Alarin potentiates ongoing epileptiform activity in rat brain slices: an *in vitro* electrophysiological study

Ömer Faruk Kalkan[1]*, Hilal Öztürk[2], Zafer Şahin[1], Harun Başoğlu[2], Selcen Aydin Abidin[2], İsmail Abidin[2]

[1] *Karadeniz Technical University, Faculty of Medicine, Department of Physiology, Trabzon, Turkey*
[2] *Karadeniz Technical University, Faculty of Medicine, Department of Biophysics, Trabzon, Turkey*
* Email: ofkalkan@ktu.edu.tr

Alarin is a newly discovered neuropeptide that belongs to the galanin peptide family with a wide range of bioactivity in the nervous system. Its function in the brain's autonomic areas has been studied, and it has been reported that alarin is involved in the regulation of excitability in hypothalamic neurons. Its role in the regulation of excitability in the hippocampus, however, is unknown. In this study, we investigated if alarin induced any synchronous discharges or epileptiform activity, and if it had any effect on already initiated epileptiform discharges. We used thick acute horizontal hippocampal slices obtained from 30- to 35-day-old rats. Extracellular field potential recordings were evaluated in the CA1 region of the hippocampus. Our data demonstrated that, alarin application did not result in any epileptiform activity or abnormal discharges. 4-aminopyridine was applied to induce epileptiform activity in the slices. We found that alarin increased the frequency of interictal-like events and the mean power of local field potentials in the CA1 region of the hippocampus, which was induced by 4-aminopyridine. These results demonstrated for the first time that alarin has a modulatory effect on synchronized neuronal discharges and showed the contribution of the neuropeptide alarin to epilepsy-like conditions.

Key words: neuropeptide, alarin, epileptiform activity, extracellular recording, hippocampus

INTRODUCTION

The central nervous system (CNS) utilises neuropeptides along with neurotransmitters to regulate the neural network (Russo, 2017). Besides the contribution of neuropeptides in physiological conditions such as reproduction, feeding, digestion and nociception, they are also associated with many pathological processes (Pinheiro Da Silva et al., 2013). Epilepsy and seizures characterized by the abnormal firing of neurons, which are some of the pathologies that neuropeptides contribute to (Kovac and Walker, 2013). Neuropeptides have a longer synaptic activity than neurotransmitters, which function on a millisecond time scale (Bhat et al., 2021). As a result of long-lasting synaptic activation, neuropeptides lead to the long-term modulation of neuronal network activity, which contributes to the modulation of the neural firing threshold (Kovac and Walker, 2013). These synaptic properties make neuropeptides a major focus of neuropathology research (Kovac and Walker, 2013). Knowledge of the nature of neuropeptides has increased significantly. However, the roles of neuropeptides in pathologies such as epilepsy and seizures are not yet fully understood.

Alarin is a recently discovered 25 amino acid neuropeptide belonging to the galanin peptide family; it is a variant transcript of the *GALP* gene, and it is widely distributed in the brain and peripheral nervous system (Oliveira Volpe et al., 2020). Alarin functions primarily in physiological processes such as feeding and water intake by activating pathways in the hypothalamus and pituitary gland (Abebe et al., 2022b; Tyczewska et al., 2022). In addition to its autonomic contribution, alarin also has extra hypothalamic effects. A recent study showed that alarin exhibited regulatory effects in depression and may be a potential therapeutic target

(Abebe et al., 2022a). Another study demonstrated the cellular effects of alarin. It was shown that alarin moderated myocardial hypertrophy by inhibiting the cyclic adenosine monophosphate/protein kinase A signalling pathway to attenuate autophagy (Shen et al., 2021). There are many studies related to circulating alarin levels in many metabolic disorders, such as type 2 diabetes and obesity (Abebe et al., 2022b). Even though alarin belongs to the galanin peptide family, it cannot exhibit its biological function *via* galanin receptor activation (Tyczewska et al., 2019). Therefore, unlike other peptides in the galanin family, it may have a different biological pattern. Alarin exerts its central nervous system effects by exciting of neural pathways, and it has a widespread bioactivity in this system (Eberhard et al., 2012). However, there is no evidence relating to how alarin affects extra hypothalamic activity and how it contributes to abnormal neuronal discharges such as those in epilepsy-like conditions.

Acute brain slices are useful "*ex-vivo*" models to study synaptic events and post-synaptic currents, as well as the function of a given neuronal circuit. They are powerful tools to study the excitability of groups of neurons and pathological conditions such as epilepsy and seizures (Avoli and Jefferys, 2016). 4-aminopyridine (4AP) is a convulsant substance that interferes with K+ channels, which include D-type and A-type K+ currents and a sub-portion of delayed rectifier currents and suppresses neuronal K+ repolarization (Fueta and Avoli, 1992). 4AP application induces a hyper excitable state that eventually leads to synchronous discharges. Two types of synchronous discharges are primarily observed in 4AP-induced activity, "ictal"-like long-lasting (>10 s) compact discharges and individual "interictal"-like events with a duration of 0.5 to several seconds. Due to the specific connectivity of the hippocampus and its interactions with other brain regions, 4AP-induced epileptiform discharges are widely used as an experimental epilepsy model activity in these slices (Gonzalez-Sulser et al., 2012; Kalkan et al., 2021). Synchronous discharges can be recorded using this method. Electrodes are placed in the region of interest (e.g., hippocampus CA1), and neuronal activity can be recorded as the extracellular field potential. Several models were developed to investigate epileptiform activity (Raimondo et al., 2017). *In vitro* hippocampus slice preparations are particularly common. By using these preparations, it is possible to focus on some of the intrinsic neuronal and synaptic features that appear to be important in the development of burst activity and hyper excitability in the epileptic brain (Schwartzkroin, 1986). Several *in vitro* studies that have been performed in hippocampal areas, it has been stated that the galanin peptide family has potent relationships to epilepsy-like conditions (Mazarati et al., 1998; 2000). Because alarin is a member of the galanin peptide family, we wanted to investigate the potential relevance of alarin in epilepsy-like conditions in hippocampal slices.

In this study, based on the knowledge that neuropeptides are involved in the regulation of excitability in various brain regions, and that alarin regulates excitability in the hypothalamus, we hypothesized that alarin could have a modulatory role in the excitability of the hippocampal region. According to our hypothesis, we formed the research question: Does alarin modulate neuronal activity under hyper-excitable conditions as in the case of ongoing epileptiform activity? We investigated the effects of alarin in acute brain slices recording extracellular field potentials. Activity was recorded from the CA1 region of the hippocampus which is involved in the induction of epileptic synchronization.

METHODS

Animals

The rats used for the experiments in this study were bred and provided by the Surgical Application and Research Centre of Karadeniz Technical University. The animals were housed in conventional polycarbonate cages and in a 12:12-h light–dark cycle. The temperature of the room was maintained at 22±1°C. The animals were fed with a standard chow diet *ad libitum.*

Our study was approved by the Karadeniz Technical University Animal Care and Ethics Committee (Research proposal approval number: 2020/32).

Slice preparation

We obtained acute brain slices from 30 to 45-day-old male Wistar albino rats. The brain slices were obtained using the method described in a previous study (Aydin-Abidin and Abidin, 2019). In summary, animals were anaesthetised with isoflurane and decapitated immediately after anaesthesia. The brain tissue was removed and placed into cold (1.5–2°C) artificial cerebrospinal fluid aCSF which contained (in mM): 125 NaCl, 2.5 KCl, 1.25 NaH_2PO_4, 25 $NaHCO_3$, 25 D-glucose, 2 $CaCl_2$, and 1.5 $MgCl_2$. The brain tissue was allowed to rest for 3-4 min. Horizontal hippocampus slices (350 μm thick) were prepared using a vibrational microtome (Leica VT100S, Germany) in ice-cold aCSF. The slices were then transferred into a chamber. In this chamber, they recovered for at least 1 h at 30 ± 1°C. Throughout the

procedure, the aCSF was oxygenated with 95% O_2 and 5% CO_2 at pH 7.4.

Electrophysiological recordings

We transferred the slices to a recording chamber for electrophysiological recordings. The submerged-type recording chamber was continuously perfused with oxygen-saturated aCSF. The temperature of the aCSF was kept at 30±1°C. We left the slices in the chamber for about 15 min. Glass pipettes were positioned at the recording area.

Extracellular field potentials were recorded from the CA1 region of the hippocampus. The analogue biological signals were fed to a differential amplifier (A-M Systems Model 1700). The signals were recorded in the AC mode, filtered between 0.1 Hz and 5 kHz and amplified at x1000. Additionally, an analogue notch filter at 50Hz was applied during the recording process. The data were digitized at 5 kHz by using a Digidata-1400 A/D converter. The pCLAMP10 Clampex software (Molecular Devices, Sunnyvale, CA, USA) was used for data acquisition. For the offline analyses, the Clampfit software was used.

Glass pipettes were used for the recording process. The resistance of the pipettes was ~ 1 MΩ. The pipettes were pulled using a puller (Sutter P-1000, Japan) made out of borosilicate capillaries (GB 150F-8P, Sutter, Japan). The pipettes were filled with aCSF. For each channel, the recording pipettes were put in contact with the tissue smoothly.

Experimental design

Alarin was dissolved in distilled water, and high-concentration small batches (0.25 ml) were prepared and mixed into aCSF before the experiments. This study included two series of independent experiments. In the first series of experiments, the effects of alarin on basal activity were evaluated. Activity in the CA1 region was recorded. Then, 1, 5, and 20 nM of alarin was applied to the bath. For the following 20 min, the activity as recorded a second time. The total powers of the field potentials were compared between time points before and after the alarin application. In the second series of experiments, the effects of alarin on epileptiform activity were evaluated. First, the basal activity in the slice was checked for any abnormal activity or discharges for about 15 min. Then, 100 µM 4AP (Sigma-Aldrich, Phoenix Pharmaceuticals Inc., CA, USA) was applied to induce epileptiform activity. During the following 20 min, synchronized discharges

named "ictal"- or "interictal-like" events developed (Aydin-Abidin and Abidin, 2019). We observed epileptiform activity 20 min later. For 45 min, neuronal activity was recorded. Then, aCSF containing 100 µM 4AP + 200 nM alarin was perfused into the recording chamber. The perfusion rate was 2.5–3 ml/min. For another 20 min, alarin was allowed to exert its effects. To evaluate these effects, recordings were taken for another 60 min. In our preliminary studies, 1 nM, 5 nM and 20 nM doses of alarin were tested. 1 nM of alarin did not change ictal properties but slightly increased the interictal frequency (n=6 for 1 nM and 5 nM). The effects of the 5 nM and 20 nM doses were similar, but the effect of the 20 nM dose was the most prominent. Hence, the 20 nM dose was used in the experiments, and the results of the other doses are not shown.

The extracellular field potentials that were recorded were then analysed offline. 50 Hz and harmonics were notch-filtered. Activity that lasted shorter than 4 s was accepted as interictal-like events, whereas those with a longer duration than 4 s was accepted as an ictal-like event (Fig. 1B, C). The duration of each event was defined as the time between deflection and return to baseline potentials. The duration between the onsets of two consecutive ictal-like events was accepted as the interval between the two events. The amplitude of an interictal-like event was defined as the height from the peak positive deflection to the peak negative deflection. Activities with amplitude 4× of the baseline amplitude were accepted as synchronous activities. The power spectrum of the recordings was calculated to characterize the effects of alarin on the mean power of local field potentials. The recordings were analysed and compared between time points before and after alarin application. The pClamp10 software was used for the analysis. The power was calculated based on 15-minute recordings. The fast Fourier transform (FFTs) of the recordings was used to calculate the power by applying the Hamming window function and root mean square power values. The sample size was 8,192 points, and the spectral resolution was 1.22 Hz. The local field potential signals were divided into 1,000-ms time windows. For each window, power *vs.* frequency distribution plots were obtained and averaged. The 1–120-Hz frequency range was used for the local field potential power calculations.

Statistical analysis

For the statistical tests, GraphPad Prism 6 software was used. The datasets were tested for normal distribution using the Shapiro-Wilk normality test. The values of each parameter before and after alarin application

were compared. The data on interictal frequency, interictal amplitude, and ictal frequency were not normally distributed. The data on interictal duration, ictal duration, and power were normally distributed. For the comparisons of the non-normally distributed data, the parametric paired-samples t-test was used. Otherwise, the Wilcoxon non-parametric paired test was used. The data are presented as mean ± SEM, and p<0.05 was accepted as statistically significant.

RESULTS

The effects of alarin on excitability were tested in the CA1 region of the hippocampus (Fig. 1A). The application of 100 μM 4AP triggered synchronous discharges which could be classified as ictal-like and interictal-like events based on their durations. A total of 17 slices, obtained from each of 8 rats were used. In 14 of these

slices, reliable epileptiform activities were observed. In the 3 remaining slices, epileptiform activities did not develop. In 10 of these slices, both inter-ictal and ictal activities were observed. In 4 of the slices, only interictal-like activities were observed (Fig. 1B, C).

During the epileptiform activity, alarin was applied to the bath. The effects of alarin were evaluated by comparing the electrophysiological properties of synchronous discharges measured before and after alarin application. The mean frequency of the interictal-like events was 0.0695±0.03 Hz (n=14), while their mean duration was 0.8191±0.1715 s and their mean amplitude was 0.8650±0.1634 mV (Fig. 2A). The application of alarin significantly increased the frequency to 0.13930±0.04526 Hz (n=14) (p=0.0031). Alarin did not change the duration of interictal activity significantly (0.9384±0.1907 s). Similarly, interictal amplitudes were not significantly affected (0.8420±0.1321 mV) (Fig. 2A). The application of alarin did not significant-
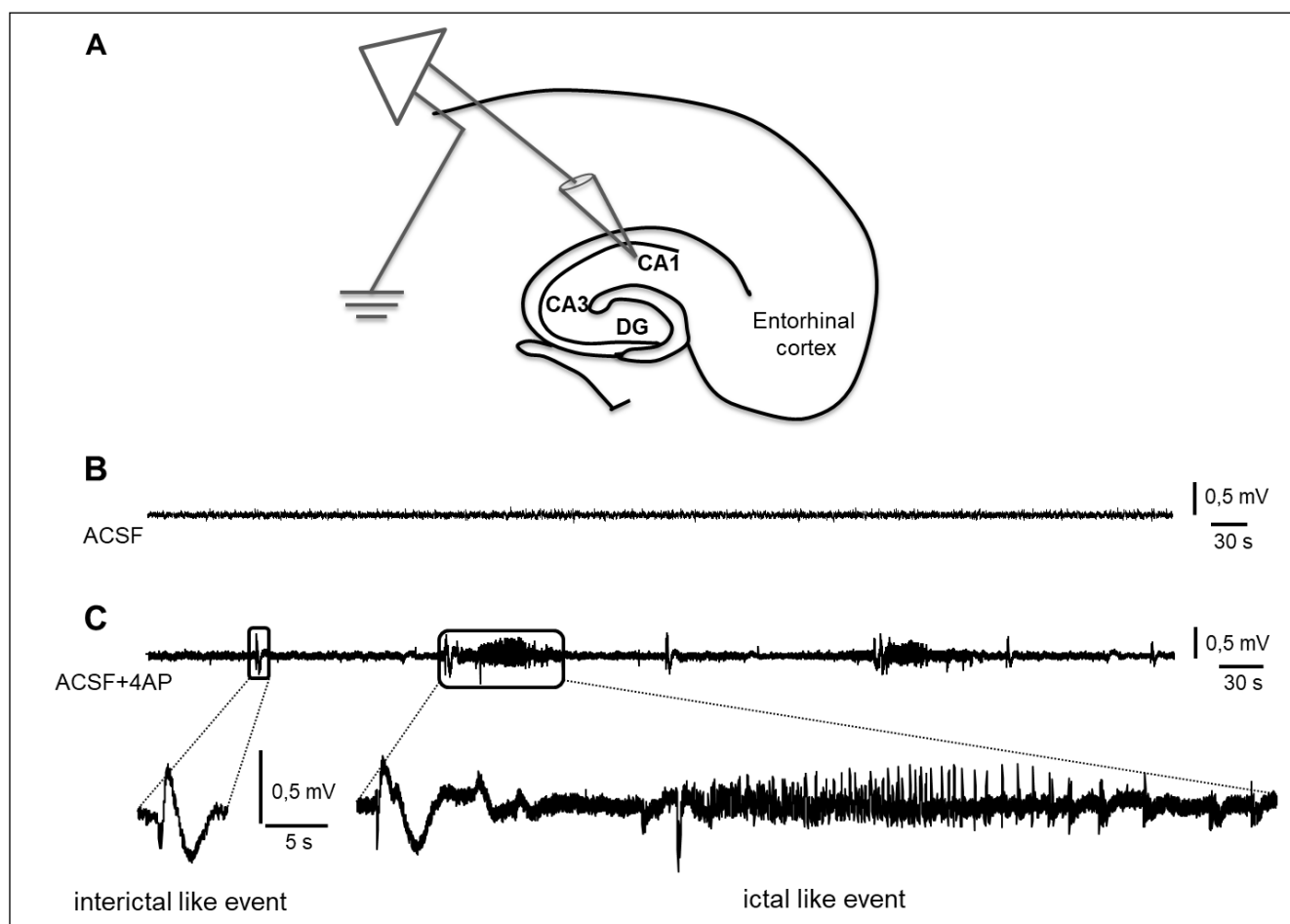

Fig. 1. Transverse hippocampal slices were used for the experiments. The slices were containing the cortical areas, hippocampi as well as the connections between. A recording glass electrode was placed on CA1 region. (A) A drawing showing the slice shape and the site of recording. (B) 4AP typically induced ictal and interictal-like epileptiform events. (C) A sample recording is shown.
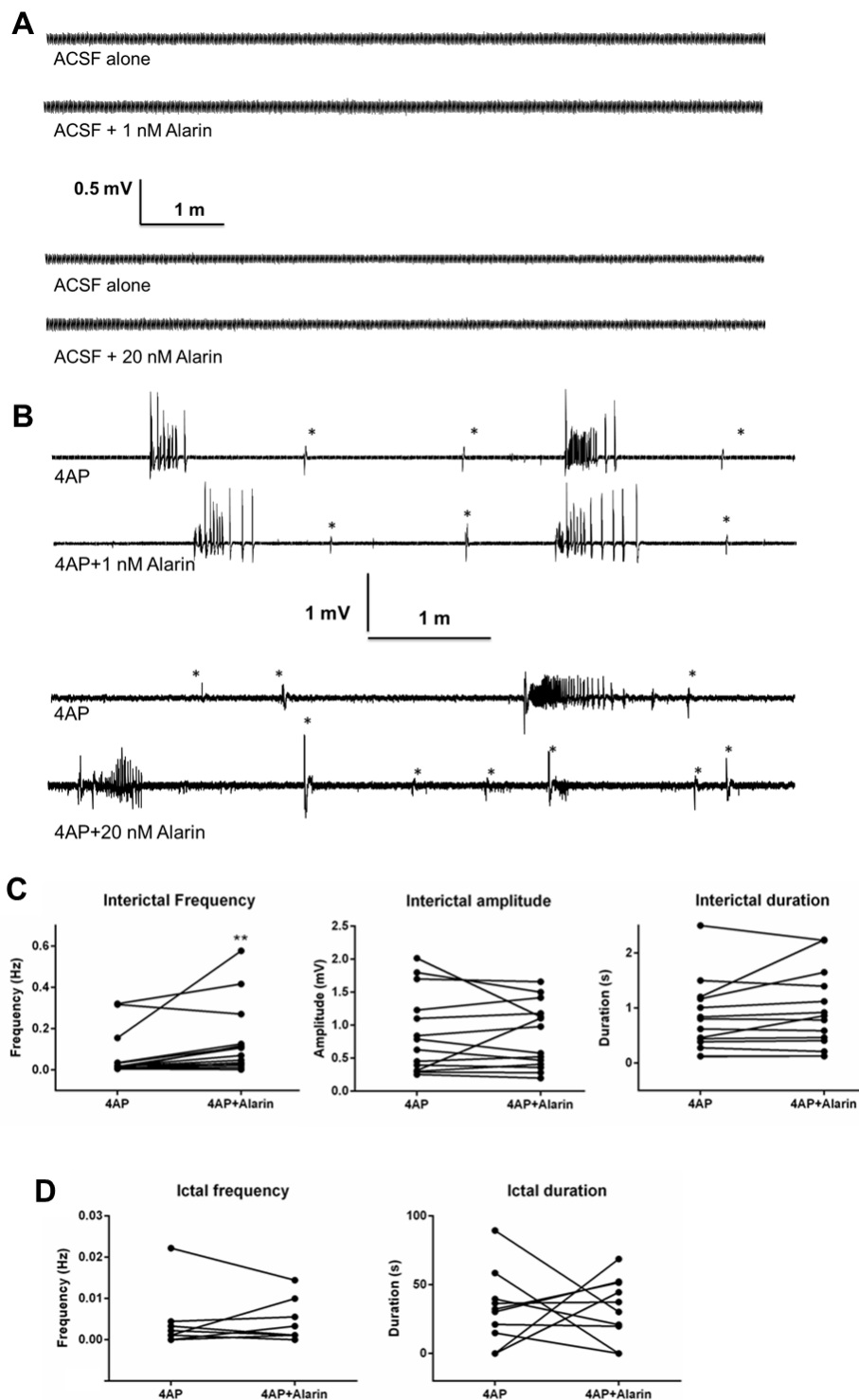
Fig. 2. The effects of 1 nM and 20 nM alarin application during basal activity and during 4AP epileptiform activity has been shown. Basal activity obtained while ACSF alone was perfused with no additional drug. (A) Basal activities of CA1. Bath-applied alarin did not induce any changes, nor 1 nM neither 20 nM had effect of basal activity. (B) On the other hand, alarin at 20 nM, increased the incidence of interictal events when applied following the induction of an epileptiform activity. 1 nM alarin had no such effect. Asterisks represents the interictal activities. (C) The frequency, amplitude and durations of interictal-like events before and after alarin application during ongoing discharges induced by 4AP. Alarin application increased the frequency of interictal-like events (**p<0.01). The duration and amplitude of interictal-like events were not altered. (D) The duration and the frequency of mean ictal-like events also not affected significantly by alarin.

ly change the properties of the ictal-like events, and while the mean duration of the ictal discharges was 34.30±8.538 s before, it was 32.56±7.18 s after alarin application (n=10; p=0.984) The frequencies of ictal-like events were also not changed by the alarin application. The mean frequency was 3.88±2 (10^{-3} Hz) before and 3.78±1.54 (10^{-3} Hz) after the alarin application (n=10; p>0.99) (Fig. 2B).

To better evaluate the effects of alarin on the excitability of CA1 circuitry, the total power of local field potentials was analysed. Fig. 3 presents, the frequency distributions of the amplitudes of a sample recording and the mean power changes (Fig. 3A, B). The total power values of 0.1-50 Hz signals were compared. The mean total power of field potentials was $4.8 \times 10^{-5} \pm 0.96 \times 10^{-5}$ mV^2. Alarin application increased the total power to $13 \times 10^{-5} \pm 2.69 \times 10^{-5}$ mV^2 (n=14; p=0.0085).

DISCUSSION

The aim of this study was to investigate the acute effects of alarin on the systemic excitability of hippocampal slices. According to this aim, the effects were studied in the basal and hyper excitable conditions of the slices. The application of 4AP induced a hyper excitable state and elicited epileptiform activities. Our findings revealed that: alarin alone did not induce any abnormal activity in synchronous discharges, and alarin increased the interictal frequency and mean power of local field potentials. The central effects of alarin on the hypothalamic regions have been shown

in several studies (Abebe et al., 2022b). Apart from these central effects, this is the first study to show that alarin modulates 4AP-induced neuronal activity in the hippocampus.

In vitro studies are critical for in understanding the nature of the pathogenesis of epilepsy and for investigating effective treatment mechanisms (Avoli et al., 2002). Among other chemicals that induce epileptiform activity, 4AP is one the most effective convulsant agents that causes synchronised discharges in rat and mouse hippocampal and cortical slices (Fueta and Avoli, 1992; Brückner and Heinemann, 2000). In our study, we observed epileptiform-like activities in the hippocampal slice preparations induced by 4AP. Fast interictal discharges were also recorded as a result of the 4AP induction. Furthermore, long-lasting ictal discharges were recorded in some slices. Our experimental findings were all compatible with previous studies (Righes Marafiga et al., 2021). By blocking various types of K+ channels, 4AP application has the potential to induce epileptiform activity (Coetzee et al., 1999). This blockage increases neural circuit excitability by increasing cytosolic calcium ($[Ca^{2+}]i$), and this increased $[Ca^{2+}]i$ causes the release of both excitatory and inhibitory neurotransmitters (Qian and Saggau, 1999). Considering the electrophysiological nature of epilepsy, it is clear that interictal-like spikes and amplitudes are widely accepted as characteristics of epilepsy (Staley and Dudek, 2006). In our study, alarin modulated 4AP-induced epileptiform activity by increasing the frequency and the mean power of the amplitudes of interictal-like events in the hippocampus. The galanin
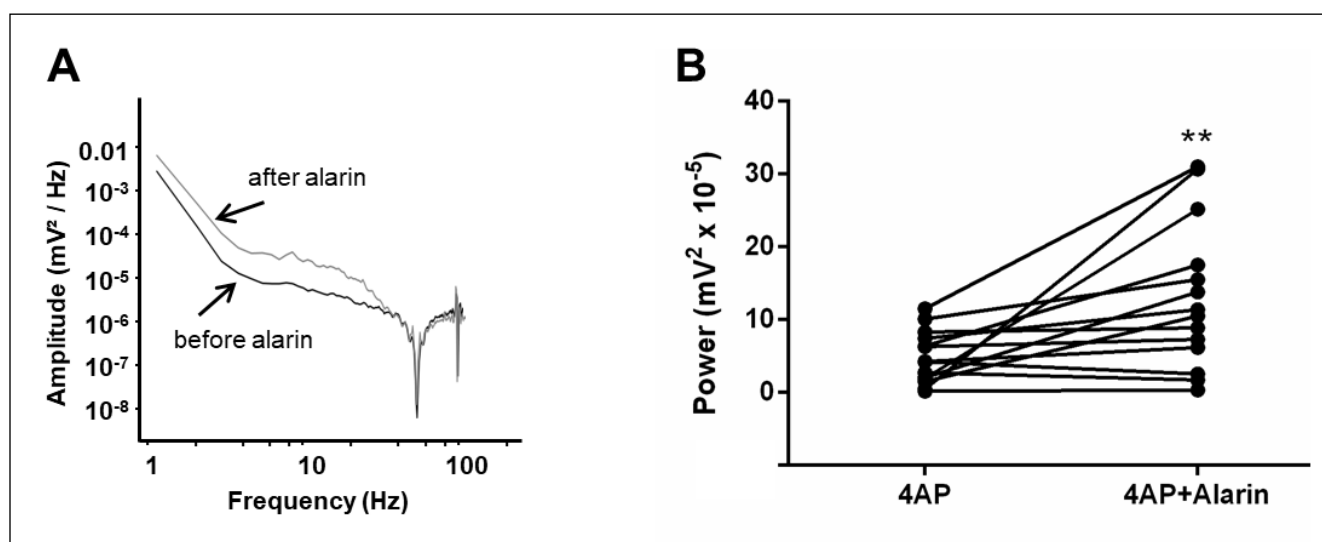

Fig. 3. Alarin enhanced the power of field potentials in the CA1 region during epileptiform activity. (A) Sample power spectra of a recording from CA1 are shown. Alarin application moved the power distribution curve up in the power axis. (B) The total power of this epileptiform activity was significantly increased upon alarin application in CA1 (n=14, ***p<0.001).

peptide family exerts a wide range of bioactivities in the CNS, as well as in the hippocampus and contributes to many different physiological functions (Gundlach et al., 2001). In a previous study, which examined the role of galanin in status epilepticus, it was found that galanin acted as an endogenous anticonvulsant *via* galanin type 2 receptors (Mazarati et al., 1998). Even though alarin is a member of the galanin peptide family, our data revealed an opposite effect relative to the aforementioned study. However, this was conceivable data, and it was compatible with current studies. Such recent studies, emphasised that even though alarin is a member of the galanin peptide family, it has been found to affect another type of receptor than those affected by galanin peptides (Tyczewska et al., 2019). This may explain why alarin induces an excitatory effect rather than an inhibitory one. There are also several studies about the cellular effects of alarin. According to these studies, alarin caused the release of neuropeptide Y (Boughton et al., 2010), the secretion of GnRH (Fraley et al., 2013), and the activation of the TrkB signalling pathway (Wang et al., 2015). These cellular effects of alarin may have a different cumulative pattern in the hippocampus and lead to an accumulated excitatory effect. However, this possibility needs to be investigated in a further study. Neuropeptides are also known to be expressed in non-neuronal cells. Accordingly, it is not surprising that glial cells also contain these peptides. The galanin peptide family, which also contains alarin, is also expressed in glial cells and especially in microglia (Ubink et al., 2003). Microglia-neuron communication is very important for the nervous system, and it involves cation influx and glutamate-mediated excitatory patterns (Luo et al., 2021). This strict microglia-neuron communication also applies to epileptic conditions (Eyo et al., 2017). Microglia-neuron communication, the expression of peptides in the galanin peptides family (which includes alarin) in microglia, and the excitatory component of microglia-neuron communication may also explain how alarin enhances epileptiform activity in the hippocampus. Our data also demonstrated that alarin alone at different concentrations did not change neural activity. This may be attributed to the limited bioactivity of the receptor distribution of alarin in the hippocampus.

CONCLUSION

In conclusion, our electrophysiological analyses indicated that alarin did not induce abnormal activity in rat brain slices. However, it increased the interictal-like events in the hippocampus. These data point to a modulatory effect of alarin in ongoing epileptiform activity.

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