Hypothalamic action of phoenixin to control reproductive hormone secretion in females: importance of the orphan G protein-coupled receptor Gpr173

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We recently identified a novel peptide in hypothalamic extracts, named phoenixin, which appeared to exert significant effects on reproductive hormone secretion (22). Although expressed in several non-CNS tissues, the major site of production was determined to be the hypothalamus and, importantly, specific binding was demonstrated in hypothalamic, pituitary gland, and ovary. These findings suggested that phoenixin might exert actions at multiple levels of the hypothalamo-pituitary-gonadal (HPG) axis. Indeed, we demonstrated that phoenixin exposure enhanced gonadotropin-releasing hormone (GnRH)-stimulated luteinizing hormone (LH) release from rat anterior pituitary cells in culture and that the peptide increased the expression of the GnRH receptor and potentiated the upregulation of that receptor by GnRH itself (22). These actions may be a reflection of the physiological effect of phoenixin endogenously produced in the pituitary gland or an action of peptide of hypothalamic origin acting as a true neuroendocrine factor.

Phoenixin immunoreactivity was identified in multiple hypothalamic sites, including the paraventricular, supraoptic, periventricular, and ventromedial hypothalamic nuclei, as well as in the median eminence, and the anterior pituitary gland (22). We were unable to determine whether the phoenixin-like immunoreactivity in median eminence was limited to the internal or external layers, and, thus, could not verify a potential neuroendocrine action of the peptide. A physiologically relevant action of phoenixin to contribute to the control of HPG axis function was strongly suggested by our observation that compromise of phoenixin production in hypothalamus using small interfering RNA (siRNA) resulted in a significant interruption in estrous cyclicity (22). This may have reflected a loss of a direct hypothalamic site of action of the peptide or the loss of its neuroendocrine effect in the pituitary gland.

Here, we provide further evidence for an important role of brain-derived phoenixin in the control of reproductive function by demonstrating the peptide’s ability to act within brain to stimulate LH secretion. We have employed our “deductive ligand-receptor matching strategy” (21) to identify a candidate receptor for phoenixin. In addition, we describe hypothalamic sites of receptor expression related to reproductive function and demonstrate that siRNA-mediated knockdown of this receptor not only results (as with compromise of phoenixin production) in the delayed appearance of estrus in cycling female rats but also blocks the stimulatory effect of phoenixin on gonadotrophs.

MATERIALS AND METHODS

Animals and Surgery

Female Sprague Dawley outbred rats were obtained from Harlan/Envigo (Indianapolis, IN) weighing 200–250 g and housed under controlled environmental conditions (12:12-h light-dark cycle, lights on 0600, 22–24°C) with free access to lab chow and tap water. An indwelling stainless-steel cannula (23 gauge; 17 mm) was implanted into the right lateral cerebroventricle using a stereotaxic apparatus, as previously described (14). All animals were given a mixture of ketamine (60 mg/ml; Ketaset, Fort Dodge Animal Health, Fort Dodge, IA) and xylazine (8 mg/ml; TransquVed, VedCo, Saint Joseph, MO) anesthetic intraperitoneally at 0.1 ml/100 g body wt with subcutaneous buprenorphine SR-LAB algasle (1.0 mg/ml, 0.1 ml/100 g body wt; ZooPharm, Laramie, WY). Following intracerebroventricular can-
nulation, rats were housed individually and vaginal smears were taken daily (0800–1000) to verify the presence of a normal 4–5-day estrous cycle. All procedures and protocols were approved by the Saint Louis University Animal Care and Use Committee.

Reagents

Rat phoxin-20 amide and GnRH peptides were purchased from Phoenix Pharmaceuticals (Burlingame, CA). siRNA constructs were designed by and purchased from Integrated DNA Technologies (Corvalle, IA), targeting either Gpr173 or enhanced green fluorescent protein (eGFP) (Table 1).

Characterization of PNX Action in Rodent Estrous Cycle

Lateinizing hormone response to PNX in vivo. On the afternoon of estrus (after animals had returned to pre-intracerebroventricular implantation weights), an indwelling jugular catheter was implanted, as previously described (10). Between 0900 and 1000, rats were moved to a quiet testing room the following day after verification of the presence of a diestrous vaginal smear. An extension line (PE-50 tubing) was connected to the jugular catheter, and animals were left undisturbed for at least 30 min. Blood samples (0.25 ml) were withdrawn into heparinized syringes at 10 and 5 min, and immediately before intracerebroventricular administration of vehicle (0.9% wt/vol sterile NaCl, 2 l) or vehicle containing 1.0 or 3.0 nmol phoxin-20 amide (Phoenix Pharmaceuticals, Burlingame, CA). Additional blood samples were taken 5, 15, 30, and 60 min after the intracerebroventricular injections. Sterile, isotonic NaCl (0.25 ml) was administered intravenously following each blood sampling. Plasma was collected following centrifugation (4°C, 2000 g, 10 min) and stored frozen (−20°C) until determination of LH content by RIA. Cannula placement in the lateral ventricle was verified by a dipsogenic response to 25 pmol ANG II (Phoenix Pharmaceuticals). Plasma LH levels were measured using the National Hormone and Pituitary Program (Torrance, CA) RIA rat LH kit (rLH-RP-3 standard). The detection limit of LH level in plasma was 0.125 ng/ml, and the intra- and inter-assay variabilities were 5 and 7%, respectively.

In Vitro Knockdown of Gpr173

Pituitary cell dispersal and culture. Anterior pituitary glands, removed from random cycling adult, female Sprague-Dawley rats (Harlan/Envigo, Indianapolis, IN) following rapid decapitation, were harvested for mRNA expression analysis. Anterior pituitary glands containing 10 nM GnRH was added to the cultures, and the cells were incubated for 2 h. Then, medium was collected for determination of lateinizing hormone (LH) content by radioimmunoassay, and cells were harvested for mRNA expression analysis.

cDNA synthesis and quantitative RT-PCR. RNA was isolated from dispersed anterior pituitary cells, arcuate nucleus, medial basal hypothalamus, anteroventral periventricular nucleus, and anterior pituitary gland using a PureLink RNA isolation kit (Life Technologies/Fisher Scientific), according to the manufacturer’s instructions. First-strand complementary DNA (cDNA) was synthesized using iScript reverse transcription followed by real-time PCR using iQ SYBRGreen Master Mix and a Bio-Rad CFX96 Real-Time System (all Bio-Rad, Hercules, CA). The following primers were designed using PrimerQuest software developed by Integrated DNA Technologies (Corvalle, IA): HPRT-1: forward, 5'-AGTCCTCGTCTGTTAGTGATGAT-3' and reverse 5'-CTCGAGGGAGCTCTTTCAGTCG-3'; and Gpr173: forward 5'-CTGCGAGGCTGGTGGAAAG-3' and reverse 5'-TCTGAGGTCCTTTAAAAACCA-3'. Primer specificity was confirmed using PrimerBlaster (NCBI, Bethesda, MD). Changes in mRNA expression were calculated using the ΔΔCt method (16), and data were normalized to the housekeeping gene HPRT-1 (NM_012583.2).

Identification of Sites of GPR173 Expression

Perfusion and histology. Diestrous female rats (n = 5) were deeply anesthetized with pentobarbital sodium and perfused transcardially with 4% paraformaldehyde (PFA). Brains were dissected, postfixed for 4 h in 4% PFA, and cryoprotected overnight at 4°C in diethylylcarboxyanine (DEPC)-treated 0.1 M PBS pH 7.4, containing 20% sucrose. The brains were cut (30-μm sections) in the frontal plane in a freezing microtome. Five series were collected and stored at −20°C in cryoprotectant, until being processed for in situ hybridization to assess the distribution of GPR173 in the rat brain.

In situ hybridization histochemistry. Single label in situ hybridization histochemistry (ISHH) for Gpr173 mRNA was performed in series of hypothalamic sections, as previously described (7, 20). Briefly, tissue sections from diestrous female rats (n = 5) were mounted onto SuperFrost plus slides (Fisher Scientific), air-dried overnight, and fixed in 4% paraformaldehyde in DEPC-treated PBS for 20 min. Tissue was dehydrated in increasing concentrations of ethanol, cleared in xylol, rehydrated in decreasing concentrations of ethanol, and placed in prewarmed sodium citrate buffer, pH 6.0. Slides were microwaved for 10 min followed by dehybridization in graded ethanol. The Gpr173 riboprobe was produced from rat brain cDNA (reference gene for rGPR173: NM_022255.2). Primers were

Table 1. Small interfering RNA (siRNA) constructs

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PHOENIXIN CONTROLS ESTROUS CYCLICITY VIA INTERACTION WITH Gpr173

R491

designed to amplify 611 bp of the Gpr173 gene starting at position 49 bp. Final primers containing T3 and T7 promoter regions are as follows: T3_rGpr173: 5'-CAG AGA TGC AAT TAA CCC TCA CTA AAG GGA GAC TGC CAT CAG CAT CCG CTT A-3' and T7_rGpr173: 5'-CCA AGC CCT CTA ATA CGA CTC ACT ATA GGG AGA ACT GCC TTC ATC TTG CGG TG-3'. For generation of antisense 33P-labeled Gpr173 riboprobes, the ampiclon was subject to in vitro transcription with 33P-UTP and T7 polymerase, according to the manufacturer protocols (Promega, Madison, WI). The nucleotide mixture was then digested with DNAase and the labeled probe was purified and collected by using resin spin columns (GE Healthcare, New York, NY). The 33P-labeled Gpr173 riboprobe was diluted to 106 cpm/ml in a hybridization solution containing 50% formamide, 10 mM Tris-HCl (pH 8.0), 5 mg RNA (Invitrogen), 10 mM diethiothreitol (DTT), 10% dextran sulfate, 0.3 M NaCl, 1 mM EDTA, and 1X Denhardt's solution. Hybridization solution with the riboprobe was applied on each slide and incubated overnight, at 57°C. Slides were washed in 2× SSC (sodium chloride sodium citrate buffer) and treated with 0.02% RNase A (Roche) for 30 min. Sections were then rinsed in decreasing concentrations of SSC containing 0.25% DTT: 1 h in 2× SSC at 50°C, 1 h in 0.2× SSC at 55°C, and 1 h in 0.2× SSC at 60°C. Tissue was dehydrated in increasing concentrations of ethanol and slides were placed in X-ray film cassettes with BMR-2 film (Kodak) for 2 days.

In Vivo Compromise of Endogenous Gpr173

Measuring changes in estrous cyclicity. To verify the physiological importance of our top candidate phoenixin receptor in the hypothalamic control of reproductive hormone secretion, cycling female rats, implanted with lateral cerebroventricle cannulas, were administered 2 µg siRNA (121 pmol) targeting either eGFP of Gpr173 in 2 µl PBS on the afternoons of estrus and diestrus, followed by daily vaginal smears to determine the time interval before the appearance of the next estrous event.

Effect on PNX ability to stimulate LH secretion. Lateral cerebroventricular cannulas were implanted in a second group of adult female rats, and estrous cyclicity monitored by daily vaginal cytology. On the afternoon of estrus, animals were divided into two groups, those receiving 121 pmol siRNA targeting eGFP, as a control, or siRNA targeting the expression of our lead candidate phoenixin receptor, the orphan G protein-coupled receptor, Gpr173 (Table 1). A second intracisternal administration of siRNA was conducted on the afternoon of diestrus, at which time, an indwelling jugular catheter was implanted, as described above. On the following morning, diestrous day 2, animals were moved to a quiet room, extension lines attached to the jugular catheters, and animals were left undisturbed for at least 30 min. Then blood samples (0.25 ml) were removed via the jugular catheters at 10 and 5 min, and immediately undisturbed for at least 30 min. Then blood samples (0.25 ml) were removed via the jugular catheters at 10 and 5 min, and immediately followed by intracerebroventricular administration of 3.0 nmol phoenixin-20. Additional blood samples were taken 5, 15, 30, and 60 min later, and saline was administered intravenously to replace blood volume removed. Following the final blood sampling, animals were killed by rapid decapitation to facilitate collection of anterior pituitary gland, and the following hypothalamic regions: arcuate nucleus (Arc), medial basal hypothalamus (MBH), and anteroventral periventricular nucleus (AVPV). Tissues were harvested for RNA isolation to measure siRNA-induced changes in mRNA levels of Gpr173.

Statistical Analysis

All RIA results and changes to estrous cyclicity were analyzed by one-way ANOVA (between groups) with Scheffé’s multiple comparisons post hoc testing. Regression analysis was conducted examining the correlation between degree of mRNA compromise and LH response to intracisternal administration of phoenixin. Gene expression data from quantitative PCR are transformed and, therefore, analyzed using the Mann-Whitney U nonparametric test. All data are represented as means ± SE and considered statistically significant if \( P < 0.05 \).

RESULTS

Central Administration of Phoenixin Significantly Stimulated LH Secretion During Diestrus

Lateral cerebroventricular administration of phoenixin on the morning of diestrus resulted in increased plasma LH levels in conscious, unrestrained rats. Significantly higher plasma LH levels were detected at 5 and 15 min in animals receiving 3.0 nmol phoenixin compared with those receiving saline vehicle \( (P < 0.001) \). At 15 min, plasma LH levels in animals administered 3.0 nmol phoenixin significantly exceeded those present in rats receiving the 1.0-nmol dose \( (P < 0.05) \) (Fig. 1). Analysis of plasma LH levels over the 60-min sampling period following intracisternal injections revealed a dose-related stimulatory effect of intracisternal administration of phoenixin (Fig. 1, inset).

Reduction in Gpr173 Expression Inhibited Potentiation of GnRH-Induced LH Secretion by Phoenixin in Dispersed Anterior Pituitary Cultures

As expected, gonadotropin-releasing hormone (10 nM) treatment resulted in significant increases in LH release into the incubation medium in all pretreatment groups. There were no significant differences in the magnitude of the LH response to

Fig. 1. Central administration of phoenixin stimulated luteinizing hormone (LH) secretion. Rats bearing a lateral ventricle cannula were injected with saline vehicle \( (n = 8) \), 1.0 nmol phoenixin (PNX)-20 amide \( (n = 9) \), or 3.0 nmol PNX-20 amide \( (n = 6) \) following a 10-min preinjection plasma collection for baseline determination by RIA. Plasma was collected at 5, 15, 30, and 60 min postinjection. At 5 and 15 min, administration of 3.0 nmol PNX resulted in a significant rise in circulating LH levels. The inset depicts significant dose-dependent increase in total LH secretion over the entire 60-min testing period. Data were analyzed by ANOVA with Scheffé’s multiple-comparison test. *\( P < 0.05 \) and **\( P < 0.01 \) vs. vehicle-treated. †\( P < 0.05 \) vs. 1.0 nmol PNX-treated.
GnRH across the four groups (Fig. 2A). No significant effects of phoxinixin alone on LH release were observed across all four groups. In cells pretreated with Lipofectamine alone (i.e., no siRNA), pretreatment with phoxinixin prior to GnRH incubation resulted in significant potentiation of the LH response compared with that observed in response to GnRH alone (P < 0.001). Similarly, in cells pretreated with the control siRNA (eGFP targeting siRNA), phoxinixin significantly potentiated (P < 0.05) the LH response to GnRH. This potentiation, however, was not observed in cells pretreated with either 10 or 100 nM Gpr173 siRNA, in which there was 15% and 36% reduction in Gpr173 mRNA expression, respectively (Fig. 2B).

**Distribution of GPR173 mRNA in the Rat Forebrain**

Coronal sections of the rat brain extending from the medial septum to the caudal limits of the mammillary bodies were hybridized with Gpr173 antisense riboprobe. Brain sites containing low-density of neuronal soma, including the corpus callosum, optic tract, fornix, and anterior commissure, were used to define the background. Brain nuclei and areas showing similar density of silver grains (hybridization signal) to those four areas were defined as negative for Gpr173 expression. We observed different degrees of hybridization signal in all forebrain areas analyzed. Moderate-to-dense hybridization signal was found in the hippocampus, in the piriform cortex, in the lateral septum, in the bed nucleus of the stria terminalis, in the medial nucleus of the amygdala, and in the paraventricular nucleus of the thalamus. In the hypothalamus, dense expression of Gpr173 was observed in the anteroventral periventricular nucleus (AVPV), in the medial preoptic nucleus (MPO), in the paraventricular and supraoptic nuclei (PVH, SON), in the ventromedial nucleus (VMH), and in the compact formation of the dorsomedial nucleus of the hypothalamus (DMH). Moderate Gpr173 expression was also found in the medial preoptic area (MPA), in the arcuate nucleus (Arc), in the lateral hypothalamic area, and in the ventral prefrontal nucleus (PMV) (Fig. 3).

**Compromise of Endogenous Gpr173 Significantly Disrupted Estrous Cyclicity**

Animals pretreated on the afternoons of estrus and diestrous day 1 with siRNA targeting eGFP (controls) displayed normal estrous cyclicity (Fig. 4A). Conversely, those pretreated with siRNA targeting Gpr173 exhibited a significant delay in the appearance of the next estrus, a lengthening of the cycle that was significant (P < 0.001) vs. eGFP targeted animals (Fig. 4B).

**Knockdown of Centrally Derived Gpr173 Abrogated Phoenixin Induced LH Secretion**

Central administration of 3.0 nmol phoxinixin, on the morning of diestrous day 2, resulted in a significant increase in plasma LH levels in rats pretreated on the afternoons of estrus and diestrous day 1, with siRNA targeting eGFP (controls); however, no increase was observed in animals pretreated with siRNA targeting Gpr173 (Fig. 4C). A significant difference in plasma LH levels was observed between groups at the 15- and 30-min sampling intervals.

Pretreatment with siRNA targeted to Gpr173 once daily for 2 days resulted in a significant decrease (~29%) in Gpr173 mRNA expression in the arcuate nucleus compared with eGFP siRNA treated (Fig. 4D). However, no significant changes were detected in the expression of Gpr173 in the anterior pituitary, MBH, or AVPV. Regression analysis revealed a positive correlation between the extent of compromise of Gpr173 mRNA levels in the arcuate nucleus and the LH

![Fig. 2. Knockdown of Gpr173 abrogated the potentitative effect of phoxinixin on gonadotrophin-releasing hormone (GnRH)-stimulated LH release. Anterior pituitary glands from random cycling, female rats were dispersed 24 h prior to Lipofectamine transfection with siRNA targeting either Gpr173 or eGFP. Cells were incubated overnight in either DMEM alone or containing 1 μM PNX-20 amide, followed by a 1-h exposure to GnRH. A: knockdown of Gpr173 prevented the ability of phoxinixin to potentiante GnRH-stimulated LH secretion. (P < 0.05, **P < 0.01, ***P < 0.001; One-way ANOVA with Scheffe’s post hoc test). B: pretreatment with 100 nM Gpr173 siRNA resulted in ~39% decrease in Gpr173 mRNA expression relative to eGFP siRNA pretreatment (***P < 0.001 vs. vehicle, †P < 0.05 vs. eGFP siRNA, using Mann-Whitney U-test).](http://ajpregu.physiology.org/ by 10.22033/ajpregu.2017)
secretory response to intracerebroventricular administered phoenixin at 15 min ($Y = -0.1605X + 0.8377, P < 0.05$) and 30 min ($Y = -0.1085X + 0.6314, P = 0.066$) (Fig. 4E).

**DISCUSSION**

Compromise of hypothalamic phoenixin levels resulted in a significant disruption of the rat estrous cycle, suggesting that this peptide participates in the neuronal circuitry controlling GnRH release into the median eminence for delivery to the anterior pituitary gland or via a direct action on gonadotrophs in the anterior pituitary gland after delivery via the hypophyseal portal vessels (22). Here, we demonstrated that central administration of phoenixin to diestrous rats resulted in a significant dose-related increase in plasma LH levels, indicating a potential direct action within the hypothalamus. In addition, phoenixin potentiated GnRH-induced LH secretion from cultured, anterior pituitary cells in vitro (22).

Reproductive hormone secretion is regulated by complex feedback mechanisms that impact not only the gonadotrophs of the anterior pituitary gland, but also the neural networks controlling GnRH release. During diestrus, a period similar to the follicular phase of the human menstrual cycle, levels of circulating reproductive hormones (with the exception of progesterone) are low (4, 15). At this time low ambient levels of estrogen exert an inhibitory action on hypothalamic reproductive centers and pituitary gonadotrophic hormone production (1, 5). We hypothesize that ovarian hormone fluctuations might similarly affect the endogenous expression of phoenixin or its cognate receptor. Additionally, although it is clear from initial studies that phoenixin is required for normal ovarian function, the mechanisms that govern its production and release remain unknown, largely due to the elusiveness of its cognate receptor.

By implementing the “deductive ligand receptor-matching strategy” used to successfully deorphanize both the C-peptide and neuronostatin receptors (21, 23), we sought to identify the phoenixin receptor.

Phoenixin lacks homology to any known, secreted peptide and, therefore, is unlikely to bind a receptor with an already identified ligand. Additionally, during phoenixin’s initial purification and characterization, one of its pharmacologic effects was to increase cyclic AMP accumulation in a rat pituitary adenoma cell line, RC-4B/C (22). This suggested that the phoenixin receptor is an orphan GPCR, of which there are ~133 (2). Using our receptor matching strategy, we generated...
a GPCR expression profile in phoenixin-responsive cell lines. We then determined overlapping expression patterns of these orphan GPCRs (21). Of the candidates, Gpr173 was the only orphan receptor to exhibit an expression pattern that coincided with tissue sites where we had described specific phoenixin binding (22). Gpr173, also designated SREB3, belongs to the SREB subfamily of G protein-coupled receptors, which are highly conserved among vertebrate species and notably expressed in the brain and ovary (12).

We then sought to demonstrate a functional relationship between phoenixin and this candidate receptor. In dispersed anterior pituitary cells, Gpr173 was required for potentiation of GnRH-induced LH secretion by phoenixin. A limitation, however, of this approach was the use of a coding siRNA duplex (here directed against eGFP, a protein not produced in the rat) for a control to avoid untoward effects of compromise of any other mammalian protein expressed in the same cell cultures. We acknowledge that in the in vitro studies, we used the siRNA duplexes directed against eGFP at a lower concentration (in vitro, 10- and 100-fold) than the duplexes targeting GPR173, as directed by the manufacturer.

Like phoenixin, Gpr173 also was required for maintenance of reproductive function, since siRNA-induced compromise of hypothalamic Gpr173 expression in cycling female rats significantly disrupted estrous cyclicity. The benefit of using siRNA is the ability to transiently decrease the mRNA encoded by a gene of interest and then allow for recovery of mRNA levels and normal function, which in this case is reentrance into the estrous cycle.

Female rats receiving siRNA-targeted to Gpr173 exhibited a significant delay in the appearance of estrus compared with those given the control eGFP siRNA. Transition to estrus requires a surge in LH secretion known to be caused by increasing frequency and intensity of the pulsatile release of GnRH (3, 13). Exogenously administered phoenixin acted in brain to stimulate a preovulatory-like surge in LH secretion. Similarly, constant hormone exposure in male rats may interfere with any local action of phoenixin to interact with GnRH. Therefore, it is possible that Gpr173 acts merely as a coreceptor or is expressed downstream in the neural circuitry activated by phoenixin signaling. On the other hand, at least in pituitary cell cultures, it is likely that phoenixin interacts directly with Gpr173 since compromise of receptor expression prevented the action of phoenixin to potentiate GnRH stimulation of LH release. Current efforts are directed toward demonstrating a physical association between phoenixin and Gpr173. We have not located an antibody to GPR173 with suitable specificity for dual-labeling histochemistry or for immunoprecipitation assays and are currently attempting to establish physical association of phoenixin with Gpr173 by biotin transfer.

Gpr173 is highly expressed in areas related to reproductive control including the MPO, AVPV, Arc, VMH, and MeA. These areas have dense expression of estrogen receptor-α (17). Also, kisspeptin, an important player in reproductive function, is expressed in the AVPV and Arc (8, 18). It will be important in future studies to examine possible colocalization of Gpr173 with kisspeptin and/or GnRH neurons in hypothalamus and determine whether those neurons respond to direct administration of phoenixin, as determined by immediate early gene expression or changes in membrane potential. Additional, nonreproductive actions of phoenixin are suggested by the observation of Gpr173 expression in the dorsomedial and ventromedial hypothalamic nuclei perhaps more related to energy homeostasis (6, 11). Furthermore, distinct labeling in supraoptic and paraventricular nuclei point to possible actions of phoenixin on vasopressin and oxytocin secretion (9).

Female rats were used exclusively in these studies to further characterize phoenixin’s involvement in maintenance of estrous cyclicity, a model that was also conducive to identifying a physiological relationship with our candidate receptor Gpr173. Although male rats do exhibit similar phoenixin expression within the brain, phoenixin did not potentiate GnRH-stimulated LH secretion from cultures of dispersed pituitary cells harvested from male rats (22). It may be that ambient gonadal steroid levels influence the ability of phoenixin to interact with GnRH. Similarly, constant hormone exposure in male rats may interfere with any local action of phoenixin in the hypothalamus, an interaction that can only be observed during cyclic fluctuations in steroid levels, as is the case during the estrous cycle.

We hypothesize that brain-derived phoenixin acts within reproductive centers of the hypothalamus (and perhaps neighboring tissues). This would explain why compromise of phoenixin production (22) and Gpr173 mRNA levels at least in the arcuate nucleus (Fig. 4) results in disruption of estrous cyclicity. In our initial discovery paper (22), we detailed the presence of immunoreactive phoenixin in pituitary gland and now know that it is produced in cells (not yet identified phenotypically) of the anterior lobe. Abundant phoenixin immunoreactivity is present in nerve endings in the neural lobe (in all likelihood

Fig. 4. Compromise of endogenous Gpr173-disrupted estrous cyclicity and inhibited PNX stimulation of LH release. A and B: cycling female rats implanted with a lateral ventricle cannula were given two consecutive injections of 2 μl of saline containing 121 pmol (2 μg) of either Gpr173 siRNA or eGFP siRNA on the afternoons of estrus and diestrus day 1. Vaginal cytology was used to monitor progression through the estrous cycle. A: each bar indicates the phase of the estrous cycle for individual animals. Rats given Gpr173 siRNA (n = 6) exhibited a significant delay in the reappearance of estrus compared with eGFP siRNA-treated (n = 6). B: average estrous cycle duration was, therefore, significantly extended by the knockdown of Gpr173. (***P < 0.001 vs. eGFP siRNA-treated; one-way ANOVA) C and D: after consecutive intracerebroventricular injections (on estrus and diestrus day 1) of either Gpr173 siRNA (n = 9) or eGFP siRNA (n = 9), all rats were administered intracerebroventricularly 3.0 nmol PNX-20 amide. Plasma were collected over 60 min for quantification of circulating LH levels by RIA and tissues [anterior pituitary, arcuate nucleus, medial basal hypothalamus (MBH), and anteroventral periventricular nucleus (AVPV)] collected for RNA isolation. C: animals pretreated intracerebroventricularly with eGFP siRNA exhibited a significant phoenixin-induced rise in plasma LH at 15 and 30 min postinjection. However, those pretreated with Gpr173 siRNA did not exhibit the stimulatory effect of phoenixin on LH release. (⁎P < 0.05 vs. eGFP siRNA-treated; one-way ANOVA). D: central administration of Gpr173 siRNA resulted in a significant reduction in the levels of Gpr173 in the arcuate nucleus (⁎P < 0.05 vs. eGFP siRNA-treated; Mann-Whitney U-test). E: relationship of the degree of GPR173 mRNA reduction and the LH response to intracerebroventricularly administered phoenixin. LH values plotted are those present 15 or 30 min following phoenixin administration.
projecting from cell bodies in the supraoptic and paraventricular nuclei) that are located in some cases adjacent to the short portal vessels that communicate blood-borne factors between the two lobes. Although phoxin-in-like immunoreactivity is present in the median eminence, we have not been able to localize it to the external laminae and, thus, do not believe that phoxin-in of hypothalamic origin is entering the hypophyseal portal vessel to act as a classical neuroendocrine peptide.

Instead, we hypothesize that the second site of phoxin-in action within the HPG axis, the adenohypophysis, reflects the presence of Gpr173-expressing cells that are activated by peptide of local production or peptide initially delivered to the neurohypophysis.

**Perspectives and Significance**

Both the hypothalamic and the adenohypophyseal actions of phoxin-in require the participation of the orphan G protein-coupled receptor, Gpr173, which is endogenously expressed in those tissues. These studies further highlight the possibility that phoxin-in is a necessary participant in the central regulation of reproductive hormone secretion and cyclic ovarian function. Importantly, the identification of a candidate phoxin-in receptor, Gpr173, will invigorate future investigation of the importance of phoxin-in in major reproductive events, such as puberty and reproductive senescence, with the potential to identify a novel therapeutic target for the treatment of reproductive disorders, such as precocious or constitutional delay of puberty, as well as impaired fertility.

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**DISCLOSURES**

No conflicts of interest, financial or otherwise, are declared by the authors.

**AUTHOR CONTRIBUTIONS**


**REFERENCES**


