Endocrine Journal 2004, **51** (2), 171–176

Effects of GABA and Bicuculline on the Electrical Activity of Rat Olfactory Placode Neurons Derived at E13.5 and Cultured for 1 Week on Multi-electrode Dishes

KUMIKO SUYAMA, SHIGEO DAIKOKU, TOSHIYA FUNABASHI AND FUKUKO KIMURA

Department of Neuroendocrinology, Yokohama City University Graduate School of Medicine, 3-9 Fukuura, Kanazawa-ku, Yokohama 236-0004, Japan

Abstract. The present study was performed to record the electrical activity of olfactory placode neurons and to check the effect of GABA and bicuculline on it. Olfactory placodes obtained at day 13.5 of gestation were cultured for 1 week on multi-electrode dishes. Olfactory placode neurons showed spontaneous firing, with firing rates of 0.77 ± 0.05 Hz (0.03–3.82 Hz, n = 12), but there was no bursting activity. Perfusion with 10 μM GABA almost immediately inhibited 8 of 11 firing activities (we could not test it in 1 activity). In contrast, perfusion with 10 μM bicuculline induced facilitation in 5 of 12 activities and did not induce any change in 7 other activities. Statistical analysis by χ^2-test showed a significant difference in the response of neurons to the two drugs. Fisher's exact probability test showed that the inhibitory effect of GABA was significant ($p<0.05$) whereas neither the facilitatory effect nor the lack of effect of bicuculline was significant ($p>0.1$). These results suggest that cultured olfactory placode neurons, even in a probably immature stage, respond to GABA with inhibition, as generally observed at mature stages.

Key words: Rat olfactory placode, Cultures, MED, Extracellular recording, GABA, Bicuculline

(*Endocrine Journal* **51**: 171–176, 2004)

WE have shown that cultured rat embryonic olfactory placodes, which contain a variety of cell types such as olfactory receptor neurons (for review, see [1]), pheromone receptor neurons (for review, see [2]) and GABA neurons [3–5], secrete GnRH into the medium in a pulsatile fashion with a mean interpulse interval of approximately 30 min [5, 6]. The GnRH secretion from cultured olfactory placodes decreased during infusion of 20 μM GABA, but did not change during infusion of 20 μM bicuculline, a $GABA_A$ receptor antagonist [5].

On the other hand, we recorded electrical activity of immortalized GnRH neurons (GT1-7 cells) cultured on a multi-electrode dish (MED), which measures action potentials by extracellular recording [7]. GT1-7 cells exhibited bursts composed of 5–7 action potentials. Similar bursting activity was found in acutely dissociated GnRH neurons genetically targeted with fluorescent protein [8]. The burst occurred intermittently at intervals of approximately 13.3 sec [7] and 1–10 sec [8], respectively. Although these studies did not detect intermittent increases of the bursting activity that may be associated with pulsatile GnRH secretion, a study that measured extracellular potentials of GT1-7 neurons showed episodes of increased firing rate with an interval of about 25 min [9], which is very similar to the interval of GnRH secretion produced by GT1-7 neurons as well as that produced by cultured olfactory placode neurons [5, 6, 10–12].

In the electrophysiological studies, further, it has been an interesting notion that GABA acts as an excitatory neurotransmitter during early development and thus plays an excitatory role in both GT1-1 and GT1-7 cells [7, 13, 14] as well as in GnRH neurons in organotypic cultures of embryonic olfactory placode [15]. The stimulatory effect of GABA in GT1 cells was

Received: October 2, 2003
Accepted: January 16, 2004
Correspondence to: Dr. Fukuko KIMURA, Department of Neuroendocrinology, Yokohama City University Graduate School of Medicine, 3-9 Fukuura, Kanazawa-ku, Yokohama 236-0004, Japan

confirmed in the measurement of GnRH secreted [16, 17].

Therefore, we became interested in recording the electrical activity of olfactory placode neurons cultured in an MED system and in checking whether there were intermittent increases in the electrical activity if recorded. The effect of GABA and bicuculline on the activity was also checked.

Materials and Methods

Collection and Culture of Olfactory Placodes

Wistar strain rats (Charles River, Yokohama, Japan) were reared in our animal facilities under controlled environmental conditions (room temperature of 22 ± 1°C and lights on from 5.00 to 19.00 h). All animal housing and surgical procedures were in accordance with guiding principles for the Care and Use of Animals by the Physiological Society of Japan and approved by the Animal Care and Use Committee of the Yokohama City University Graduate School of Medical Sciences.

Embryos were obtained at 13.5 days of gestation (E13.5) after killing the parent animal by cervical dislocation without anesthesia, and kept in 4°C Gey's buffer saturated with O_2. Embryo heads were resected into a flat plastic dish filled with Gey's buffer at 4°C. After removing the brain, the head was dissected sagittally through each of the nasal passages taking care not to damage their medial walls, which contain developing vomeronasal organs, using a pair of ophthalmic scissors under a dissecting microscope. Finally, the nasal septum was sectioned medially, so that two medial placodes were obtained from one embryo as reported elsewhere [6].

The medial placodes were further trimmed to preserve the growth of the vomeronasal organ and the nasal fiber bundles; the placodal tissue plates were cut along the rostral, ventral, and caudal borders of the vomeronasal organ and the dorsal portion of the plate was cut beneath the cribriform plate. The trimmed tissue plates were a trapezoids with a broad dorsal base. A pair of olfactory placode tissues from an animal were transferred to a MED (Panasonic, Kyoto, Japan). The transplants were embedded into chick plasma (Equitec-bio, Ingram, TX, USA) clot coagulated with thrombin (Int. Reagents, Co., Kobe, Japan). After 25 min, the tissues were covered with culture medium and kept in petri dishes stored in an incubator (37°C, 5% CO_2, humidified). The culture medium was DMEM/F12 (GIBCO BRL, Rockville, MD, USA) and was supplemented with 5% fetal bovine serum (GIBCO BRL), 100-units/ml penicillin, and 100-μg/ml streptomycin. The culture medium was changed every 3–4 days.

Recording

Electrical activity of cultured olfactory placode neurons was recorded about a week after the start of culture by means of an extracellular recording system with MEDs. The system has been described in detail elsewhere [7, 18]. Cells on the MED were perfused with the culture medium at a flow rate of 1 ml/min at 37°C. After recording for more than 20 min, GABA (Sigma Chemical Co., St. Louis, Mo., USA) or bicuculline (bicuculline methiodide, Sigma Chemical Co.), each at a 1 : 1000 dilution in saline to make a 10 μM concentration, was added to the medium by bath perfusion for a period of 20 min. It took about 2 min for the drug to reach the MED after the start of perfusion, as indicated in Fig. 1. A recovery period of more or less than 20 min, depending on the individual culture, was allowed after cessation of GABA or bicuculline perfusion. Recording was continued also throughout the recovery period. Both GABA and bicuculline were administered in a solution of physiological saline.

Data Analysis

When the extracellular potential exceeded a preset threshold value of 50% greater than noise, it was counted as a single spike [9]. The number of spikes recorded in a computer was counted once every min. Changes in the firing rate greater than 30% of that in the preceding period were regarded as significant [19, 20]. The data were statistically analyzed by χ^2-test and Fisher's exact probability test, and differences were considered to be significant at $p<0.05$.

Results

We started a total of 64 cultures for recording electrical activity on the MED and attempted recording 1 week after the start of each culture. We succeeded in

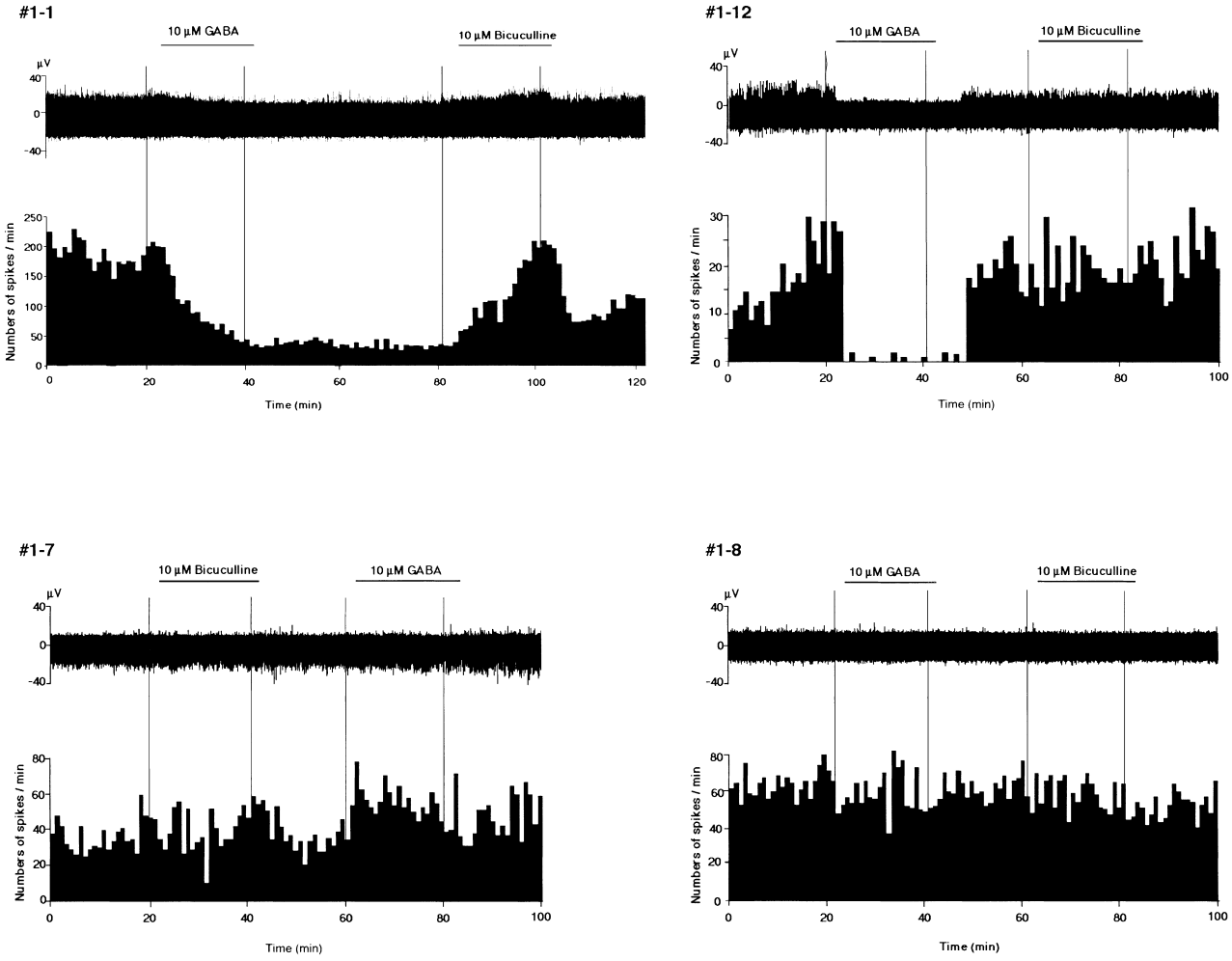

Fig. 1. Examples of the effect of perfusion (horizontal bars) of GABA and bicuculline on the spontaneous electrical activity of olfactory placode neurons in one-week cultures. The number of the electrical activity corresponds with the number in Table 1. The start and end of the drug perfusion are shown by vertical lines. It took about 2 min for the drug to reach the MED after the start of perfusion so that the horizontal line showing the perfusion starts after 2 min. For further details, see text.

making recordings from only 19 one-week cultures, and were eventually able to check drug effects in 10 cultures. Although each MED contained 64 microelectrodes, we were able to record extracellular electrical activity from only 1 electrode in each of 8 cultures, and from 2 electrodes in the remaining 2 one-week cultures. In other words, in the majority of cultures only a single electrode gave us cell activity, showing that this study was extremely consumptive of time and effort.

Examples of the spontaneous firing pattern of cultured olfactory placode neurons and of the effects of GABA and bicuculline are illustrated in Fig. 1. Olfactory placode neurons demonstrated spontaneous action potentials with mean spontaneous firing rates of 0.77 ± 0.05 Hz (0.03–3.82 Hz, n = 12), which were calculated for electrical activity during the control periods; mean firing rates were first calculated for individual activities and then for all 12 activities. We could not find any sign of bursting activity with intervals of approximately 10 sec such as seen in GT1-7 cells [7] and GnRH neurons [8] in either the control periods or in the testing periods to set every recording conditions just after starting the recording experiments. An attempt was also made to find an episodic increase with intervals of approximately 25 min, such as seen in GnRH neurons [9], in the firing activity in the control periods. This was done by calculating the mean firing rate + 2σ (population standard deviation) value for each activity in the 20-min control period and the testing periods

Table 1. Effects of perfusion of GABA or bicuculline on the spontaneous firing rate

Treatments	Effects	Electrical activity												Total
		#1–1*	#1–2	#1–3	#1–4	#1–5	#1–6	#1–7*	#1–8*	#1–9	#1–10	#1–11	#1-12*	n
10 μM GABA**	Facilitation							+						1
	Inhibition	+	+	+	+	+				+		+	+	8***
	No response						+		+					2
10 μM Bicuculline	Facilitation	+		+						+	+	+		5
	Inhibition													0
	No response		+		+	+	+	+	+				+	7

Effects of drugs are shown by + at the site corresponding to facilitation, inhibition, or no response.
Effects of drugs in neurons shown with * are illustrated in Fig. 1.
** indicates a statistical significance by χ^2-test ($p<0.01$).
*** indicates a statistical significance by Fisher's exact probability test ($p<0.05$).

and checking whether there was any firing with rate greater than this value; but we found no such activity in any of the control or testing periods, indicating that these olfactory placode neurons cultured for 1 week did not fire episodically.

The effects of the drug on the 12 activities are summarized in Table 1. Perfusion with 10 μM GABA almost immediately inhibited firing in 8 out of 11 activities (in #1–10, we could not test the effect of GABA); it took about 2 min for the drug to reach the MED and the effect occurred almost instantly after its arrival. The return of the firing to control level took more than 10 min after the cessation of GABA perfusion, and even more than 40 min in #1–1 (Fig. 1). GABA increased the firing in only 1 activity (#1–7), and in 2 activities (#1–6 and #1–8), it did not cause any change at all. In contrast, perfusion with 10 μM bicuculline induced facilitation in 5 of 12 activities, and no change in 7 activities. Statistical analysis by χ^2-test showed a significant difference in the response of neurons between 10 μM GABA and 10 μM bicuculline treatment ($p<0.01$). Fisher's exact probability test sowed that the inhibitory effect of GABA was significant ($p<0.05$) but that neither facilitatory effect nor the lack of effect of bicuculline was significant ($p>0.1$).

Discussion

Olfactory placode neurons had spontaneous firing activity with firing rates ranging from 0.03 to 3.82 Hz (0.77 ± 0.05 Hz). These frequencies were almost similar to those for mouse GnRH neurons; in GnRH neurons derived from 11.5-day-old mouse embryos and cultured for 1–2 weeks, the frequencies of spontaneous firing rate were smaller than 4 Hz [15], and in single GnRH neurons derived from 15–20-day-old mice, the frequencies were 0.02–1.0 Hz (0.4 ± 0.1 Hz) [21, 22]. Although we did not determine in the present study whether the recorded neurons were GnRH neurons or not, it is likely that each activity was recorded from 1 or at most 2 neurons.

Contrary to our expectation, in the present study, the spontaneous firing of these olfactory placode neurons did not generate spontaneous bursts of activity at intervals of about 10 sec. In earlier studies, spontaneous, intermittent bursting at intervals 1–10 sec was observed in the electrical activity of GnRH neurons which were dissociated acutely after being obtained from pubertal female mice [8], and in GT1-7 neurons [7]. Episodic increases that could be associated with pulsatile GnRH secretion also could not be detected. The reason for the lack of bursting activity at intervals of 1–10 sec or episodic increase at intervals of 25 min is unclear, but it is possible either that the neurons were too immature to show such activity or that none of the neurons from which we recorded were GnRH neurons.

Eventhough, the maturation of GnRH neurons has been discussed in relation with their response to GABA, and thus the response of electrical activity to GABA may indicate the maturation of neurons. Han *et al.* [23] observed that effects of GABA on the mouse GnRH neurons switched from depolarization to hyperpolarization around postnatal 31 days, i.e., at puberty, suggesting that the time of maturation is correlated with developmental conditions. It is generally thought that GABA hyperpolarizes adult rat brain

neurons, while depolarizes them during the early neonatal period [24]. GnRH neurons derived from E10.5-11.5 mouse olfactory placode and cultured for 6 days showed depolarization (= excitatory = increase in firing) [4, 15]. In the present study, which employed E13.5 rat olfactory placodes cultured for 7 days, GABA exhibited inhibitory effects by decreasing spike firing in most neurons (8 out of 11 activities, 73%), except in one case where increased firing (= depolarization) was seen. It is possible that the GABA effect on the rat olfactory placode neurons changes somewhere around 20.5 (= 13.5 + 7 for rats in our study) days, at least in *in vitro*. However, recently a contradictory report was published by DeFazio *et al.* [25]. They reported that GABA depolarized (= excited) all GFP-tagged mouse GnRH neurons regardless of their developmental status, due to high contents of Cl ions in these cells. The discrepancy between the data of Han *et al.* [23] and the data of DeFazio *et al.* [25] has not yet been solved. In the present study, we used rat olfactory placodes. There may be a difference between the two animal species regarding the effect of GABA on CNS neurons at different developmental stages.

In the present study, the perfusion of 10 μM bicuculline induced facilitation in 5 of 12 activities and did not cause any change in 7 other activities, without any significance for facilitation or the lack of effect. This phenomenon may be related, first, to the possible existence of $GABA_B$ receptors, in addition to $GABA_A$ receptors, on cultured olfactory placode neurons, although in immature GnRH neurons only $GABA_A$ receptor subtypes have been shown to be expressed [21, 26, 27]. Second, the concentration of bicuculline was probably too small and a larger concentration might have significant effects. However, the concentration of 10 μM has been employed in most electrophysiological studies on juvenile GnRH neurons, GT1-7 neurons, and also other neonatal CNS neurons, although the inhibition of firing activity by bicuculline was observed only in GT1-7 neurons [7] and hippocampal neurons during the first postnatal week [24] and the effect on juvenile GnRH neurons was not tested [21]. It was for this reason that we considered it appropriate to employ this concentration in our studies concerning developmental changes in GABA actions on neurons.

In view of the possibility that the GABA effects observed in the present study were due to the embryonic olfactory placode neurons derived at E13.5 and cultured for 1 week being just in transition from an immature to a mature stage, the insignificance of the facilitatory effect of 10 μM bicuculline is not unreasonable. It is probable that the facilitatory effect will become statistically significant as the duration of culture increases. Even so, the GnRH secretion from olfactory placode neurons derived at E13.5 and cultured for 2 weeks did not change during infusion of 20 μM bicuculline, although it decreased during infusion of 20 μM GABA [5]. Further studies are needed to determine whether facilitatory effects become significant if cultures are continued for a longer period of time.

Acknowledgment

The present study was supported by a Grant-in-Aid for Exploratory Research (10877013 and 13877011 to FK) from the Ministry of Education, Culture, Sports, Science, and Technology, Japan.

References

1. Farbman AI (1994) Developmental biology of olfactory sensory neurons. *Semin Cell Biol* 5: 3–10.
2. Halpern M (1987) The organization and function of the vomeronasal system. *Annu Rev Neurosci* 10: 325–362.
3. Tobet SA, Chickering TW, King JC, Stopa EG, Kim K, Kuo-Leblank V, Schwarting GA (1996) Expression of gamma-aminobutyric acid and gonadotropin-releasing hormone during neuronal migration through the olfactory system. *Endocrinology* 137: 5415–5420.
4. Wray S, Fueshko SM, Kusano K, Gainer H (1996) GABAergic neurons in the embryonic olfactory pit/vomeronasal organ: maintenance of functional GABAergic synapses in olfactory explants. *Dev Biol* 180: 631–645.
5. Funabashi T, Daikoku S, Suyama K, Mitsushima D, Sano A, Kimura F (2002) Role of gamma-aminobutyric acid neurons in the release of gonadotropin-releasing hormone in cultured rat embryonic olfactory placodes. *Neuroendocrinology* 76: 193–202.
6. Funabashi T, Daikoku S, Shinohara K, Kimura F (2000) Pulsatile gonadotropin-releasing hormone (GnRH) secretion is an inherent function of GnRH

neurons, as revealed by the culture of medial olfactory placode obtained from embryonic rats. *Neuroendocrinology* 71: 138–144.

7. Funabashi T, Suyama K, Uemura T, Hirose M, Hirahara F, Kimura F (2001) Immortalized gonadotropin-releasing hormone neurons (GT1-7 cells) exhibit synchronous bursts of action potentials. *Neuroendocrinology* 73: 157–165.
8. Kuehl-Kovarik MC, Pouliot WA, Halterman GL, Handa RJ, Dudek FE, Partin KM (2002) Episodic bursting activity and response to excitatory amino acids in acutely dissociated gonadotropin-releasing hormone neurons genetically targeted with green fluorescent protein. *J Neurosci* 22: 2313–2322.
9. Nunemaker CS, DeFazio RA, Geusz ME, Herzog ED, Pitts GR, Moenter SM (2001) Long-term recordings of networks of immortalized GnRH neurons reveal episodic patterns of electrical activity. *J Neurophysiol* 86: 86–93.
10. Krsmanovic LZ, Stojilkovic SS, Merelli F, Dufour SM, Virmani MA, Catt KJ (1992) Calcium signaling and episodic secretion of gonadotropin-releasing hormone in hypothalamic neurons. *Proc Natl Acad Sci USA* 89: 8462–8466.
11. Martinez de la Escalera G, Choi AL, Weiner RI (1992) Generation and synchronization of gonadotropin-releasing hormone (GnRH) pulses: intrinsic properties of the GT1-1 GnRH neuronal cell line. *Proc Natl Acad Sci USA* 89: 1852–1855.
12. Wetsel WC, Valenca MM, Merchenthaler I, Liposits Z, Lopez FJ, Weiner RI, Mellon PL, Negro-Vilar A (1992) Intrinsic pulsatile secretory activity of immortalized luteinizing hormone-releasing hormone-secreting neurons. *Proc Natl Acad Sci USA* 89: 4149–4153.
13. Hales TG, Sanderson MJ, Charles AC (1994) GABA has excitatory actions on GnRH-secreting immortalized hypothalamic (GT1-7) neurons. *Neuroendocrinology* 59: 297–308.
14. Spergel DJ, Catt KJ, Rojas E (1996) Immortalized GnRH neurons express large-conductance calcium-activated potassium channels. *Neuroendocrinology* 63: 101–111.
15. Kusano K, Fueshko S, Gainer H, Wray S (1995) Electrical and synaptic properties of embryonic luteinizing hormone-releasing hormone neurons in explant cultures. *Proc Natl Acad Sci USA* 92: 3918–3922.
16. Favit A, Wetsel WC, Negro-Vilar A (1993) Differential expression of gamma-aminobutyric acid receptors in immortalized luteinizing hormone-releasing hormone neurons. *Endocrinology* 133: 1983–1989.
17. Martinez de la Escalera G, Choi AL, Weiner RI (1994) Biphasic GABAergic regulation of GnRH secretion in GT1 cell lines. *Neuroendocrinology* 59: 420–425.
18. Oka H, Shimono K, Ogawa R, Sugihara H, Taketani M (1999) A new planar multielectrode array for extracellular recording: application to hippocampal acute slice. *J Neurosci Methods* 93: 61–67.
19. Nishihara M, Matsukawa T, Kimura F (1986) Responses of arcuate neurons to some putative neurotransmitters in perfused rat hypothalamic slices: effects of in vivo and in vitro estrogen treatments. *Jpn J Physiol* 36: 683–697.
20. Osada T, Nishihara M, Kimura F (1991) Sex differences in the basal firing rate and the responsiveness to opioid peptides of rat hippocampal neurons. *Jpn J Physiol* 41: 843–850.
21. Sim JA, Skynner MJ, Pape JR, Herbison AE (2000) Late postnatal reorganization of $GABA_A$ receptor signaling in native GnRH neurons. *Eur J Neurosci* 12: 3497–3504.
22. Sim JA, Skynner MJ, Herbison AE (2001) Heterogeneity in the basic membrane properties of postnatal gonadotropin-releasing hormone neurons in the mouse. *J Neurosci* 21: 1067–1075.
23. Han SK, Abraham IM, Herbison AE (2002) Effect of GABA on GnRH neurons switches from depolarization to hyperpolarization at puberty in the female mouse. *Endocrinology* 143: 1459–1466.
24. Cherubini E, Gaiarsa JL, Ben-Ari Y (1991) GABA: an excitatory transmitter in early postnatal life. *Trends Neurosci* 14: 515–519.
25. DeFazio RA, Heger S, Ojeda SR, Moenter SM (2002) Activation of A-type gamma-aminobutyric acid receptors excites gonadotropin-releasing hormone neurons. *Mol Endocrinol* 16: 2872–2891.
26. Jung H, Shannon EM, Fritschy JM, Ojeda SR (1998) Several $GABA_A$ receptor subunits are expressed in LHRH neurons of juvenile female rats. *Brain Res* 780: 218–229.
27. He D, Mitsushima D, Uemura T, Hirahara F, Kimura F (2000) Change in the number of $GABA_A$ receptor positive GnRH neurons in immature and mature male rats. *Jpn J Physiol* (*Suppl*.) 50: S203.