NEURAL REGENERATION RESEARCH
Volume 7, Issue 31, November 2012

www.nrronline.org

doi:10.3969/j.issn.1673-5374.2012.31.002 [http://www.crter.org/nrr-2012-qkquanwen.html]
Ren XX, Wang SJ, Rong PJ, Zhu B. Activation of hypothalamic gono-like neurons in female rats during estrus. Neural Regen Res. 2012;7(31):2413-2423.

Activation of hypothalamic gono-like neurons in female rats during estrus☆

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Abstract

In mammals, gonadal function is controlled by the activity of hypothalamic gonadotropin-releasing hormone neurons, which control the secretion of adenohypophyseal and gonadal hormones. However, there are a number of unanswered questions in relation to gonadal function. It is currently unknown how erotogenic stimulation of the genitals influences the subpopulation of hypothalamic medial preoptic area neurons, antidromically identified as projecting to the median eminence at different periods of the estrous cycle. Additionally, the distinctiveness of hypothalamic medial preoptic area neurons, with respect to methods of feedback control by exogenous hormones, is also unknown. In this study, spontaneous discharges from individual neurons encountered within the medial preoptic area, gono-like neurons, were recorded extracellularly using glass microelectrodes. To confirm the cellular and histochemical properties of the recording units, antidromic stimulation was performed using a side-by-side bipolar stimulating electrode placed into the median eminence, alongside microiontophoretic injections of the conventional tracer, horseradish peroxidase. In addition, further immunohistochemical analyses were performed. Results showed that elevated gono-neuron activity was accompanied by increased background activity and greater responses to erotogenic stimuli during estrus. Application of clitoral traction stimulation resulted in increased activation of the gono-like neurons. This neuronal activity was noticeably inhibited by β-estradiol administration. Immunohistochemical analyses revealed the presence of gonadotropin-releasing hormone-reactive protein in hypothalamic cells in which electrophysiological recordings were taken. Thus, medial preoptic area neurons represent the subset of hypothalamic gonadotropin-releasing hormone neurons described from brain slices *in vitro*, and might serve as a useful physiological model to form the basis of future *in vivo* studies.

Key Words

medial preoptic area; gonadotropin-releasing hormone; erotogenic stimuli; estrous cycle; neuronal discharge; feedback control; neural regeneration

Research Highlights

(1) Spontaneous discharges from individual neurons encountered within the medial preoptic area were recorded extracellularly using glass microelectrodes.
(2) Antidromic stimulation was performed using a side-by-side bipolar stimulating electrode placed into the median eminence. Individual neurons at the recording site in the medial preoptic area in female rats were found to be the subpopulation of gonadotropin-releasing hormone neurons.
(3) Spontaneous discharges were significantly increased in female rats during proestrus/estrus after sexual stimuli, and displayed characteristics of exogenous hormone feedback inhibition.

Abbreviations

mPOA, medial preoptic area; GnRH, gonadotropin-releasing hormone

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Received: 2012-08-20
Accepted: 2012-10-08
(N201120424003/WJ)

INTRODUCTION

The medial preoptic area (mPOA) of the hypothalamus is important for the integration and regulation of a variety of physiological functions and behaviors, including reproductive behavior. Stimulation of the mPOA in anesthetized rats evokes the urethrogenital reflex, vaginal vasocongestion, and increases intracavernosal pressure[1-2]. The mPOA is also critical for the initiation of sexual behavior in males, as males that receive mPOA lesions are unable to mount, intromit, or ejaculate[3]. Therefore, activation of the mPOA in both sexes induces important responses that are related to sexual behavior. Hypothalamic mPOA gonadotropin-releasing hormone (GnRH) neurons play essential roles in reproduction and the hypothalamo-pituitary-gonadal axis by forming the final common pathway in the central control of fertility. These neurons project to the median eminence, where GnRH is secreted in a pulsatile pattern to regulate the reproductive axis[4]. This pulsatile pattern is crucial for maintenance of fertility, as continuous GnRH signals suppress gonadotropin synthesis and release[5-6]. As is the case for other neurons, the discharge pattern of action potentials is important for GnRH neurons to function properly. Despite the obvious differences in ovarian and uterine structures and sexual behavior observed among mammals, in all species examined the estrous cycle appears to be regulated by gonadal hormone under the direct control of GnRH neurons. Augmentation of GnRH secretion has been identified as the initiating agent in pubertas praecox and estrus[7]. Damage to the mPOA disturbed GnRH secretion in female rats, which in turn caused the cessation of both estrus and preovulatory delivery of luteotropic hormone[8-9]. Among the many inputs that GnRH neurons receive, estrogens play the most important role, as they provide both negative and positive feedback. In females, estrogen influences the activity and output of GnRH neurons to generate the preovulatory luteinizing hormone surge and ovulation.

Approximately 20 years ago, a new technique to record electrical activity of the GnRH pulse generator was developed in a collaboration between the Kawakami and Knobil laboratories[10]. Striking increases were observed in the multiunit activity of the medial basal hypothalamus of ovariectomized rats and rhesus monkeys, which were under continuous anesthesia with thiopental during recording[10-11]. Each volley was coincident with the initiation of a luteinizing hormone pulse, suggesting the existence of an electrophysiological correlation between the pulsatile pattern of GnRH secretion and a certain neuronal oscillator. The authors hypothesized that this neuronal oscillator is the GnRH pulse generator, although its neuronal composition and supracellular organization remain undetermined. In the present study, we explored the effect of erotogenic stimulation of genital organs on the subpopulation of hypothalamic mPOA neurons corresponding to GnRH cells observed *in vitro*. In addition, we sought to characterize the distinctiveness of these neurons with respect to susceptibility to feedback control by exogenous hormones.

RESULTS

Quantitative analysis of experimental animals

A total of 49 female Sprague-Dawley rats were used in this study. Every morning of the experiment, a drop of female rat vaginal secretion diluted in saline was imbibed in the vaginal orifice, smeared on a slide, and stained with hematoxylin-eosin after exsiccation. The estrous cycle was classified on the basis of the cast-off cells present in the secretion. Rats were randomly assigned to two groups: proestrus/estrus group (n = 18) and metestrus/diestrus group (n = 31). All rats were included in the final analysis.

General properties of recorded neurons in the mPOA

A total of 928 hypothalamus neurons in the mPOA were recorded in 49 female rats, 743 of which did not respond to sexual stimulation. In the 185 responsive neurons, 120 displayed excitatory and 65 displayed inhibitory responses to erotogenic stimulation applied to the clitoris. The majority (> 90%) of units exhibited spontaneous activity. However, a certain number of silent cells progressively developed residual activity while their responses to stimulation of reproductive organs were being tested.

In the present study, we focused on the gono-like neurons, *i.e*. the units that responded to erotogenic input. A total of 120 erotogenic neurons were studied. Most of these neurons exhibited slow-firing rates (less than 4 Hz) and could generally be activated by antidromic stimuli applied to the region of the median eminence at a constant current of 50–80 μA, for a 0.1 ms duration, at 0.5 Hz. Cells displaying spontaneous activity were found either after long periods of recording or following testing of previous units for their responses to clitoral stimulation. The gono-like neurons exhibited a random firing pattern composed of a mixture of rapid bursts and irregular single spikes; there was a higher degree of variability in the firing rate in gono-like neurons because firing

occurred in episodes separated by quiescent periods. The firing patterns of gono-like neurons typically consisted of long or short periods of quiescence, brief firing bursts separated by a few seconds, and a mean firing rate of 0.3–4.0 Hz. Table 1 summarizes the cumulative results for responsiveness at different periods of the estrous cycle.

Table 1 Comparison of gono-neuron responsiveness to erotogenic stimuli in the different periods of the estrous cycle

Estrous phase	Total neurons (*n*)	Type of cells	Neurons (*n*)	Type (%)
Proestrus/estrus (*n* = 18)	93	Excitatory	72	77.42
		Inhibitory	21	22.58
Metestrus/diestrus (*n* = 31)	92	Excitatory	48	52.17
		Inhibitory	44	47.83

Data indicate the number (*n*) of various kinds of neurons. Type (%) indicates the proportion of the number (*n*) of type of cells and total neurons.

The background firing rate of gono-like neurons in the hypothalamus was also variable throughout the estrous cycle. The mean spontaneous firing rates were 1.81 ± 0.44 spikes/second (*n* = 45) and 0.96 ± 0.25 spikes/second (*n* = 39) in the periods of proestrus/estrus and metestrus/diestrus, respectively. Neuronal discharges were significantly higher in periods of proestrus/estrus compared with periods of metestrus/diestrus ($P < 0.01$).

Responses of gono-like neurons to erotogenic stimulation

Gono-like neurons were activated by both erotogenic (clitoral friction or vibrating baculum in the vagina and cervix) and dorsal clitoral nerve stimulation. In periods of proestrus/estrus, application of touch-tap stimulation on the clitoris for 30 seconds resulted in a phasic response prior to a short period of adaptation, which was followed by a tonic discharge lasting throughout the period of stimulation. A representative example from an individual excitatory neuron is shown in Figure 1. Application of a vibrating baculum to the vagina/cervix area caused gono-like neurons to exhibit a clear monotonic stimulus-response relationship. Electronic stimulation of 20 Hz, 0.5 ms, and 120 μA for a period of 30 seconds on the dorsal clitoral nerves resulted in high neuronal firing rates for the first few seconds, followed by sustained discharge throughout the period of stimulation (Figure 2).

Responses to the application of erotogenic irritation were quantitatively evaluated during the 30-second period of stimulation, summarized in Table 2. Significantly higher response *values* of neurons were obtained during the proestrus/estrus period *versus* the metestrus/ diestrus period ($P < 0.01$, $P < 0.05$; Table 2).

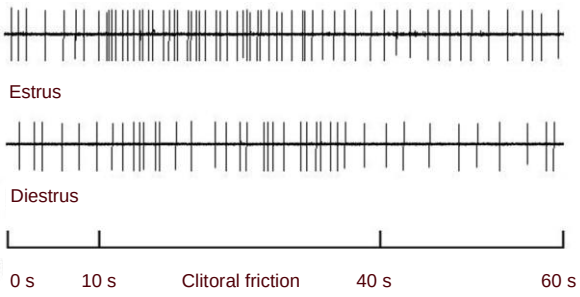

Figure 1 Spontaneous discharges of hypothalamic gono-neuron and responses to clitoral friction in estrus and diestrus.

In excitatory gono-neurons during estrus, higher background activity and increased responses to erotogenic stimuli relative to the corresponding values obtained during diestrus were observed. s: Second.

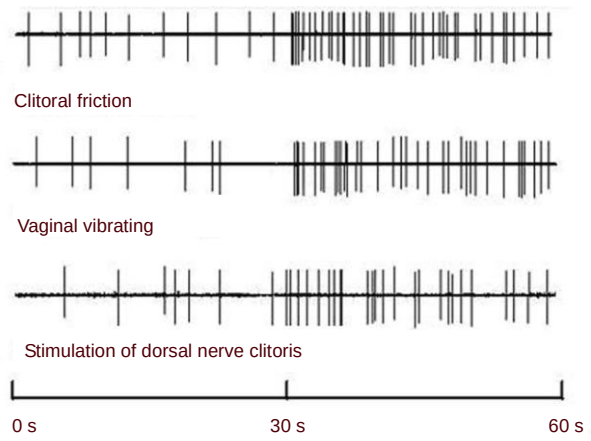

Figure 2 Responses of a representative excitatory gono-neuron to erotogenic stimulation.

Application of clitoral friction stimulation resulted in elevated activity of gono-neurons, whereas vaginal-cervical stimulation with vibrating baculum caused moderate activation, and electrical stimulation applied to the dorsal clitoral nerves resulted in marginal neuronal discharges. s: Second.

Interestingly, clitoral friction resulted in higher neuronal firing rates, especially during proestrus/estrus (Table 2). In each case, the mean number of spikes evoked by erotogenic stimuli was recorded at different phases of the estrous cycle. Differences between the responses from proestrus/estrus and those from metestrus/diestrus were significant ($P < 0.01$, $P < 0.05$). The majority of inhibitory gono-like neurons responded to only one type of erotogenic stimulation, and had higher spontaneous discharges compared with excitatory units ($P < 0.05$).

Table 2 Comparison of gono-neuron responses to three types of erotogenic stimuli at different phases of the estrous cycle

Stimulus	Estrous phase	Neurons (*n*)	Response values (spikes/s)	
			Background	Response
Clitoral friction	Proestrus/estrus	54	1.80±0.36	7.84±1.19[a]
	Metestrus/diestrus	66	0.94±0.15	3.81±0.32[a]
Vibrating baculum	Proestrus/estrus	29	1.97±0.31	4.85±0.71[a]
	Metestrus/diestrus	25	1.12±0.23	3.13±0.47[b]
Dorsal clitoral nerve	Proestrus/estrus	16	2.14±0.52	4.18±0.65[a]
	Metestrus/diestrus	13	1.28±0.47	3.22±0.39[b]

Data are expressed as mean ± SEM in each group. [a]$P < 0.01$, [b]$P < 0.05$, *vs*. background (one-way analysis of variance and Tukey HSD analysis).

Clitoral friction ($n = 65$) and vibrating baculum ($n = 38$) stimuli gave rise to a decreasing discharge rate and a rapid silencing effect, followed by a low rate of firing that lasted throughout the period of stimulation (from 3.29 ± 0.69 to 0.95 ± 0.21 spikes/second for clitoral friction, and from 3.12 ± 0.73 to 1.79 ± 0.58 spikes/ second for the vibrating baculum, $P < 0.01$). These responses were followed, in several cases, by long-lasting inhibition; this effect was particularly clear when the vibrating baculum was used, but was also noticeable with clitoral friction.

Negative feedback certification of gono-like neurons by hormones

In the experiments testing hormone-induced negative feedback of gono-like neurons, neurons exhibiting high spontaneous activity were selected for observation. If erotogenic stimulation induced excitatory or inhibitory responses higher than 100%, negative feedback effects were assessed by intravenously administrating three exogenous hormones; β-estradiol (30–50 ng/kg), testosterone (8 mg/kg), and hydrocortisone (20 mg/kg), in a random sequence.The majority of excitatory gono-like neurons (27/33) ceased firing upon administration of estrogen (Table 3). Figure 4 shows an example in which β-estradiol treatment inhibited neuronal firing rates for approximately 5–10 minutes after infusion of the hormone. During the administration of β-estradiol, a mean inhibition of 2.05 ± 0.36 to 0.97 ± 0.22 spikes/second and a mean inhibitory rate of 45.81% were observed (Table 3; $P < 0.01$). Among the other six gono-like neurons, four showed no response and two exhibited marginally increased firing rates when estrogen was administered. The animals of these six units were in proestrus, which is characterized by high luteinizing hormone secretion. The majority of inhibitory gono-like neurons also suppressed firing upon administration of estrogen (Table 3). Gono-like neurons of female rats, whether excitatory (Figure 4) or down-firing, also induced inhibitory discharges during and after administration of testosterone. For the whole population of cells, testosterone was effective, but elicited less suppression (1.68 ± 0.28 to 1.03 ± 0.31 spikes/second), and had a lower inhibitory rate (27.25%, $P < 0.05$; Table 3) compared with β-estradiol. In contrast, gono-like neurons did not respond to hydrocortisone. Figure 4 and Table 3 show a representative example. The combined results of the whole population showed that corticosteroid was completely ineffective. GnRH immunohistochemistry revealed that the site of recorded neurons detected by electrophysiology was consistent with the site of GnRH-immunoreactive responses in brain slices (Figure 4).

DISCUSSION

Identification of hypothalamic gono-like neurons in physiological and endocrinological assays

Attempts to elucidate the *in vivo* physiological properties of GnRH neurons using electrophysiological approaches are difficult because of the diffuse distribution pattern and paucity of this neuronal subtype (600–2 500 in the mammalian brain) in the hypothalamus-preoptic area[12-13]. To overcome this obstruction, GnRH neurons in mice and rats were genetically labeled by promoter-driven transgenic manipulations so that GnRH neurons could be visualized for identification before electrical recording.

This approach has been highly successful in mice and rats[3, 14-18]. Nevertheless, these observations were all performed in brain slices *in vitro*, and the properties of GnRH neurons in physiological conditions remain obscure. In the present experiments, we recorded and characterized the activities of a subpopulation of hypothalamic neurons, some of which were influenced (185/928, 19.94%) by erotogenic stimulation of the genital organs, and were controlled by negative feedback with exogenous estrogenic hormones. These neurons exhibited unique characteristics of gono-like neurons, or those classified as GnRH units *in vitro*[15-16].

Table 3 Gono-like neuron responses to exogenous hormones

Hormone	Type	Neurons (*n*)	Response values (spikes/s)		Inhibition (%)
			Background	After spikes	
β-estradiol	Excitatory	33	2.05±0.36	0.97±0.22[a]	45.81
	Inhibitory	15	1.28±0.21	0.43±0.11[a]	67.36
Testosterone	Excitatory	13	1.68±0.28	1.03±0.31[b]	27.25
	Inhibitory	11	1.59±0.40	1.11±0.37[b]	38.58
Hydrocortisone	Excitatory	13	1.24±0.14	1.09±0.15	8.70

Data are expressed as mean ± SEM. [a]$P < 0.01$, [b]$P < 0.05$, *vs*. background using one-way analysis of variance and Tukey HSD analysis.

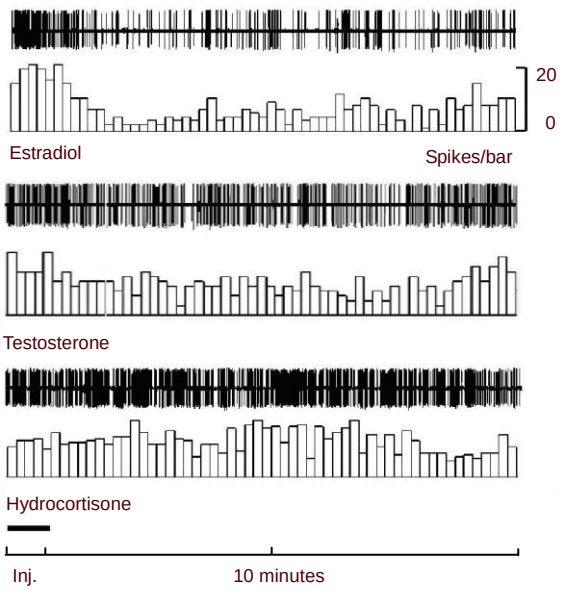

Figure 3 Gono-like neuron responses to exogenous hormone administration.

Gono-like neuron firing was inhibited by β-estradiol, partially suppressed by testosterone, and not affected by hydrocortisone administrations. Inj.: International injection of hormore.

The current understanding of the putative mechanisms linking neuronal activity and GnRH release is primarily based on extracellularly recorded multiple unit electrical activity in the arcuate region of the medial basal hypothalamus[19-20]. Multiple unit activity is an electrophysiological correlate of the GnRH pulse generator, and is characterized by episodes of spike activity associated with luteinizing hormone release. The episodic multiple unit firings are separated by protracted intervals of relative quiescence, corresponding to the interpulse interval of serum luteinizing hormone levels. The intermittent profile of multiple unit activity is also reminiscent of the secretory pattern of GnRH[3, 21]. Previous studies of GnRH neurons have primarily relied upon either immortalized transformed GnRH cell lines (GT1-7 cells)[22-23] or cultured embryonic GnRH neurons[24]. In the present study, we observed that a subpopulation of hypothalamic neurons (approximately 20%) were especially responsive to erotogenic inputs. Mallick and colleagues observed mPOA neurons in male rats that responded to stimulation of the dorsal penile nerve with increased firing (excitatory in our delineation)[25].

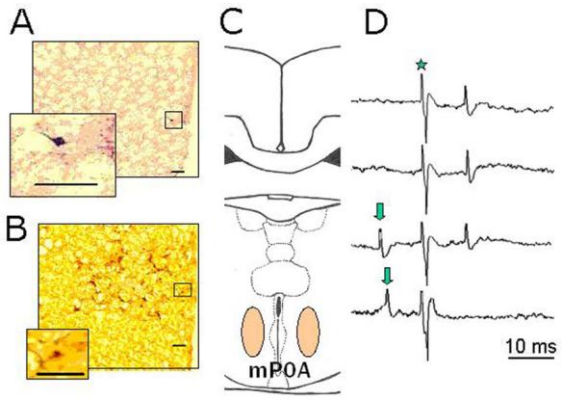

Figure 4 A labeled horseradish peroxidase (HRP) and GnRH immunohistochemical positive hypothalamic gono-like neuron in the electrophysiological recording site.

(A) Labeled HRP-positive hypothalamic gono-neuron. Photomicrograph of the horizontal section of the hypothalamic neuron showed an intense and homogeneous distribution of HRP within the cell body.

(B) Photomicrograph of GnRH immunohistochemical staining in another slice from the same area. GnRH-immunoreactive product is abundant in the hypothalamic unit. The insets show magnification of the HRP-positive and GnRH-positive neurons. Scale bars in A and B: 50 μm.

(C) The shaded region in the schematic coronal section in (B) illustrates the approximate location of mPOA neurons satisfying the identification criteria.

(D) Extracellular electrophysiological recordings from a spontaneously active mPOA neuron that was antidromically activated from the median eminence with a constant onset latency of 9 ms (top two traces). A spontaneous action potential (arrow) of the mPOA neuron triggered a stimulated spike (stimulus artifact), and the antidromic spike occurred after the refractory period of the cell elicited a constant antidromic action potential (third trace).The antidromic spike occurred within the refractory period of the cell cancels out the antidromic potential of the cell (bottom trace).

GnRH: Gonadotropin-releasing hormone; mPOA: medial preoptic area.

This subpopulation of hypothalamic cells was inhibited by exogenous estrogenic hormone *via* negative feedback control, whereas corticosteroid was wholly ineffective. It is possible that these defined units are indeed gono-like neurons, equivalent to the GnRH cells recorded *in vitro*. Nunemaker *et al* [26] examined the negative feedback effect of estrogen on the firing activity of green fluorescent protein-labeled GnRH-1 neurons by comparing ovariectomized mice, bearing an estradiol capsule for 1 week, with ovariectomized controls. Results indicated that estradiol-stimulated firing frequency altered the episodic rhythm (low-frequency rhythm) by increasing the duration of the quiescence period in a subset (50%) of GnRH-1 neurons. The episodic rhythm altered by estrogen appears to represent the negative feedback effect in mouse GnRH-1 neurons[26]. Some researchers observed that estradiol infusion rapidly increased firing frequency, spikes per burst, and burst duration of primate GnRH-1 neurons[1]. Kimura *et al* [27] found that the administration of estrogen to ovariectomized rats decreased the frequency of multiunit activity volleys.

Evidence suggests that estrogen-receptive neurons participate in the generation of reproductive behaviors and convey the estrogen message to GnRH neurons. Estrogen exerts its effects in the brain by binding to and activating two members of the nuclear receptor family, estrogen receptor (ER-α and ER-β). Estrogen is an important feedback regulator for GnRH release. Luteinizing hormone pulses and volleys of hypothalamic electrical activity, two markers of GnRH secretion, and GnRH release itself are each modulated by estradiol[4, 28-33]. Debate continues, however, over the sites and mechanisms of estradiol action on GnRH release. Specifically, it is unclear whether estradiol acts directly on GnRH neurons or through estrogen-sensitive afferents. Historically, no evidence for estrogen accumulation[34] or receptor expression[35-36] has been found in GnRH neurons, suggesting that estradiol feedback is conveyed to GnRH neurons *via* estrogen-sensitive afferents[37]. Recent studies, using more sensitive methods, indicate that GnRH neurons express ERβ, indicating that direct action is also possible[38-39]. Both direct and transsynaptic mechanisms may cooperate in the regulation of GnRH release. These findings reveal that certain subpopulations of estrogen- receptive neurons in hypothalamic areas known to regulate reproductive behavior and GnRH neurons are glutamatergic in the female rat. Thus, the estrogen signal could be propagated through glutamate neurons to distant sites, and influence the activity of postsynaptic neurons[40].

Hypothalamic gono-like neuron activity and the estrous cycle

Although it has been assumed that synchronized firing of GnRH neurons is necessary for pulsatile GnRH secretion, no clear evidence exists for this hypothesis. In the present experiments, hypothalamic gono-like neurons changed their spontaneous firing patterns throughout the estrous cycle, such that significantly more spontaneous discharges were observed during proestrus/estrus compared with metestrus/diestrus. Both the number of responsive gono-like neurons in single animals and the percentage of excitatory gono-like neurons were greater, and the number of inhibitory cells was smaller during proestrus/estrus compared with metestrus/diestrus. In studies of multiple unit activity recorded from the hypothalamus, GnRH neuronal activities were consistent with synchronistic and pulsatile discharges from multiple cells, the direct consequence of which was the pulsatile release of GnRH, which controls the function of the estrous cycle. An episodic increase in multiple unit activity recorded from the medial basal hypothalamus correlates with pulsatile luteinizing hormone release[19, 41-42], and has been proposed to reflect activity of luteinizing hormone releasing hormone-1 neurons (LHRH-1). *In vitro* calcium imaging studies have indicated that LHRH-1 neurons appear to possess an intrinsic pulse-generating mechanism[43-44], because they exhibit spontaneous intracellular calcium oscillations, which synchronize at a frequency similar to LHRH release[45-47]. Nonetheless, the electrophysiological properties of LHRH-1 neurons responsible for pulsatile LHRH-1 release remain elusive. Funabashi and colleagues recorded simultaneous spontaneous action potentials from multiple cells using immortalized GnRH neurons cultured on a multi-electrode dish. The spontaneous discharges of GnRH cells resembled bursts composed of 5–10 action potentials that were recorded almost simultaneously from several microelectrodes, suggesting synchronized electrical activity of GnRH cells. Periodic bursts were completely and reversibly blocked by 1–5 μM tetrodotoxin, indicating that voltage-dependent Na^+ channels were involved in their generation. Although the precise mechanism of synchronized burst patterns requires clarification, gap junction communication among GnRH cells is at least partially involved[48]. Tetel *et al* [49] observed that stimulation of the vagina and cervix, provided by the male during copulation or manually with a probe, causes many behavioral and endocrine changes associated with female reproduction. Moreover, vaginal-cervical stimulation from mating or manual probing increases the expression of Fos immunoreactivity in discrete populations of neurons in

the preoptic area, mediobasal hypothalamus, and midbrain in rats, suggesting that these neurons respond to this type of stimulation[49]. Steroid hormones may elicit some of their effects on female reproductive behavior and physiology by altering the responsiveness of ventromedial nucleus neurons of the hypothalamus to vaginal-cervical stimulation[50]. Sexual stimulation (copulation with intromission or vaginal-cervical stimulation) induces c-fos mRNA and Fos-like immunoreactivity within GnRH neurons of the female rat preoptic area[51]. Estrogen and progesterone can augment the responsiveness of certain GnRH neurons to vaginal-cervical stimulation, consistent with the effects of sexual activity on GnRH release[52].

Hypothalamic gono-like neurons *in vivo* and GnRH neurons *in vitro*

In our observations, the great majority of gono-like neurons exhibited spontaneous activity and a random firing pattern composed of a mixture of rapid bursts and irregular single spikes. Additionally, the firing rate was highly variable. The firing patterns of gono-like neurons typically consisted of long or short periods of quiescence, brief firing bursts separated by a few seconds, and a mean firing rate of 0.5–3.0 Hz. This frequency is similar to that described *in vitro* in transgenic mouse GnRH-1 neurons[13, 53], cultured GnRH-1 neurons from mouse olfactory placodes[24], and in primate GnRH neurons. Extracellular recording studies revealed mean firing rates of 7.18 ± 7.91 Hz in mPOA units that responded to dorsal penile nerve stimulation in male rats[25]. Nunemaker and Moenter [16, 54] also showed that mouse GnRH-1 neurons exhibit a firing pattern consisting of bursts (trains of multiple action currents) occurring at less than 20-second intervals, clusters (trains of multiple bursts) that occur at 6–8 minute intervals, and episodes (trains of multiple clusters) that occur at 20–30 minute intervals. The authors categorized burst, cluster, and episodes as high, intermediate, and low-frequency rhythms, respectively. The firing patterns of GnRH neurons in brain slices *in vitro* are similar to those observed in the gono-like neurons in our *in vivo* experiments with female rats. In addition, histological and immunohistochemical studies showed an intense and homogeneous distribution of horseradish peroxidase reaction product and abundant GnRH-immunoreactive product in the electrophysiologically recorded hypothalamic unit, influenced by erotogenic stimulation of the genital organs and controlled by negative feedback with exogenous estrogenic hormones. Thus, this group of neurons likely represent the subset of hypothalamic GnRH neurons described in brain slices *in vitro*.

The electrophysiological method used in these experiments can be applied to observe further physiological and pathological characteristics and changes, and the response to some interventions (such as drugs or acupuncture) of this subset of hypothalamic GnRH neurons *in vivo*.

Moreover, the studies presented here may serve as a useful physiological model of this hypothalamic subpopulation in future *in vivo* studies.

MATERIALS AND METHODS

Design

A randomized, controlled, animal experiment.

Time and setting

Experiments were performed at the Physiology Laboratory of Institute of Acupuncture and Moxibustion, China Academy of Chinese Medical Sciences, Beijing, China, from May 2005 to May 2006.

Materials

A total of 49 healthy, 9–11 weeks old, virgin female Sprague-Dawley rats, weighing 240–280 g and exhibiting normal estrous cycles, were provided by the Institute of Genetics and Developmental Biology, Chinese Academy of Sciences, China (license No. SCSK-2002-0016). All procedures were performed in accordance with the *Guidance Suggestions for the Care and Use of Laboratory Animals*, formulated by the Ministry of Science and Technology of China[55].

Methods

Surgical preparation

Rats were initially anesthetized with an intraperitoneal injection of urethane (1.0–1.2 g/kg; Duxin-Fine Preparation Factor, Beijing, China). Rats were tracheotomized to prevent aspiration of saliva and, when required, to perform artificial ventilation. Catheters were inserted into the left carotid artery and the jugular vein for blood pressure monitoring and drug injection, respectively. Heart rate and blood pressure were continuously monitored and body temperature was maintained around 37°C by means of a feedback-controlled homeothermic blanket. The animal was placed in a stereotaxic head frame (KOPF-900; Tokyo, Japan); the skull was fixed and leveled between bregma and lambda. A craniotomy was performed to remove bone overlying the cortex, and this allowed electrodes to be lowered into the hypothalamus and covered with liquid

paraffin. The locations of the mPOA of the hypothalamic region and the median eminence were defined based on the brain atlas of Paxinos and Watson[56]. The stereotaxic coordinates for the mPOA and median eminence were 0.5–1.8 mm posterior to bregma, 0.4–0.9 mm lateral to the midline, 7.0–8.0 mm below the skull surface and 2.5–3.0 mm posterior to bregma, at the midline, 9.5–10.0 mm below the skull surface, respectively.

Unit recordings

Spontaneous discharges from individual neurons encountered within the mPOA were recorded extracellularly using glass microelectrodes filled with a solution of 1.0 M NaCl and pontamine sky blue (tip impedance 10–20 MΩ). To certify the cellular and histochemical properties of the recording units, in the last studied neuron of 13 experimental animals, recording micropipette electrodes with 1.5 mm glass capillaries (impedance 20–40 MΩ) filled with 4% horseradish peroxidase, 0.5 M KCl in 0.05 M Tris buffer, and pH 7.25 at 25°C, were used to deposit horseradish peroxidase at the recording site with the following iontophoresis parameters: 50 nA (positive) pulses (150 ms duration) at 4 Hz for 5 minutes. This procedure allowed further immunohistochemical analysis of labeled neurons. The cortex was covered with agar gel to minimize the movements caused by respiration. The isolated action potentials were fed into a window discriminator and displayed on an oscilloscope screen (VC-10, Nihon Kohden, Tokyo, Japan). The outputs of the window discriminator and amplifier were fed into a data collection system and a personal computer data acquisition system (Power Lab) to compile peristimulus time histograms or wavemark files for further analysis. The spontaneous firing rate of mPOA neurons was continuously monitored for 5 to 10 minutes before and after stimulation. As there was often moment-to-moment fluctuation in mPOA neuronal excitability that was associated with a change in background activity, impulse counts in association with a stimulation procedure were compared with background activity for the 30-second period immediately before the stimulation took place (“net response”). If the impulse counts during stimulation were 20% more or less than the background activity, the neuron was considered to have excitatory or inhibitory responses, respectively.

To identify mPOA neurons with axonal projections to the median eminence, antidromic stimulation was performed using a side-by-side bipolar stimulating electrode placed into the median eminence. Antidromic activation of mPOA neurons was performed by applying square-wave current pulses (0.5 ms) at 1 Hz with varying amplitude (initially 0.6 mA) to determine threshold intensity. When a gono-like neuron displayed a constant onset latency during antidromic stimulation, additional tests were performed to confirm its antidromic nature as previously described[57]. The unit responses of mPOA cells to stimulation of the median eminence were subjected to the following criteria to identify tested neurons as tuberoinfundibular neurons in the hypothalamus-adenohypophyseal system: (1) a constant latency of the antidromic spike, (2) ability to follow high frequency stimulation at approximately 200 Hz at a 1:1 ratio, and (3) collision or cancellation of the stimulus-evoked spike by a spontaneous action potential. For extremely short latency responses (< 5 ms), high frequency stimulation could not always be demonstrated because of interference from the stimulus artifact. Collision of stimulus-evoked spikes with spontaneous action potentials was not performed in a subset of neurons because of a lack of spontaneous activity[57]. All gono-like neurons that responded to erotogenic stimulation in the present study satisfied at least two of the criteria.

Reproductive organ stimulus

In all experiments, manual touch-tap stimulation on the clitoris was performed with a lubricated cotton ball for 30 seconds. Vaginal-cervical stimulation was manually performed with a vibrating baculum probe (6 Hz) with copulation-like intromission and vellication to all female rats. In 11 female rats, the dorsal clitoral nerves were exposed bilaterally and mounted on a pair of electrodes. Electrical stimulation was delivered by a constant-current stimulator (SEM-7203, Nihon Kohden, Tokyo, Japan) and consisted of unipolar square waves of 0.5 ms duration at an intensity of 100–200 μA for 30 seconds at 20 Hz.

Experimental procedures

Unit electrical activity of neurons at the recording mPOA site induced by sexual stimuli

When cell activity was isolated from background noise, a standard protocol was employed to determine the effects of different sexual stimulations. If sexual stimulation induced excitatory or inhibitory responses of the defined population of hypothalamic neurons greater than 100% of the unstimulated level, a feedback intervention test was performed using intravenous infusion of β-estradiol (30–50 ng/kg; Sigma, St. Louis, MO, USA), testosterone (8 mg/kg, AcroSeal, NJ, USA), or hydrocortisone (20 mg/kg; GuoYao, Shanghai, China). Neurons that met the following criteria were identified as genital-related

neurons in the hypothalamus: (1) stable and responsive activation (including excitatory or inhibitory) upon stimulation of the genital organs of receptive female rats by clitoral friction, vibrating baculum in the vagina, and cervix or dorsal clitoral nerve irritation; (2) feedback inhibition by intravenous infusion of estradiol or testosterone, but not hydrocortisone. Consequently, these neurons were identified as hypothalamo-hypophyseal neurosecretory cells.

Histological and immunohistochemical verification of recording sites

To assess the histochemical properties of neurons that were characterized electrophysiologically, microiontophoretic injections of horseradish peroxidase and subsequent immunohistochemical analysis were performed in 27 recorded cells from 13 animals. This method labeled one, two, or at most three neurons (and/or occasionally glia) at physiological recording sites *in vivo*. Because the labeled cell types cannot be known with certainty a priori, and physiological response properties cannot be ascribed to particular cells, physiological response properties at the labeling site could only be inferred. Animals surviving for 3 hours after injection of horseradish peroxidase were deeply anesthetized with 10% urethane, and a thoracotomy was quickly conducted and intubation was performed from the left ventricle to the aorta. The animal was then perfused with 50–150 mL 0.9% NaCl (until the liver whitened), followed by 500 mL of 4°C 1.0% paraformaldehyde, 1.25% glutaraldehyde in 0.1 M PBS, at pH 7.4, rapidly at first and then slowly, for no longer than 30 minutes. The brainstem with attached cerebellum was quickly removed and stored overnight in 0.1 M PBS containing 30% sucrose. To locate positive neurons, 40 μm sections were cut on a cryotome. Sections were treated with horseradish peroxidase according to the tetramethylbenzidine-sodium tungstate protocol and mounted on neutral gum-coated slides.

Every other slice was counterstained for GnRH using the ABC method following the manufacturer's instructions (PK-6101, Vector Laboratories, Burlingame, USA). Briefly, brain sections were fixed with 4% paraformaldehyde in PBS (pH 7.4) for 25 minutes, and then washed three times in a mixture of Tris-buffered saline and Tween-20 (TBST) (20 mM Tris-HCl buffer, pH 7.4, 150 mM NaCl, 0.1% Tween-20) followed by quenching in 2% H_2O_2 in TBST for 30 minutes. Sections were subsequently blocked with 2% bovine serum albumin and 10% normal goat serum in TBST for 1 hour, then incubated overnight at 4°C with rabbit anti-phospho-GnRH antibody (#AB1567, Chemicon American) at 1:2 000. The next day, sections were treated for 2 hours with the biotinylated anti-rabbit antibody at 1:200, followed by 30 minute incubation with avidin-biotin-horseradish peroxidase complex (PK-6101, Vector Laboratories). Staining was visualized using 2,3'-diaminobenzidine tetrahydrochloride (DAB, 0.5 mg/mL in PBS containing 0.1% H_2O_2). To confirm the specificity of each antibody, several sections were concurrently processed without primary antibodies. All sections were examined under bright field microscopy. Photomicrographs were taken with a digital photomicroscope (Olympus, Tokyo, Japan).

Statistical analysis

Data were expressed as mean ± SEM. Homogeneity of variances, one-way analysis of variance, and Turkey HSD tests were performed to determine significant differences between values (SPSS 13.0 software, SPSS, Chicago, IL, USA). $P < 0.05$ was considered statistically significant.

Funding: This work was supported by the National Basic Research Program of China (973 Program) No. 2011CB505200, 2012CB518503 and grants from the National Natural Science Foundation of China, No. 30371804.

Author contributions: Peijing Rong and Bing Zhu designed the study and were responsible for obtaining approval from the Animal Care Committee of Institute of Acupuncture and Moxibustion, China Academy of Chinese Medical Sciences. Peijing Rong and Xiaoxuan Ren contributed to the protocol setting and manuscript preparation. Xiaoxuan Ren and Shaojun Wang participated in data collection, and prepared the figures for the manuscript.

Conflicts of interest: None declared.

Ethical approval: The study was approved by the Animal Care Committee of Institute of Acupuncture and Moxibustion, China Academy of Chinese Medical Sciences.

Author statements: The manuscript is original, has not been submitted to or is not under consideration by another publication, has not been previously published in any language or any form, including electronic, and contains no disclosure of confidential information or authorship/patent application disputations.

REFERENCES

[1] Giuliano F, Allard J, Compagne S, et al. Vaginal physiological changes in a model of sexual arousal in anesthetized rats. Am J Physiol. 2001;281(1):R140- 149.

[2] Marson L. Lesions of the periaqueductal gray block the medial preoptic area-induced activation of the urethrogenital reflex in male rats. Neurosci Lett. 2004; 367(3):278-282.
[3] Meisel RL, Sachs BD. The Physiology of Male Sexual In: The Physiology of Reproduction. 2nd ed. New York: Raven Press. 1994.
[4] Moenter SM, Caraty A, Locatelli A, et al. Pattern of gonadotropin-releasing hormone (GnRH) secretion leading up to ovulation in the ewe: existence of a preovulatory GnRH surge. Endocrinology. 1991;129(3): 1175-1182.
[5] Kanasaki H, Purwana I, Oride A, et al. Extracellular signal-regulated kinase (ERK) activation and mitogen-activated protein kinase phosphatase 1 induction by pulsatile gonadotropin-releasing hormone in pituitary Gonadotrophs. J Signal Transduct. 2012;2012:198527.
[6] Ciccone NA, Xu S, Lacza CT, et al. Frequency-dependent regulation of follicle-stimulating hormone beta by pulsatile gonadotropin-releasing hormone is mediated by functional antagonism of bZIP transcription factors. Mol Cell Biol. 2010;30(4):1028-1040.
[7] Perera AD, Plant TM. Ultrastructural studies of neuronal correlates of the pubertal reaugmentation of hypothalamic gonadotropin-releasing hormone (GnRH) release in the rhesus monkey (Macaca mulatta). J Comp Neurol. 1997; 385(1):71-82.
[8] Ronnekleiv OK, Kelly MJ. Luteinizing hormone-releasing hormone neuronal system during the estrous cycle of the female rat: effects of surgically induced persistent estrus. Neuroendocrinology. 1986;43(5):564-576.
[9] Van der Beek EM, Horvath TL, Wiegant VM, et al. Evidence for a direct neuronal pathway from the suprachiasmatic nucleus to the gonadotropin-releasing hormone system: combined tracing and light and electron microscopic immunocytochemical studies. J Comp Neurol. 1997;384(4):569-579.
[10] Hotchkiss J, Knobil E. The Menstrual Cycle and its Neuroendocrine Control. In: The Physiology of Reproduction. 2nd ed. New York: Raven Press. 1994.
[11] Kawakami M, Uemura T, Hayashi RF. Electro-physiological correlates of pulsatile gonadotropin release in rats. Neuroendocrinology. 1982;35(1):63-67.
[12] Parhar IS. Cell migration and evolutionary significance of GnRH subtypes. Prog Brain Res. 2002;141:3-17.
[13] Suter KJ, Song WJ, Sampson TL, et al. Genetic targeting of green fluorescent protein to gonadotropin-releasing hormone neurons: characterization of whole-cell electrophysiological properties and morphology. Endocrinology. 2000;141(1):412-419.
[14] Christian CA, Mobley JL, Moenter SM. Diurnal and estradiol-dependent changes in gonadotropin-releasing hormone neuron firing activity. Proc Natl Acad Sci USA. 2005;102(43):15682-15687.
[15] DeFazio RA, Moenter SM. Estradiol feedback alters potassium currents and firing properties of gonadotropin-releasing hormone neurons. Mol Endocrinol. 2002;16(10): 2255-2265.
[16] Nunemaker CS, Straume M, DeFazio RA, et al. Gonadotropinreleasing hormone neurons generate interacting rhythms in multiple time domains. Endocrinology. 2003;144(3):823-831.
[17] Nunemaker CS, DeFazio RA, Moenter SM. A targeted extracellular approach for recording long-term firing patterns of excitable cells: a practical guide. Biol Proced Online. 2003;5(1):53-62.
[18] Suter KJ, Wuarin JP, Smith BN, et al. Whole-cell recordings from preoptic/hypothalamic slices reveal burst firing in gonadotropin-releasing hormone neurons identified with green fluorescent protein in transgenic mice. Endocrinology. 2000;141(10):3731-3736.
[19] Tanaka T, Ozawa T, Hoshino K, et al. Changes in the gonadotropinreleasing hormone pulse generator activity during the estrous cycle in the goat. Neuroendocrinology. 1995;62(6):553-561.
[20] Hiruma H, Nishihara M, Kimura F. Hypothalamic electrical activity that relates to the pulsatile release of luteinizing hormone exhibits diurnal variation in ovariectomized rats. Brain Res. 1992;582(1):119-122.
[21] Caraty A, Locatell A. Effect of time after castration on secretion of LHRH and LH in the ram. J Reprod Fertil. 1988;82(1):263-269.
[22] Van Goor F, Krsmanovic LZ, Catt KJ, et al. Control of action potential-driven calcium influx in GT1 neurons by the activation status of sodium and calcium channels. Mol Endocrinol. 1999;13(4):587-603.
[23] Costantin JL, Charles AC. Spontaneous action potentials initiate rhythmic intercellular calcium waves in immortalized hypothalamic (GT1–1) neurons. J Neurophysiol. 1999;82(1):429-436.
[24] Kusano K, Gueshko S, Gainer H, et al. Electrical and synaptic properties of embryonic luteinizing hormone-releasing hormone neurons in explant cultures. Proc Natl Acad Sci U S A. 1995;92(9):3918-3922.
[25] Mallick HN, Manchanda SK, Kumar VM. Sensory modulation of the medial preoptic area neuronal activity by dorsal penile nerve stimulation in rats. J Urol. 1994; 151(3):759-762.
[26] Nunemaker CS, DeFazio RA, Moenter SM. Estradiol-sensitive afferents modulate long-term episodic firing patterns of GnRH neurons. Endocrinology. 2002; 143(6):2284-2292.
[27] Kimura F, Jinnai K, Sano A. LHRH pulse generator is stimulated by naloxone in the pentobarbital-blocked proestrous rat. J Neuroendocrinol. 1995;7(12):917-922.
[28] Zhang C, Kelly MJ, Rønnekleiv OK. 17 β-estradiol rapidly increases ATP-sensitive potassium channel activity in gonadotropin-releasing hormone neurons [corrected] via a protein kinase signaling pathway. Endocrinology. 2010; 151(9):4477-4484.
[29] Goodman RL, Gibson M, Skinner DC, et al. Neuroendocrine control of pulsatile GnRH secretion during the ovarian cycle: evidence from the ewe. Reprod Suppl. 2002;59:41-56.

[30] Wang Y, Garro M, Kuehl-Kovarik MC. Estradiol attenuates multiple tetrodotoxin-sensitive sodium currents in isolated gonadotropin-releasing hormone neurons. Brain Res. 2010;23(1345):137-145.
[31] Mori Y, Nishihara M, Tanaka T, et al. Chronic recording of electrophysiological manifestation of the hypothalamic gonadotropin-releasing hormone pulse generator activity in the goat. Neuroendocrinology. 1991;53(4):392-395.
[32] Arreguin-Arevalo JA, Ashley RL, Wagenmaker ER, et al. Membrane-initiated actions of estradiol (E2) in the regulation of LH secretion in ovariectomized (OVX) ewes. Reprod Biol Endocrinol. 2010;8:40.
[33] Kato A, Hiruma H, Kimura F. Acute estradiol modulation of electrical activity of the LHRH pulse generator in the ovariectomized rat: restoration by naloxone. Neuroendocrinology. 1994;59(5):426-431.
[34] Shivers BD, Harlan RE, Morrell JI, et al. Absence of oestradiol concentration in cell nuclei of LHRH-immunoreactive neurones. Nature. 1983;304 (5924):345-347.
[35] Herbison AE, Theodosis DT. Localization of oestrogen receptors in preoptic neurons containing neurotensin but not tyrosine hydroxylase, cholecystokinin or luteinizing hormone-releasing hormone in the male and female rat. Neuroscience. 1992;50(2):283-298.
[36] Lehman MN, Karsch FJ. Do gonadotropin-releasing hormone, tyrosine hydroxylase-, and ß-endorphin-immunoreactive neurons contain estrogen receptors? A double-label immunocytochemical study in the Suffolk ewe. Endocrinology. 1993;133(2):887-895.
[37] Herbison AE. Multimodal influence of estrogen upon gonadotropin-releasing hormone neurons. Endocr Rev. 1998;19(3):302-330.
[38] Skynner MJ, Sim JA, Herbison AE. Detection of estrogen receptor α and β messenger ribonucleic acids in adult gonadotropin-releasing hormone neurons. Endocrinology. 1999;140(11):5195-5201.
[39] Hrabovszky E, Steinhauser A, Barabas K, et al. Estrogen receptor-ß immunoreactivity in luteinizing hormone-releasing hormone neurons of the rat brain. Endocrinology. 2001;142(7):3261-3264.
[40] Eyigor O, Lin W, Jennes L. Identification of neurones in the female rat hypothalamus that express oestrogen receptor-alpha and vesicular glutamate transporter-2. J Neuroendocrinol. 2004;16(1):26-31.
[41] Wilson RC, Kesner JS, Kaufman JM, et al. Central electrophysiologic correlates of pulsatile luteinizing hormone secretion in the rhesus monkey. Neuroendocrinology. 1984;39(3):256-260.
[42] Nishihara M, Takeuchi Y, Tanaka T, et al. Electrophysiological correlates of pulsatile and surge gonadotrophin secretion. Rev Reprod. 1999;4(2):110-116.
[43] Terasawa E. Luteinizing hormone-releasing hormone (LHRH) neurons: mechanism of pulsatile LHRH release. Vitam Horm. 2001;63:91-129.
[44] Terasawa E. Pulse generation in LHRH neurons. In: Handa RJ, Hayashi S, Terasawa E, et al. Neuroplasticity, development, and steroid hormone action. Boca Raton: CRC Press. 2002.
[45] Moore JP Jr, Shang E, Wray S. In situ GABAergic modulation of synchronous gonadotropin releasing hormone-1 neuronal activity. J Neurosci. 2002;22(20): 8932-8941.
[46] Terasawa E, Keen KL, Mogi K, et al. Pulsatile release of luteinizing hormone-releasing hormone (LHRH) in cultured LHRH neurons derived from the embryonic olfactory placode of the rhesus monkey. Endocrinology. 1999;140(3):1432-1441.
[47] Terasawa E, Schanhofer WK, Keen KL, et al. Intracellular Ca2+ oscillations in luteinizing hormone-releasing hormone neurons derived from the embryonic olfactory placode of the rhesus monkey. J Neurosci. 1999;19(14): 5898-5909.
[48] Funabashi T, Suyama K, Uemura T, et al. Immortalized gonadotropin-releasing hormone neurons (GT1–7 cells) exhibit synchronous bursts of action potentials. Neuroendocrinology. 2001;73(3):157-165.
[49] Tetel MJ, Getzinger MJ, Blaustein JD. Fos expression in the rat brain following vaginal-cervical stimulation by mating and manual probing. J Neuroendocrinol. 1993;5(4): 397-404.
[50] Tetel MJ, Getzinger MJ, Blaustein JD. Estradiol and progesterone influence the response of ventromedial hypothalamic neurons to tactile stimuli associated with female reproduction. Brain Res. 1994;646(2):267-272.
[51] Pfaus JG, Jakob A, Kleopoulos SP, et al. Sexual stimulation induces Fos immunoreactivity within GnRH neurons of the female rat preoptic area: interaction with steroid hormones. Neuroendocrinology. 1994;60(3): 283-290.
[52] Pfaus JG, Marcangione C, Smith WJ, et al. Differential induction of Fos in the female rat brain following different amounts of vaginocervical stimulation: modulation by steroid hormones. Brain Res. 1996;741(1-2):314-330.
[53] Spergel DJ, Kruth U, Hanley DF, et al. GABA- and glutamate-activated channels in green fluorescent protein-tagged gonadotropin-releasing hormone neurons in transgenic mice. J Neurosci. 1999;19(6):2037-2050.
[54] Moenter SM, DeFazio RA, Pitts GR, et al. Mechanisms underlying episodic gonadotropin-releasing hormone secretion. Front Neuroendocrinol. 2003;24(2):79-93.
[55] The Ministry of Science and Technology of the People's Republic of China. Guidance Suggestions for the Care and Use of Laboratory Animals. 2006-09-30.
[56] Paxinos G, Watson C. The Rat Brain in Stereotaxic Coordinates. 6th ed. San Diego: Elsevier Academic Press. 2005.
[57] Lipski J. Antidromic activation of neurones as an analytic tool in the study of the central nervous system. J Neurosci Meth. 1981;4(1):1-32.

(Edited by Xu WM, Li HF/Yang Y/Song LP)