Archives of Razi Institute, Vol. 77, No. 2 (2022) 689-695

DOI: 10.22092/ARI.2022.357297.2014

Original Article

Central Phoenixin Protective Role on Pentylenetetrazol-Induced Seizures during Various Stages of the Estrous Cycle among Rats

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Received 13 December 2021; Accepted 15 January 2022
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Abstract

It is known that phoenixin-14 (PNX-14) has a mediatory role in reproduction; however, there is no report on the role of the PNX-14 on epilepsy. Therefore, this study aimed to investigate the antiepileptic effects of the PNX-14 on the pentylenetetrazol (PTZ)-induced epilepsy in the stages of the estrous cycle among rats. A total of 168 adult female Wistar rats were randomly divided into seven groups, including control (intracerebroventricular injection was performed with saline), PNX-14 (5 µg), PNX-14 (10 µg), bicuculline (competitive antagonist of $GABA_A$ receptors; 5 nmol)+PNX-14 (5 µg), bicuculline (BIC) (5 nmol)+PNX-14 (10 µg), saclofen (competitive antagonist of $GABA_B$ receptors; 2.5 µg)+PNX-14 (5 µg), and saclofen (2.5 µg)+PNX-14 (10 µg) in proestrus, estrus, metestrus, and diestrus. Afterward, the control and treatment groups were followed by intraperitoneal administration of 80 mg/kg PTZ. Initiation time of myoclonic seizures (ITMS), initiation time of tonic-clonic seizures (ITTS), seizure duration (SD), and mortality rate (MR) were monitored and recorded for 30 min. According to the results, PNX-14 alone significantly reduced the SD and seizure mortality in all phases of estrus (P<0.05). The injection of PNX-14 with BIC significantly reduced SD and seizure mortality in all estrus phases (P<0.05). PNX-14 alone increased both ITMS and ITTS in all phases of estrus (P<0.05). Furthermore, the injection of PNX-14 with BIC significantly reduced the effects of the PNX-14 on ITMS and ITTS in all estrus stages (P<0.05). These results showed that the antiepileptic activity of PNX-14 was probably mediated by $GABA_A$ receptors, and this effect was more prominent during the luteal phase than the follicular phase.

Keywords: Estrus cycle, Pentylentetrazole, Phoenixin, Rat

1. Introduction

Epilepsy is considered one of the oldest brain-related disorders. Catamenial epilepsy is a type of seizure configuration associated with the menstrual period which happens in some epileptic women during the menstrual cycle (1). It is regarded as one of the earliest neurological disturbances in females suffering from focal or general epilepsies (2). Variation in ovarian hormones during the menstrual period, as well as electrolyte imbalance, are the main factors of menstrual epilepsy (3). Since P4 produces anticonvulsant effects and E2 shows proconvulsant properties, steroid hormones can have modulatory effects on the onset of seizure and propagation. In the rodents, an association was observed between serum estradiol/P_4 levels and the incidence of seizure in ovulatory cycles. The highest

seizure activity was correlated with increased estrogen level (4). Pentylenetetrazol (PTZ) has been used widely for catamenial epilepsy seizures. It is antagonized by the gamma-aminobutyric acid-ergic (GABAergic) inhibition of the $GABA_A$ receptor (5). It is known that sex hormone-related seizure is mediated through neurotransmitters in the central nervous system. P4 neuroactive metabolites, namely allopregnanolone (AP) and pregnanolone (PREG) can affect the GABAergic receptors in the brain (1).

Phoenixin (PNX) neuropeptide is recently identified (6). It is cleaved from the C-terminal of small integral membrane protein 20. Its most prevalent isoforms include the phoenixin-14 amide (PNX-14) and phoenixin-20 amide (PNX-20) acid peptides with analogous activities. PNX can produce a modulatory effect on both the nervous system and women's reproductive system (7). It is expressed in a number of hypothalamic nuclei, including the paraventricular nucleus (PVN), supraoptic nucleus, arcuate nucleus, nucleus of the solitary tract, and anterior pituitary gland (8). PNX-14 is identical in five species, including mice, rats, dogs, pigs, and humans. Only one amino acid differs in PNX-20 among the coding regions of human, canine, and porcine sequences (9). PNX can be expressed in several regions related to the brain and peripheral tissues in mammals. It is highly expressed in the hypothalamus and can play an important role in the stimulation of reproductive functions through acting on the hypothalamic-pituitary-gonadal (HPG) axis (10). Intracerebroventricular (ICV) administration of the PNX can enhance the plasma levels of the luteinizing hormone (LH) in a dose-related manner, and amplify GnRH-induced LH secretion from the anterior pituitary cells cultured *in vitro* (11).

Given the role of the sex hormones in catamenial epilepsy, as well as the physiological function of the PNX on the reproductive system, and no report on the role of the PNX in catamenial epilepsy, this study aimed to investigate the antiepileptic effects of the PNX-14 on PTZ-induced epilepsy during the different stages of the estrous cycle in rats.

2. Materials and Methods

2.1. Animals

In total, eight experiments were performed on 48 female Wistar rats (200±50 g) to investigate the possible antiepileptic effect of PNX-14 during the various stages of the estrous cycle. Animals were maintained under the normal laboratory conditions (22±1°C, 12 h dark/light cycle) in accordance with the European community regulations for laboratory animals (12). Prior to the study, sexual puberty was approved using the vaginal smears, and two regular estrous cycles were employed to select the rats, followed by estrus synchronization (13).

2.2. Synchronization of the Estrous Cycle

Before performing trials, vaginal smears were obtained each day to estimate the stage of the estrous cycle based on the most common cell type, including proestrus large round nucleated cells, estrus masses of the cornfield squamous epithelial cells, metestrus round nucleated epithelial cells with the leukocyte infiltration, and diestrus consisting of a predominance of leukocytes (13-15).

2.3. Surgery Procedure

Following the one-week adaptation period, all rats were anesthetized with intraperitoneal injection of a combination of ketamine (80 mg/kg) and xylazine (10 mg/kg). They were then placed in a stereotaxic device (Stoelting, Wood Lane, IL, USA). The skull was leveled off around the bregma after incising the scalp. A stainless-steel guide cannula (23 gauge, 12 mm length) was placed in the right lateral ventricle of the brain. The tip of the cannula was performed as Ap=-0.8 mm, DV=-3.3 mm, ML= +1.6 mm, and the cannula was placed in the right lateral cerebral ventricular (16). Following that, the cannula was then fixed to the skull. A -12.5-mm stylet was placed in the cannula to keep it from blocking before the administration. Prior to initiating experiments, animals were allowed a one-week recovery period.

2.4. Study Procedure

This study was designed to investigate the antiepileptic effects of the PNX-14 on PTZ-induced

epilepsy during the different stages of the estrous cycle in rats. A total of 168 adult female Wistar rats were randomly allocated into seven groups as control (saline), PNX-14 (5 µg), PNX-14 (10 µg), BIC (competitive antagonist of $GABA_A$ receptors; 5 nmol)+PNX-14 (5 µg), BIC (5 nmol)+PNX-14 (10 µg), saclofen (competitive antagonist of $GABA_B$ receptors; 2.5 µg)+PNX-14 (5 µg), and saclofen (2.5 µg)+PNX-14 (10 µg), each containing proestrus, estrus, metestrus, and diestrus (Table 1). Subsequently, the ICV administration of saline or vehicle was followed by intraperitoneal administration of a dose of 80 mg/kg PTZ in each group. After the seizure was induced, the behavior of animals was monitored for 30 m in order to assess seizure duration (SD), mortality rate (MR), initiation time of myoclonic seizures (ITMS), and initiation time of tonic-clonic seizures **(**ITTS) (17, 18). All experiments were conducted at 9 to 12 a.m. in order to reduce the impact of circadian rhythm on the susceptibility of the seizure (17). Drug doses were selected based on previous research or pilot studies (19, 20).

Table 1. Treatment procedure in experiment 1

Group Estrous Cycle	First injection	Second injection*
Control	Normal saline	PTZ (80 mg/kg)
PNX(5µg)	PNX(5 µg)	PTZ (80 mg/kg)
PNX(10µg)	PNX(10 µg)	PTZ (80 mg/kg)
BIC(5 nmol)+PNX(5µg)	BIC(5 nmol)+ PNX(5 µg)	PTZ (80 mg/kg)
BIC(5 nmol)+PNX(10µg)	BIC(5 nmol)+ PNX(10 µg)	PTZ (80 mg/kg)
Saclofen(2.5 µg)+PNX(5µg)	Saclofen(2.5 µg)+PNX(5 µg)	PTZ (80 mg/kg)
Saclofen(2.5 µg)+PNX(10µg)	Saclofen(2.5 µg)+PNX(10 µg)	PTZ (80 mg/kg)

*30 min after the first injection
PTZ: Pentylenetetrazol

2.5. Cannula Verification

At the end of the experiments, the placement of the cannula in the lateral ventricle was determined by the ICV administration of 10 µL methylene blue, and they were deeply anesthetized with a high dose of pentobarbital and decapitated. In the next stage, the placement of the tip of the cannula and the diffusion of the dye into the lateral ventricle were visually monitored (21).

2.6. Statistical Analysis

The data analysis was performed using one-way analysis of variances (ANOVA), followed by Tukey-Kramer multiple comparison post hoc test. The data were expressed as mean±SD (P<0.05).

3. Results

3.1. Seizures Duration

Table 2 shows the effects of BIC (5 nmol) and the combination of BIC+PNX-14 and saclofen (2.5 µg)+PNX-14 on SD at various stages of the estrous cycle. It can be observed that the injection of PNX-14 alone (5 and 10 µg) significantly reduced the SD in all phases of estrus, compared to the controls (P<0.05). The co-injection of PNX-14 with BIC significantly reduced SD in all estrus phases, compared to the controls (P<0.05). Furthermore, the co-injection of PNX-14 with saclofen did not significantly affect SD in all estrus phases, compared to the controls (P>0.05). It seems that the effects of the PNX-14 are mediated via $GABA_A$ receptors.

3.2. Seizures mortality

As can be observed in table 3, the injection of PNX-14 alone (5 and 10 µg) significantly reduced seizure mortality in all estrous phases, compared to the controls (P<0.05). Moreover, the co-injection of PNX-14 with saclofen did not significantly influence seizure mortality in all estrus phases in comparison with the controls (P>0.05). However, a statistically significant

difference was observed between the co-injection of PNX-14 and BIC injection, compared to the controls (P<0.05). The effects of the PNX-14 are likely mediated via GABAA receptors.

3.3. Initiation Time of Myoclonic Seizures

The antiepileptic effects of BIC (5 nmol) and combination of BIC and saclofen (2.5 µg) with PNX-14 (5 and 10 µg) on the onset of ITMS seconds at different stages of the phallic cycle are presented in figure 1. As can be illustrated, the injection of PNX-14 alone (5 and 10 µg) increased the onset of ITMS in all phases of estrus in comparison with the controls (P<0.05). The co-injection of PNX-14 with BIC significantly reduced the effects of the PNX-14 on ITMS in all estrus phases, compared to the controls (P<0.05). The injection of PNX-14 with saclofen using the same injection location cannot alter the effects of the PNX-14 on ITMS in all estrus phases, compared to the control group (P>0.05) (Figure 1).

3.4. Initiation time of Tonic-Clonic Seizures

As shown in figure 2, the injection of PNX-14 (5 and 10 µg) alone increased the onset of ITTS in all phases of estrus, compared to the controls (P<0.05). The injection of PNX-14 with BIC using the same injection location significantly decreased the effects of the PNX-14 on the onset of ITTS in all phases of the estrus in comparison with the controls (P<0.05); however, no statistically significant difference was found by the co-injection of the PNX-14 with saclofen in comparison with the controls (P>0.05). The effects of the PNX-14 may be mediated via $GABA_A$ receptors.

Table 2. Effects of phoenixin (5 and 10 µg), as well as the combination of bicuculline (5 nmol)+phoenixin (5 and 10 µg) and saclofen (2.5 µg)+phoenixin (5 and 10 µg) on the seizure duration (sec) during various phases of the estrous cycle. Different letters (a, b, or c) in each column indicate significant differences at the P<0.05 among various treatments in each phase of the estrous cycle. Data are presented as mean±SEM

Group Estrous Cycle	Proestrus	Estrous	Metestrus	Diestrus
Control	812±47^a	796±52^a	534±44^a	563±42^a
PNX(5 µg)	610±49^b	558±36^b	437±41^b	427±40^b
PNX(10 µg)	399±38^c	430±44^c	265±29^c	250±29^c
BIC(5 nmol)+PNX(5 µg)	765±41^a	761±63^a	554±38^a	548±38^a
BIC(5 nmol)+PNX(10 µg)	743±62^a	776±68^a	554±60^a	609±56^a
Saclofen(2.5 µg)+PNX(5 µg)	587±41^b	549±25^b	457±34^b	401±31^b
Saclofen(2.5 µg)+PNX(10 µg)	407±54^c	438±51^c	232±49^c	257±52^c

Table 3. Effects of phoenixin (5 and 10 µg), as well as the combination of bicuculline (5 nmol)+phoenixin (5 and 10 µg) and saclofen (2.5 µg)+phoenixin (5 and 10 µg) on the mortality rate (MR) of seizures (%) during various phases of the estrous cycle. Different letters (a, b, or c) in each column indicate significant differences at the P<0.05 among various treatments in each phase of the estrous cycle. The data are presented as mean±SEM.

Group Estrous Cycle	Proestrus	Estrous	Metestrus	Diestrus
Control	35.4^a	36.1^a	18.6^a	22.1^a
PNX(5 µg)	18.9^b	37.3^a	17.5^a	19.9^a
PNX(10 µg)	4^c	20.2^b	3^b	5^b
BIC(5 nmol)+PNX(5 µg)	37.3^a	38.1^a	21.1^a	22.4^a
BIC(5 nmol)+PNX(10 µg)	6^c	20.7^b	7^b	4^b
Saclofen(2.5 µg)+PNX(5 µg)	20.1^b	37.6^a	21.7^a	20.5^a
Saclofen(2.5 µg)+PNX(10 µg)	8^c	19.9^b	2b	4^b

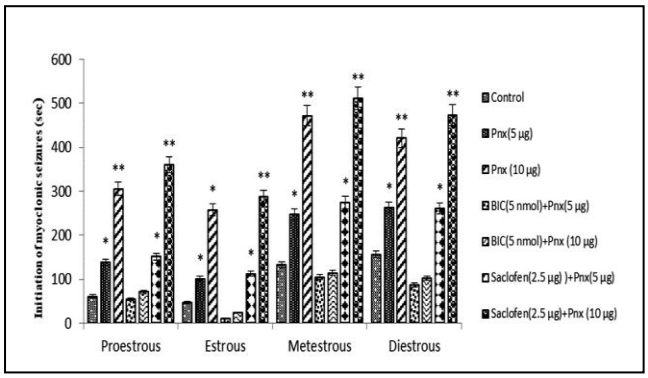

Figure 1. Antiepileptic effects of bicuculline (5 nmol), saclofen (2.5 µg), and phoenixin (5 and 10 µg), as well as the combination of bicuculline (5 nmol) and saclofen (2.5 µg) with phoenixin (5 and 10 µg) on the initiation time of myoclonic seizures (sec) during various phases of the estrous cycle.
*Asterisks indicate a significant difference in each phase of the estrous cycle, compared to the control group (P<0.05). Data are presented as mean±SEM.

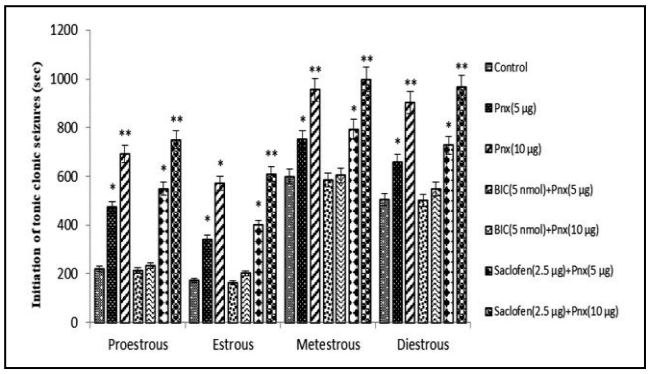

Figure 2. Antiepileptic effects of bicuculline (5 nmol), saclofen (2.5 µg), and phoenixin (5 and 10 µg), as well as the combination of bicuculline (5 nmol) and saclofen (2.5 µg) with phoenixin (5 and 10 µg) on the initiation time of tonic-clonic seizures (sec) during various phases of the estrous cycle.
*Asterisks indicate a significant difference in each phase of the estrous cycle, compared to the control group (P<0.05). Data are presented as mean±SEM.

4. Discussion

According to the results, PNX-14 alone increased the onset of myoclonic seizures and reduced the seizure time in all phases of estrus, compared to the control group. PNX-14 alone significantly reduced seizure mortality in all estrous phases. It is known that both steroid hormones and their metabolites can play a mediating role in the occurrence of seizures (22). P4 increases the seizure threshold (ST) by attenuating neuronal excitability, while E2 has inverse effects. During the estrous cycle, the threshold of seizures is positively correlated with estradiol concentrations (22). Seizure frequency increases in the follicular phase, while it is declined during the luteal phase. An inverse relationship was observed in the epileptic rats with respect to estradiol/P4 ratio, whereas estradiol concentration was enhanced in parallel to a reduction in P4 concentrations; therefore, increasing the estradiol levels can promote epileptogenesis, while P4 may be used for the preservation. Seizure incidence increases by a decrease in P4 level (23). It is reported that the injection of PTZ can induce seizures in both follicular and luteal stages of the estrous cycle in rats (17). A decrease in P4 in epileptic women might be related to its metabolites, AP, PREG, and GABA-positive allosteric modulators (24). AP administered to female rats produces the antiepileptic effects during diestrus in comparison with estrus (25).

As observed in the current study, the co-injection of PNX-14 with saclofen significantly reduced seizure mortality in all estrus phases. PNX-14 alone increased the onset of ITMS and ITTS in all phases of estrus. No statistically significant difference was reported by the injection of the BIC with saclofen. PNX as a new peptide with hormone-like actions can regulate reproduction in mammals. It acts on the HPG axis, as well as testis and ovary. GnRH levels in the hypothalamus of zebrafish are increased with the injection of PNX-20 (26). ICV injection of the PNX-20 potentiates GnRH-induced LH secretion and plasma LH levels in rats (11). Moreover, PNX leads to increased kisspeptin gene expression which means it interacts with other neurons in the CNS. The upregulation of the kisspeptin system in the gonads is considered another possible mechanism by which PNX-20 affects reproduction (26).

Our results demonstrated that the antiepileptic activity of PNX-14 was mediated by $GABA_A$ receptors, and it

was more prominent during the luteal phase than the follicular phase. GABA is an inhibitory neurotransmitter in CNS which can lead to an increase in the ST via the ionotropic $GABA_A$ receptors and activate chloride ion-selective channels and chloride influx (27). PNX produces a direct excitatory effect on magnocellular neurons and may further excite the PVN via inhibiting the GABAergic inhibitory interneurons (28). No data are demonstrating the effect of the PNX on controlling seizures, and this finding can be used as the base information. PNX showing dose-dependent antiepileptic effects may be mediated via $GABA_A$ which are more prominent during the luteal phase, compared to the follicular phase; moreover, it can be regarded as a promising natural candidate to prevent epilepsy in females.

Authors' Contribution

Study concept and design: M. Z.
Acquisition of data: N. P., A. A.
Analysis and interpretation of data: M. Z.
Drafting of the manuscript: M. Z. and J. K.
Critical revision of the manuscript for important intellectual content: M. Z. and A.A

Ethics

All experiment procedure was approved by the Faculty of Veterinary Medicine, Islamic Azad University, Science and Research Branch, Tehran, Iran.

Conflict of Interest

The authors declare that they have no conflict of interest.

Grant Support

This study was supported by Science and Research Branch, Islamic Azad University, Tehran, Iran.

Acknowledgment

The authors would like to express their deepest gratitude to the central laboratory (Dr. Rastegar Lab.) of the Faculty of Veterinary Medicine, the University of Tehran for cooperation. This study was obtained from PhD thesis performed by the first author.

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