heterogeneous neurological disorder resulting from the interplay of genetic susceptibility and environmental risk factors (Singhi, 2011; Campbell et al., 2014; Wang et al., 2017; Mukhtar, 2020; Cano et al., 2021). Despite the availability of numerous antiepileptic drugs, approximately one-third of patients continue to experience uncontrolled seizures (Zuberi et al., 2022), and many treatments are associated with adverse effects that impair quality of life (de Kinderen et al., 2014). This substantial therapeutic gap underscores the need for a deeper understanding of the mechanisms underlying seizure initiation and propagation to enable the development of more effective and targeted interventions.

Temporal lobe epilepsy (TLE) is the most prevalent form of focal, drug-resistant epilepsy and is characterized by recurrent seizures arising from mesial temporal structures, including the hippocampus, entorhinal cortex, and amygdala (Lévesque & Avoli, 2013; Jefferys et al., 2016). Among these regions, the hippocampus has been studied most extensively because its highly interconnected excitatory circuitry makes it particularly susceptible to seizure generation and propagation (Bragin et al., 1999; Zhao et al., 2015).

Animal models play a key role in elucidating the mechanisms of TLE and are broadly categorized into

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pharmacological and electrical paradigms. Pharmacological approaches replicate several major pathological features of temporal lobe epilepsy (Lévesque & Avoli, 2013; Reddy & Kuruba, 2013), but often exhibit considerable variability in seizure expression and network dynamics (Löscher, 2017), whereas electrical stimulation paradigms provide greater experimental reproducibility and reliably model hippocampal epileptiform activity and epileptogenesis (Bragin et al., 1997; Morimoto et al., 2004; Fujiwara-Tsukamoto et al., 2010; Marshall et al., 2021; Choy et al., 2022).

The pathophysiology of epilepsy remains incompletely understood, reflecting the complexity of its underlying mechanisms (Sumadewi et al., 2023; Zaitsev & Khazipov, 2023; Zhao et al., 2024). At the cellular level, epilepsy is commonly attributed to an imbalance between excitation and inhibition within neural networks (Scharfman, 2007; Trevelyan & Schevon, 2013). While glutamate and γ-aminobutyric acid (GABA) are central to maintaining this balance (Engel, 1996; Akyuz et al., 2021), additional neuromodulatory systems also influence seizure susceptibility (Salgado & Alkadhi, 1995; Anschel et al., 2004; Mishra & Goel, 2019; Akyuz et al., 2021). These systems are regulated by the orexinergic network, suggesting that orexins may serve as integrative modulators of seizure dynamic (Scammell & Winrow, 2011).

Orexins are hypothalamic neuropeptides whose signaling regulates arousal, cognition, and homeostasis (Lecea et al., 1998; Sakurai et al., 1998; Sakurai, 2003). Orexin-dependent regulation of bidirectional long-term synaptic plasticity has been demonstrated (Selbach et al., 2010), and in our previous studies, orexin-induced long-term depression of NMDA receptor–mediated responses in the CA1 region was observed in hippocampal slices (Doreulee et al., 2009). Conversely, dysregulation of the orexin system has been implicated in several neurodegenerative disorders, including Alzheimer's, Parkinson's, and Huntington's diseases (Berhe et al., 2020). The role of orexins in epilepsy, however, remains unresolved, as existing data yield conflicting conclusions. Animal studies suggest that orexins may exert proconvulsive effects, thereby exacerbating epileptogenesis. Attenuation of seizure activity through inhibition of orexin receptors or downregulation of orexin levels has been demonstrated (Erken et al., 2012; Roundtree et al., 2016; Kordi Jaz et al., 2017; Berteotti et al., 2023; Xue et al., 2023).

The antiepileptic effects of orexins have been discussed in other studies. *In vitro* experiments have demonstrated that orexin-A decreases the duration and amplitude of multiple population spike discharges in hippocampal slices, inhibits spontaneous epileptiform afterdischarges induced by bicuculline methiodide in the CA1 region (Doreulee et al., 2010), and modulates the discharge frequency of bursting neurons in the CA3 area (Selbach et al., 2004). *In vivo* studies have shown that intraventricular administration of exogenous orexin in kainate-induced epileptic rats produces effects that differ from those observed under control conditions, possibly due to morphological changes in the hippocampus and hypothalamus during epileptic status (Kapanadze et al., 2025). Moreover, orexin agonists have demonstrated beneficial effects in alleviating absence seizures (Toplu et al., 2023). Other studies further support a neuromodulatory role for orexin-A; specifically, amelioration of spatial learning and memory deficits in PTZ-kindled epileptic rats *via* OX1R-dependent, ERK1/2-induced neurogenesis in the dentate gyrus has been demonstrated (Zhao et al., 2014).

In the studies described above, both the antiepileptic and proconvulsant effects of orexin were evaluated using different epilepsy models, and the sites of exogenous orexin administration varied, with application either to the cerebral cortex or directly to the hippocampus. Despite the widespread presence of orexinergic projections and receptors in both the neocortex and hippocampus (Marcus et al., 2001), orexin receptor subtypes display differential regional expression (Elahdadi Salmani et al., 2022; Krause et al., 2024), suggesting site-specific effects on epileptic activity. Considering the neuronal circuits linking the neocortex and hippocampus, as well as evidence that the entorhinal cortex can modulate hippocampal epileptic activity (Ren et al., 2014), it is important to assess *in vivo* how exogenous orexin influences seizures of hippocampal origin when applied to the cortex versus directly to the hippocampus. This question constituted the primary objective of the present study.

METHODS

Animals

In vivo electrophysiological experiments were performed on Wistar rats (230-250 g, both sexes). Animals were housed under controlled conditions (23 ± 2°C; 50-60% humidity) with ad libitum access to tap water and standard chow, and maintained on a 12:12 h light/dark cycle (lights on 08:00-20:00). Experiments were conducted during the light phase (10:00-17:00).

Ethics Statement

All procedures were conducted in accordance with national legislation (Law of Georgia on Health Care)

and Directive 2010/63/EU of the European Parliament and of the Council of 22 September 2010 on the protection of animals used for scientific purposes. The study protocol was approved by the Institutional Animal Care and Use Committee of Tbilisi State University. Every effort was made to minimize animal suffering and to use the minimum number of animals required for reliable data acquisition. Sample size was estimated based on prior studies and statistical power calculations to ensure detection of biologically relevant effects while minimizing the number of animals used.

Experimental Procedures

Epileptiform discharges were induced by high-frequency electrical stimulation (HFES) of the dorsal CA1 hippocampus in anesthetised rats. General anesthesia was achieved with Calypsol (ketamine; Gedeon Richter) administered intraperitoneally at 100 mg/kg. Local anesthesia at the surgical site was provided by topical application of novocain during preparatory procedures. Metal (constantan) bipolar electrodes were stereotaxically implanted bilaterally into the right and left dorsal CA1, using the following coordinates (relative to bregma): (1) AP –4.3 mm, L 3.4 mm, H 2.8 mm; or (2) AP –3.8 mm, L 2.8 mm, H 2.8 mm, according to the Paxinos and Watson's stereotaxic atlas (2013). The electrodes were adhered to the skull with dental cement.

I.C.V. administration of orexin-A or B (Sigma Aldrich, # 06012/06262) at a dose of 10 µg dissolved in 10 µl saline – 0.5 µL/min (or same volume saline in the control experiments) was used to ensure gentle delivery, optimize diffusion, and minimize intracranial pressure shifts. Orexin-A or B was injected using a chemitrode into the lateral ventricle through the hole made in the cranial bone according to stereotaxic coordinates AP -0,3 mm; L 1.2mm; H 3.5mm. For the cortical administration of orexins, the skull was trephined, and a cannula was implanted at the location projecting to the frontal cortex. The physiological solution was given to the control group under the same volume and conditions.

Data were amplified (10×) and filtered (1 kHz) by using an amplifier INTRACELLULAR PRE (S/N: 010711, USA). Signals were passed through an analogue-to-digital interface (ADInstruments ML866 PowerLab 4/30, Australia) digitized at a sampling rate of 1 kHz and analyzed by means of software Chart5,5 with spike histogram extension; The data were statistically analyzed *via* GraphPad Prism and presented as the mean values and standard errors of mean. Statistical analyses were performed using paired Student's t-test for within-animal comparisons before and after orexin application, and two-way ANOVA with Fisher's LSD *post hoc* test where appropriate, a value of $P<0.05$ was considered statistically significant. Unless otherwise indicated, n represents the number of animals included in the analysis. In analyses involving spike quantification within defined recording epochs, n refers to the number of analyzed recording segments (10-s epochs) extracted from electrophysiological recordings. Minor differences in n values reflect the exclusion of recordings affected by technical artifacts or unstable baseline activity during the post-application period. In rare cases, recordings were also excluded due to the loss of the animal during the HFES experiment.

At the end of each experiment, electrode placement was verified histologically. A marking lesion was created at the tip of each implanted electrode by passing a high current. Brains were removed, fixed in 5% formalin, and coronally sectioned. Electrode tracks and lesion sites within the hippocampal fields were identified by microscopic examination (Fig. 1).

In each experiment, the stimulus intensity required to evoke epileptiform activity was first determined using stimulus–response assessments and adjusted individually. Rectangular pulses were delivered with a Master-8 pulse stimulator (A.M.P.I., Jerusalem, Israel). High-frequency stimulation consisted of 1500–2000 pulses (0.2–0.3 ms pulse duration, 4–8 V), with an inter-stimulus interval of 15–20 min. The duration of electrically evoked epileptiform discharges (EEDs) was measured from the stimulation artifact to the end of epileptiform activity. Discharge frequency and amplitude were analyzed in two 10-s epochs separated by a 10-s interval. For spike analysis, epileptiform activity was quantified within these defined recording epochs (segments), and n refers to the number of analyzed segments rather than the number of animals. The experimental design is graphically illustrated in Fig. 2.

RESULTS

The effects of HFES on the background electrical activity of the hippocampal CA1 field

High-frequency electrical stimulation reliably evoked epileptiform discharges lasting 30–100 s. Animals exhibiting responses outside this range were excluded from analysis. The mean stimulation intensity required was 5–6 V, yielding a mean discharge duration of 67.8 ± 4.9 s (n=13). In control rats, repeated HFES produced a progressive, stimulation-dependent potentiation of discharge duration, increasing from 53.9 ± 4.8 s after the first stimulation to 73.1 ± 6.8 s at 100 min (n=13; Fig. 4B), consistent with the induction of hippocampal circuit plasticity.

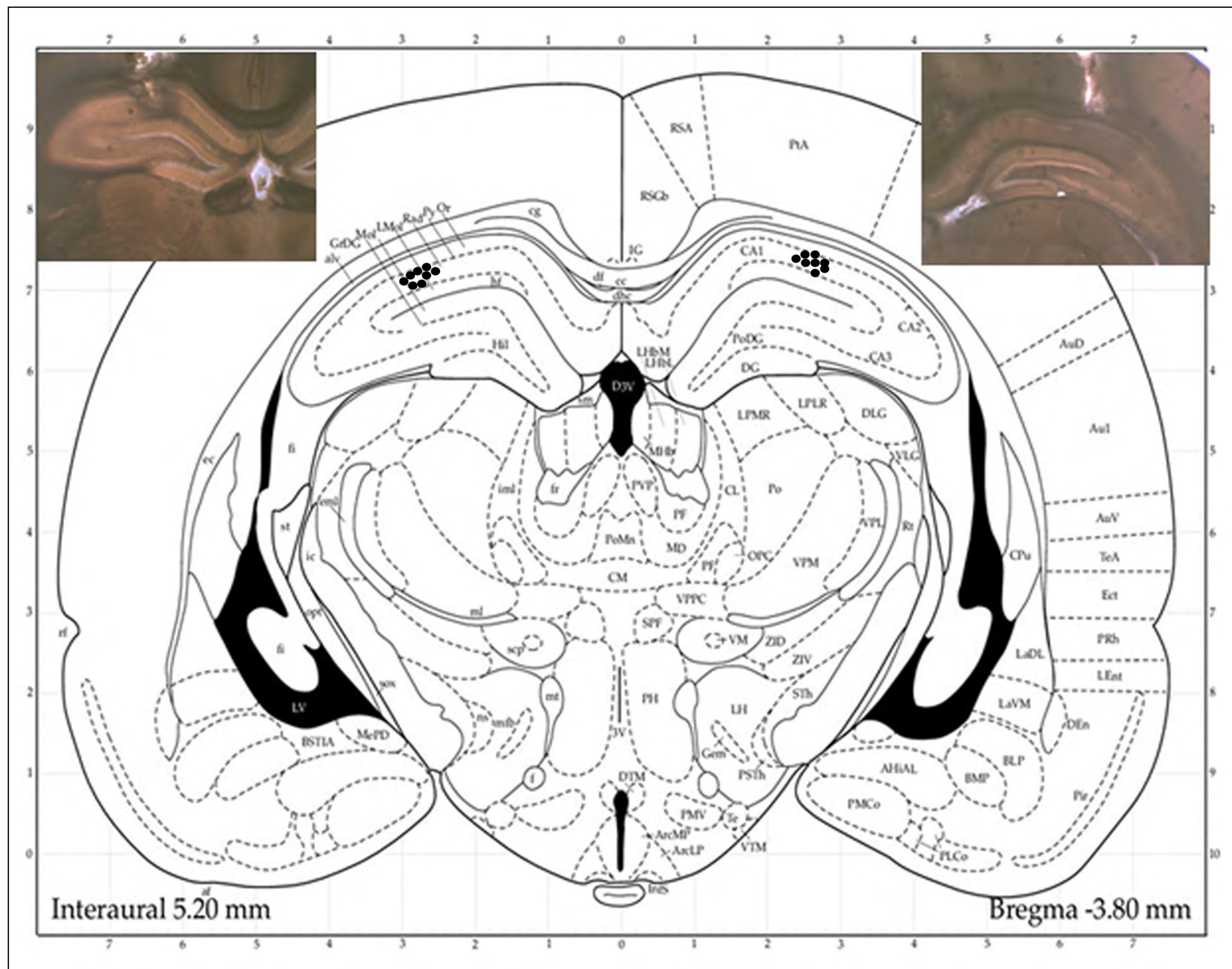

Fig. 1. Schematic graph of rat brain coronal sections and original pictures of brain slices (20 µm) from experimental rats to display locations of implanted electrodes in the hippocampal CA1 field; Atlas Source: Paxinos George and Charles Watson (*The Rat Brain in Stereotaxic Coordinates*, 2013).

The effect of intraventricular application of orexin-A and B on duration of epileptiform discharges

The effects of intraventricular administration of orexin-A/B on EEDs were assessed at 20-minute intervals over a 100-minute recording period. Orexin-A produced a transient, significant attenuation of epileptiform discharge duration, resulting in an approximate 10% reduction at 20 minutes post-application (99.45 ± 1.003 %, n=11, before; 90.33 ± 3.149 %, n=12, after; P<0.05) (Fig. 3A, 3D, 4A). The slight difference in sample size reflects exclusion of several recording segments that did not meet predefined quality criteria, for example, the amplitude of the recorded signal exceeded the predefined recording range. The progressive increase in discharge duration elicited by repeated HFES, evident in control animals, was no longer present in orexin A-treated animals, where a statistically significant difference was observed approximately 60 minutes post-administration compared with controls (Fig. 4B).

The inhibitory effects of orexin-A on electrically evoked epileptiform activity in the CA1 field could potentially be mediated by either OX1 or OX2 receptors. To clarify receptor involvement, orexin-B was applied in subsequent experimental series. Orexin-B administration did not alter the duration of epileptiform discharges (96.5 ± 7.274%, n=4, before; 102.6 ± 8.07%, n=5, after; P =0.605) (Fig. 3B), suggesting a possible involvement of OX1 receptor signaling in mediating the effects of orexin-A.

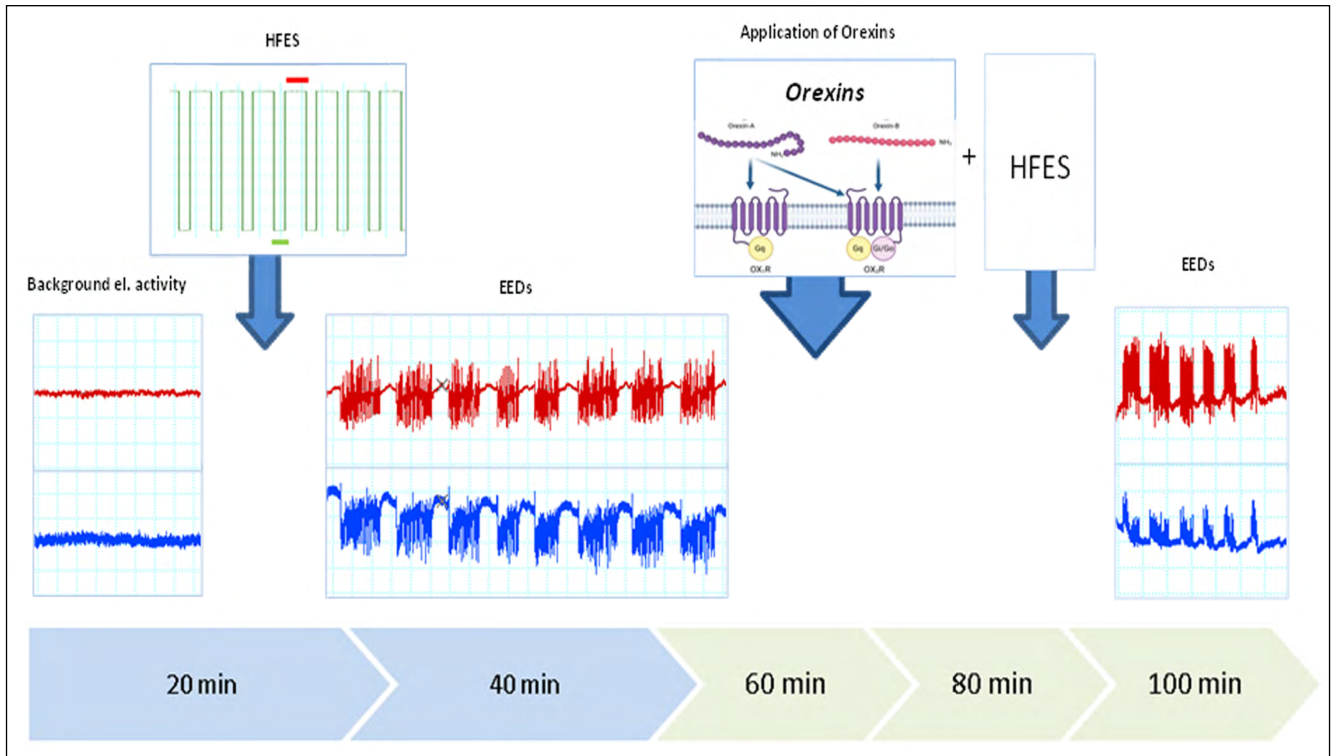

Fig. 2. Schematic representation of the experimental design; High-frequency stimulation (HFES) parameters: single spike duration 0.2-0.3 msec (green line), inter-spike interval 3 msec (red line).

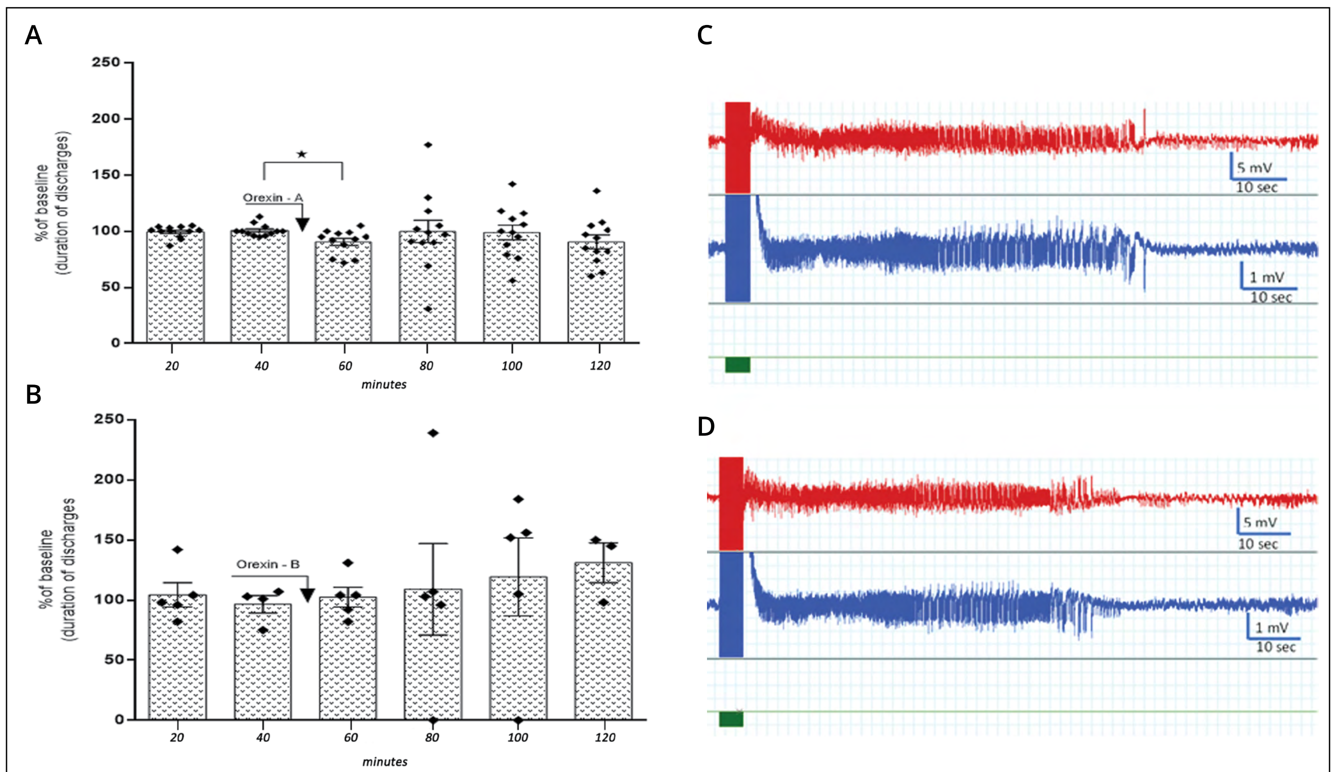

Fig. 3. The effects of intraventricular administration of (A) orexin-A and (B) orexin-B on the percent change in epileptiform discharge duration relative to baseline. Histograms show mean values with SEM. Original recordings represent EEDs (C) before and (D) after orexin-A application; Calibration: red trace – 5 mV, 10 sec; blue trace – 1 mV, 10 sec.

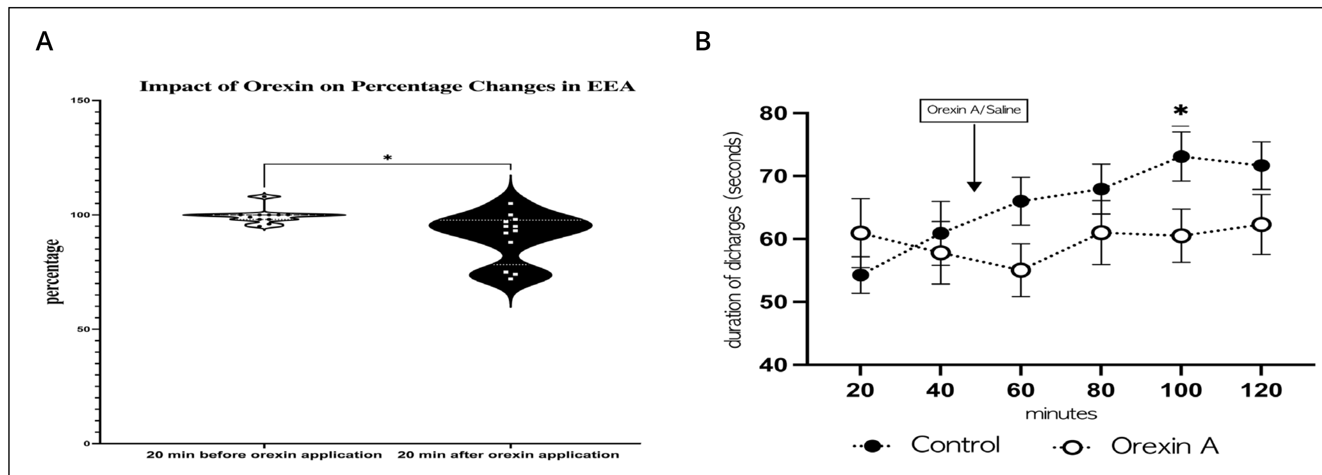

Fig. 4. The violin plots represent the effect of intraventricular application of orexin-A on percent change of duration of epileptiform discharges after 20 min of administration, paired t test was used to evaluate statistical differences between two groups (*P<0.05); (B) Histograms represent dynamics of duration of discharges in control and orexin-A injected group, two-way ANOVA with uncorrected Fisher's LSD test was used for statistical analysis (*P<0.05).

The effects of cortical application of orexin-A/B on HFES-induced epileptiform discharges in CA1

To assess the effects of cortical administration of orexins on evoked hippocampal epileptiform discharges, the compounds were applied to the frontal cortical surface. Analysis of the obtained results demonstrated that neither orexin-A (100.7 ± 1.529 %, n=12, before; 98.83 ± 6.518%, n=12, after; (P=0.786) nor orexin-B (91.40 ± 2.542%, n=5, before; 102.6 ± 8.207%, n=5, after; (P=0.228) produced statistically significant changes in the duration of EEDs relative to pre-application responses. However, cortical administration of orexin-A selectively reduced the incidence of high-amplitude (7–10 mV) population spikes during EEDs measured in 10-s epochs of epileptiform activity (from 4.3 ±1.2 before orexin-A application to 0.98 ±0.2 after application (n=15 recording segments before and n=15 recording segments after orexin-A application; each segment represents a 10-s epoch of epileptiform activity used for spike analysis) (Fig. 5).

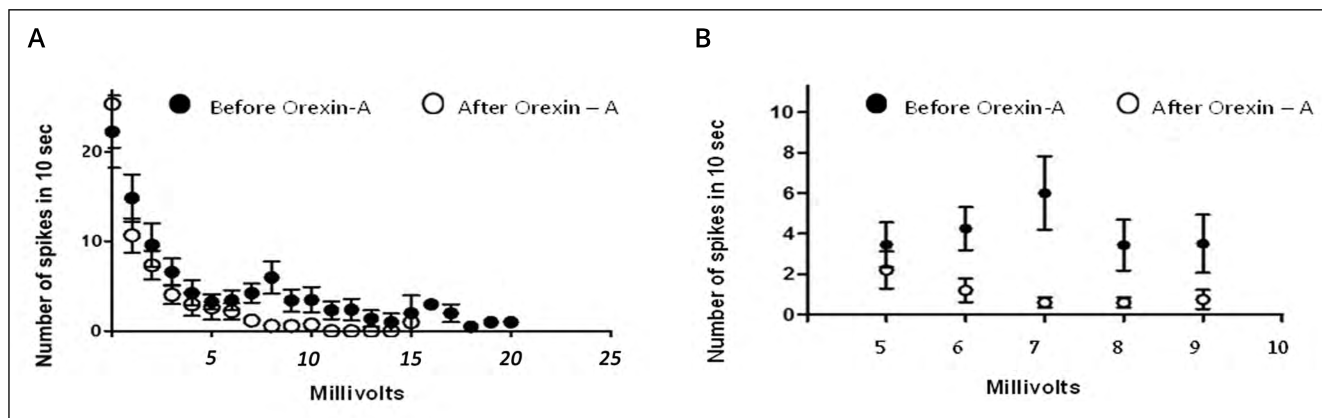

Fig. 5. The effects of cortical application of orexin-A on a number of high amplitude pop-spikes. (A) The number pop-spikes of different ampliture before (filled symbols) and after orexin-A application (open symbols). (B) Enlarged view from panel A showing the ampliture ranges where there is a statistically significant difference between the control and orexin-A effects.

DISCUSSION

In our experiments, HFES (1500–2000 pulses) of the hippocampus evoked epileptiform discharges with a mean duration of 67.8 ± 4.9 s, likely due to excessive synchronization of excitatory pyramidal neurons and transient impairment of inhibitory control (Scharfman, 2007). In control animals, repeated HFES of the hippocampus at 20-min intervals resulted in a gradual potentiation of EEDs duration, with an average enhancement of 10% per stimulation.

HFES acts primarily through long-term potentiation (LTP), strengthening existing synapses and, over longer periods, promoting the recruitment of new neuronal pathways through structural changes and enhanced network excitability. HFS induces strong, persistent increases in synaptic efficacy by reinforcing synaptic connections and frequently recruiting previously silent or inactive neuronal pathways (Hayashi, 2022). This synaptic plasticity has been extensively studied at glutamatergic synapses onto CA1 pyramidal cells of the hippocampus, where multiple forms of LTP can be induced through activation of N-methyl-D-aspartate receptors (NMDARs) (Bliss & Collingridge, 1993; Malenka & Nicoll, 1999) metabotropic glutamate receptors (mGluRs) (Bashir et al., 1993), and voltage-dependent Ca^{2+} channels (Cavuş & Teyler, 1996). Interestingly, a similar repetitive stimulation protocol used to induce LTP at glutamatergic synapses can also trigger long-term depression (LTD) at GABAergic synapses in CA1 pyramidal neurons (Ahumada et al., 2013). These data may explain the potentiation of epileptiform discharge duration induced by repetitive intrahippocampal high-frequency stimulation shown in our experiments. Repeated stimulation led to increased recruitment and synchronization of larger neuronal populations, generation of NMDA-dependent responses, and long-term depression of inhibitory interneurons, resulting in prolonged epileptiform discharges.

Our experiments show that intraventricular administration of orexin-A significantly attenuated hippocampal CA1 EEDs duration at 20 min post-application and abolished the stimulation-dependent progressive potentiation of discharge duration characteristic of control animals. The lack of effect observed with orexin-B suggests a possible involvement of OX1 receptor signaling in mediating the effects of orexin-A. However, this interpretation remains indirect, as experiments using selective receptor antagonists were not performed in the present study. The inhibitory effect of orexin-A on HFES-induced EEDs observed in our study is consistent with *in vitro* findings demonstrating that orexin-A decreases the duration and amplitude of multiple population spikes, inhibits bicuculline methiodide–induced spontaneous epileptiform afterdischarges (Doreulee et al., 2010) and causes long-lasting depression of isolated NMDA responses (Doreulee et al., 2009).

In the present study, cortical application of orexin-A did not significantly affect the duration of hippocampal CA1 epileptiform discharges; however, it induced a significant reduction in the number of high-amplitude (7–10 mV) population spikes within CA1 epileptiform activity. These findings may be explained by studies demonstrating that cortical application of orexin enhances the activity of orexin-sensitive GABAergic neurons in deep cortical layers. This orexin-induced stimulation of GABAergic neurons reduces spiking responses to larger-amplitude current steps in the medial prefrontal cortex (Messore et al., 2025). In another study, it was shown that the entorhinal cortex, the main gateway to the hippocampus, modulates epileptiform activity within hippocampal circuits; ictal discharges in the entorhinal cortex appear necessary for the hippocampus to generate ictal activity (Ren et al., 2014). Conversely, hippocampal inputs also play an important role in cortical network activity (Bustamante & Valdés, 2025; Feng et al., 2025). The attenuation of high-amplitude hippocampal population spikes following cortical application of orexin-A in our experiments may therefore be explained by orexin-mediated activation of cortical GABAergic interneurons, which likely suppress excitatory cortical output neurons, thereby reducing glutamatergic drive from the cortex to the hippocampus.

Several methodological considerations should be taken into account when interpreting the present findings. The receptor specificity cannot be definitively established in the absence of selective receptor antagonists. Although the differential effects observed for orexin-A and orexin-B are consistent with a potential contribution of OX1 receptor signaling, further experiments employing receptor-selective pharmacological tools will be required to confirm this mechanism. The intracerebroventricular administration of orexins does not provide strict anatomical specificity, and therefore the possibility that orexins influenced multiple brain regions cannot be excluded. Although electrophysiological recordings were obtained directly from the hippocampal CA1 field and the analyzed parameters reflect local network activity within this structure, the interpretation that the observed effects result from direct hippocampal receptor activation should be considered cautiously. Future studies employing region-specific microinjection or receptor-selective pharmacological approaches will be required to determine the precise anatomical locus of orexin action. The relatively small sample size in the orexin-B group reflects ethical considerations aimed at minimizing the use of experimen-

tal animals (3Rs principle). In the initial experiments (n=4–5), orexin-B did not produce statistically significant changes in the measured electrophysiological parameters. Therefore, additional experiments were not performed in order to avoid unnecessary animal use. Both male and female animals were included in the study in order to increase the generalizability of the findings. However, the experimental design was not specifically intended or statistically powered to evaluate sex-dependent differences in orexinergic modulation of epileptiform activity. Because the number of animals within each experimental group was relatively limited, stratified analysis by sex was not performed. Future studies specifically addressing sex as a biological variable may help clarify whether orexinergic modulation of hippocampal excitability exhibits sex-specific features. The experiments were conducted under ketamine anesthesia, which acts as an NMDA receptor antagonist and may influence neuronal excitability and synaptic plasticity. NMDA receptor signaling plays an important role in hippocampal network dynamics and synaptic plasticity associated with epileptiform activity. All experimental groups were studied under identical anesthetic conditions, ensuring valid relative comparisons. Moreover, ketamine was administered only prior to electrode implantation surgery, and recordings began approximately 2 hours later. No additional ketamine was given during recordings; local anesthesia was applied as required.

Taken together, the present findings provide evidence that orexinergic signaling can modulate hippocampal epileptiform activity and highlight a potential role of hypothalamic neuromodulatory systems in regulating seizure-related network dynamics. Notably, the modulatory effects of orexin appear to depend, at least in part, on the primary site of its action, whether exerted directly within the hippocampus or indirectly *via* cortical mechanisms. Further studies combining receptor-selective pharmacology and region-specific manipulations will be necessary to clarify the precise mechanisms by which orexin signaling influences hippocampal-related epileptiform activity and epileptogenesis.

CONCLUSION

Our *in vivo* findings demonstrate that orexins regulate hippocampal epileptiform activity through distinct, site-specific mechanisms. Intraventricular administration of orexin-A reduced electrically evoked epileptiform discharge duration and prevented potentiation of EEDs induced by repeated HFES, suggesting modulation of hippocampal network activity, although contributions from other brain regions cannot be excluded. Whereas cortical application of orexin-A selectively attenuated high-amplitude population spikes *via* cortico-hippocampal mechanisms. The lack of effect of orexin-B implies a dominant contribution of OX1 receptors to orexinergic modulation of hippocampal excitability.

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