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Research report

Effects of Phoenixin-14 on anxiolytic-like behavior in mice

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HIGHLIGHTS

- Centrally injected PNX-14 evoked anxiolytic-like responses in mice.
- This anxiolytic effect of PNX-14 was antagonized by Cetrorelix, but not Atosiban.
- PNX-14 injected in the AHA, but not amygdala, exerted anxiolytic effects.
- PNX-14 increased the expression level of GnRH mRNA and plasma GnRH concentration.
- Centrally injected PNX-14 and PNX-20 reduced the core temperature.

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ABSTRACT

Phoenixin is an amidated neuropeptide, which is widely distributed in brain and periphery regions and is known for its key role in reproduction. Phoenixin-14 (PNX-14), one of the endogenous active isoforms, was reported to regulate pituitary gonadotrophin secretion by increasing the expression of the GnRH receptor mRNA. Studies showed that GnRH could regulate brain responses to anxiety. However, the role of PNX-14 in anxiety was largely unclear. Here, we investigated that the effects of PNX-14 in anxiety-related behavior in adult mice via the open field and elevated plus maze. PNX-14 was administered intracerebroventricularly (i.c.v.) in different doses (5, 10, 25 and 50 nmol), and dose-dependently induced anxiolytic effects. Then this anxiolytic action was presented after PNX-14 injected into the anterior hypothalamic area (AHA), while PNX-14 infused into the amygdala did not exert anxiolytic effects. GnRH receptor antagonist (Cetrorelix) could significantly antagonize the anxiolytic effects of PNX-14, while Atosiban, a competitive vasopressin/oxytocin receptor antagonist could not. Moreover, PNX-14 could significantly lower the core temperature and Cetrorelix could block this effect of PNX-14. Additionally, the AHA infusion of PNX-14 (5 nmol) increased the expression level of the GnRH mRNA in the hypothalamus and plasma concentrations of GnRH. Similarly, i.c.v. injection of PNX-20 also reduced the core temperature and exerted anxiolytic effects. Taken together, centrally injected PNX-14 generates anxiolytic effects in mice, via the activation of the AHA GnRH system.

1. Introduction

Phoenixin (PNX), referred to herein as PNX-20 and PNX-14, are different molecular forms of PNX. They were first identified and isolated from the rat hypothalamus and bovine heart, respectively [1]. PNX has now been found to be highly conserved among multi-species, including human, rat, mouse, porcine and canine, and PNX-20 amide acids sequences differ in one amino acids among human, porcine and canine [1]. These peptides have similar biological activity and share a common C-terminal fourteen-amino acids segment that is essential for biological activity. PNX-14 is a C-terminally amidated neuropeptide and its amide acids sequence is DVQPPGLKVWSDPF-amide. Meanwhile, sequence of PNX-20 is AGIVQEDVQPPGLKVWSDPF-amide, an N-terminal extended six-amino acids peptide [2]. PNX is widely distributed in rat tissues, including the hypothalamus, heart, thymus, oesophagus, stomach, spleen and pituitary. Whereas, the tissue with the highest abundance is the hypothalamus [1]. Regrettably, the receptor of PNX has not been reported so far.

The most important biological function of PNX is reproductive action. We know that gonadotropin releasing hormone (GnRH) regulates reproductive system [3,4]. Recent researchers report that small interfering RNA of PNX in vivo could lead to the delayed appearance of oestrus and a reduction of the expression of GnRH receptor mRNA in the pituitary [1]. In addition, some researches

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suggest that PNX-14 is likely to modulate the expression of the GnRH receptor mRNA to regulate pituitary gonadotrophin secretion, which modulate reproductive processes [1,5].

Interestingly, a number of publications have demonstrated that GnRH may involve in brain responses to anxiety [6–8]. No data is yet available about action of PNX-14 on anxiety, and less is known about the direct effect of PNX-14 on the central nervous system [2]. Therefore, in this study, we investigated the central action of PNX-14 on the anxiety response and behavior, which is likely to be regulated by the hypothalamus where PNX is found in abundance [1].

2. Materials and methods

2.1. Animals

Male Kunming strain of Swiss mice was obtained from the Experimental Animal Center of Lanzhou University, China. Each mouse was used in the experiments only once. The animals were housed in cages (8 animals/cage) in a room maintained at 22 ± 2 °C and on a 12-h light-dark cycle with free access to tap water and standard laboratory food. All the protocols in this study were approved by the Ethics Committee of Lanzhou University, China.

2.2. Surgical procedure

Surgical implantation of cannula into the lateral ventricle or the bilateral anterior hypothalamic area (AHA) or the bilateral amygdala was conducted according to our previous report [9]. Each mouse (20–24 g) was anesthetized with sodium pentobarbital (Sigma-Aldrich Co., United States, at a dose of 70 mg/kg) and placed in a stereotaxic frame (Leica, Germany). Based on the atlas of Paxinos and Franklin (2001) [10], 8 mm 26-gauge stainless-steel guide cannulas, closed by stylets, were implanted over the lateral ventricle (0.5 mm posterior to bregma, 1.0 mm lateral to midline, 2.0 mm ventral to skull surface); or the bilateral anterior hypothalamic area (0.4 mm posterior to bregma, 0.4 mm lateral to midline, 4.0 mm ventral to skull surface); or the bilateral amygdala (1 mm posterior to bregma, 3.1 mm lateral to midline, 4.0 mm ventral to skull surface). After surgery, the mice were housed individually and allowed 5–7 days to recover from surgery before any administration. All experiments were carried out between 9:00 a.m. and 6:00 p.m.

At the end of the experiments, the correct position and the permeability of the cannula were checked. In the behavioral studies, each mouse was sacrificed by cervical dislocation, but before that methylene blue was injected via the implanted cannula. Data exhibiting the diffusion of methylene blue in the ventricles were analyzed in the statistical evaluation. However, the AHA and amygdala position were checked by histology. Briefly, whole brains were fixed in 4% paraformaldehyde overnight at 4 °C. Coronal sections (60 μm) were cut in a vibratome and stained with HE. Slides were observed under microscope to examine the cannula placements. A representative photomicrograph of a needle track terminating within the AHA (Fig. S5) or amygdala (Fig. S6).

2.3. Treatment

PNX-14 or PNX-20 was synthesized by a standard Fmoc-based solid-phase synthetic method, and the crude peptides obtained were purified to homogeneity with preparative HPLC. The purity of peptides was ascertained by analytical HPLC (Figs. S1 and S3), and the structure assignment was performed by ESI-TOF MS (Figs. S2 and S4). Purified PNX was dissolved in artificial CSF (aCSF) containing (in mM) 126.6 NaCl, 27.4 $NaHCO_3$, 2.4 KCl, 0.5 KH_2PO_4, 0.89 $CaCl_2$, 0.8 $MgCl_2$, 0.48 Na_2HPO_4, and 7.1 glucose, pH 7.4. Cetrorelix and Atosiban bought from sigma (Sigma-Aldrich Co., United States), was diluted with aCSF and infused into the lateral ventricle (2 μl; 1 μl/min) or the bilateral AHA or the bilateral Amygdala (0.5 μl, 0.25 μl/min).

Different doses of PNX-14 or aCSF were infused over a period of 2 min via two 10 μl Hamilton syringe mounted on a Microdrive pump (KD Scientific). All drugs and aCSF were injected using a 32-gauge stainless steel injector placed in and projecting 0.5 mm below the tip of the cannula. Infusion cannula remained in place for 1 min after infusion to allow for drug diffusion. Fifteen minutes after PNX-14 administration, the mouse was subjected to behavioral testing. In addition, the selection of the doses was based on the previous reports and our preliminary observations [2].

2.4. Elevated plus maze test

The elevated plus maze equipment consisting of two open arms and two enclosed arms is a test for the selective identification of anxiolytic and anxiogenic drug effects in mice. The maze is a plus-shaped platform which elevated 50 cm above the floor. A 60 W light bulb provide the illumination at a height of 80 cm. Each mouse were placed in the center of the maze facing toward either one of open arms, the number of entries into each arms, the time spent in per arm and the distance cover per arm were recorded for 5 min. The ratio of time spent in open arms to total time spent, the ratio of entries to open arms to total number of entries and the ratio of distance entries in open arms to total distance were presented in the figures. All experiments were conducted between 10:00 a.m. and 6 p.m. [11,12].

2.5. Open field test

The open field instrument is a an open field box which measured 60 cm × 60 cm and the center area of an open field box marked into 10 cm × 10 cm square. The standard source of illumination was a 60 W bulb at a height of 80 cm. In the open field test, novelty-induced horizontal locomotor activity was assessed during 30 min, but anxiolytic activity was assessed during 5 min. In this 5 min period, the observed parameters were the ratio of time spent in center area to total time, the ratio of distance entries in center area to total distance and the number of entries in center area. In addition, consistent with the elevated plus maze, each mouse was placed at the center area and the operator need sit approximately 1.5 m away from the apparatus in this period. The box was cleaned between each experiment with 96% ethanol absolute and all experiments were conducted between 10:00 a.m. and 6 p.m. [12,13].

2.6. Core temperature measurement

Different doses of PNX-14 (10, 25, 50, 100 nmol), PNX-20 (25 nmol) and aCSF were injected into the lateral ventricles in conscious mice, between 9:00 and 10:00 a.m., respectively. The mice had previously been fixed to the body temperature detection device. The temperature sensor was inserted into the rectum of mice for 2.5 cm. Physiological experimental system (BL-420E + type; TaiMeng, China) connected with sensor recorded the temperature of mice. Basal body temperature of each mouse was measured (once every 10 min, three times), and PNX or aCSF were injected to the lateral ventricles. Finally, the body temperature of each mouse was recorded at different time points in 10, 20, 30, 40, 50 and 60 min after drug treatment.