

# Characterization of Voltage-Gated Calcium Currents in Gonadotropin-Releasing Hormone Neurons Tagged with Green Fluorescent Protein in Rats

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**Functional analysis of GnRH neurons is limited, although these neurons play an important role in neuroendocrine regulation. Therefore, we decided to conduct cell physiological analysis of GnRH neurons. To identify GnRH neurons, we tagged the neurons with green fluorescence protein by a transgenic technique. A dispersed culture of GnRH neurons was prepared from the transgenic rats. After overnight culture, a perforated patch clamp was applied to the identified GnRH neurons to analyze the  $Ca^{2+}$  currents. In neonatal GnRH neurons, high voltage-activated  $Ca^{2+}$  currents were clearly observed, but low voltage-activated  $Ca^{2+}$  current was negligible. Nimodipine (L-type channel blocker) and  $\omega$ -conotoxin GVIA (N-type channel blocker) each attenuated the cur-**

**rent by approximately 20%. The R-type channel blocker SNX-482 attenuated the current by approximately 55%. Inhibition by the P/Q-type channel blocker  $\omega$ -agatoxin IVA was small. In GnRH neurons around puberty, however, both high and low voltage-activated  $Ca^{2+}$  currents were observed. Inhibitions by nifedipine,  $\omega$ -conotoxin GVIA, and SNX-482 were similar to those in the neonatal neurons, whereas the inhibition by  $\omega$ -agatoxin IVA was clearly seen in 40–61% of the GnRH neurons examined. These results indicate that GnRH neurons functionally express L-, N-, P/Q-, R-, and T-type channels. Expressions of P/Q- and T-type channels are developmentally regulated. (*Endocrinology* 144: 5118–5125, 2003)**

GnRH NEURONS PLAY an essential role in reproductive neuroendocrine regulation. Despite the importance of GnRH neurons, functional analysis of these neurons is limited. This is mainly due to difficulty in identifying GnRH neurons in electrophysiological experiments. Recently, however, transgenic mice were produced for specific labeling of GnRH neurons with enhanced green fluorescence protein (EGFP) (1–3), which has facilitated the cell physiological study of GnRH neurons. Firing patterns of GnRH neurons were studied in whole cell patch-clamp recordings (2, 4, 5), and the basic membrane properties were studied in current clamp analysis (3). Responses to glutamate and  $\gamma$ -aminobutyric acid were also reported on EGFP-tagged GnRH neurons (1, 5, 6). Because most of these studies were carried out in the current clamp mode, the voltage-gated currents remain to be analyzed. Kusano *et al.* (7) reported that mouse GnRH neurons in olfactory pit explant cultures express both low voltage-activated and high voltage activated  $Ca^{2+}$  currents. This report is to date the only one on the voltage-gated  $Ca^{2+}$  current in GnRH neurons analyzed by the voltage clamp experiments despite the fact that the voltage-gated  $Ca^{2+}$  channels play important roles in  $Ca^{2+}$ -dependent cellular functions such as transmitter release, cell excitability, protein phosphorylation, enzyme activity, and gene transcription. We therefore decided to study the voltage-gated  $Ca^{2+}$  currents in rat GnRH neurons. We first produced transgenic rats for the identification of GnRH neurons.

Here are two reasons why we chose rats instead of mice. First, there are already several mouse lines of EGFP-tagged GnRH neurons. If we produced a transgenic rat, we could compare GnRH neurons in mice and rats. Second, rats have been and are still commonly used for experiments on reproductive neuroendocrinology, as a consequence of which there is an accumulation of useful data on rats.

In the present study we investigated the expression profile of voltage-gated  $Ca^{2+}$  currents in neonatal and pubertal GnRH neurons by the method of perforated patch recording configuration with amphotericin B.

## Materials and Methods

All experiments were performed with the approval of Nippon Medical School animal care committee.

### Transgenic rats

The rat GnRH promoter (–3026 to +116; a gift from Dr. M. E. Wierman, University of Colorado Health Science Center, Denver, CO) (8) was used to express a transgene consisting of the intron of rabbit  $\beta$ -globin (640 bp; a gift from Dr. J. Miyazaki, Osaka University, Osaka, Japan), the coding sequence for EGFP (739 bp; CLONTECH Laboratories, Inc., Tokyo, Japan), and the polyadenylation signal. The excised transgene was injected into the pronucleus of fertilized oocytes obtained from Wistar rats (YS New Technology, Tochigi, Japan). Six transgenic founders were identified through Southern blot analysis of DNA harvested from tail snips of 112 pups with a  $^{32}P$ -labeled EGFP probe. The offspring of these 6 transgenic lines were cytologically examined, and one transgenic line, which had high and specific expression of EGFP in GnRH neurons, was selected for physiological experiments. The other five lines were not used because they had weak EGFP fluorescence. For cytological observation, brains were fixed with 4% paraformaldehyde. Forty-micrometer frozen sections of the fixed brain were cut and immunostained with antisera to GnRH (a gift from Dr. K. Inoue, Saitama

Abbreviations: Aga-IVA,  $\omega$ -Agatoxin IVA; APW, action potential waveform; EGFP, enhanced green fluorescence protein; GVIA,  $\omega$ -conotoxin GVIA; MVIIC,  $\omega$ -conotoxin MVIIC; OVLT, organum vasculosum of the lamina terminalis.

University, Saitama, Japan) and Cy3-labeled second antibody (Jackson ImmunoResearch Laboratories, West Grove, PA).

### Primary culture

The brains were excised from either 1- to 7-d-old pups or 35- to 40-d-old rats under ether anesthesia. The former were used to prepare neonatal neurons, and the latter were used for the neurons around puberty. The latter could include prepubertal animals because we did not check for the onset of puberty. Medial septum, diagonal band of Broca, organum vasculosum of the lamina terminalis (OVLT), and medial preoptic area were cut out with a razor and surgical blades. The sections were minced and treated with papain (21 U/ml; Funakoshi, Tokyo, Japan) for 30–60 min at 30 C with gentle agitation. The tissues were triturated with a 5-ml plastic pipette after several washes with MEM (Life Technologies, Inc., Tokyo, Japan). The cell suspension was applied to discontinuous Percoll density gradient centrifugation to remove debris. The cells were obtained from the middle layer of the density gradient centrifugation composed of 1.0, 1.023, and 1.078 g/ml layers and were plated on poly-lysine-coated coverslips and incubated overnight in Neurobasal-A medium (Life Technologies, Inc.) supplemented with 0.5 mM L-glutamine and B-27 (Life Technologies, Inc.) at 37 C. Most of the dissociated GnRH neurons were round, but some were spindle-shaped. These neurons did not change their shape during the overnight culture.

### Electrophysiology

The List EPC-9 patch-clamp system (Physio-Tech, Tokyo, Japan) was used for electrophysiological recordings and data analysis. Whole cell currents were measured by the perforated patch-clamp technique (9) at room temperature (25 C). The final concentration of amphotericin B (Seikagaku Corp., Tokyo, Japan) in the pipette solution was 0.05 mg/ml. The pipette solution consisted of 95 mM cesium aspartate, 47.5 mM CsCl, 1.0 mM MgCl<sub>2</sub>, 0.1 mM EGTA, and 10 mM HEPES (pH 7.2), and the osmolality was adjusted to 270 mosmol. The extracellular solution consisted of 116.3 mM NaCl, 10 mM tetraethylammonium chloride, 5 mM CsCl, 10 mM CaCl<sub>2</sub>, 0.8 mM MgCl<sub>2</sub>, 0.6 mM NaHCO<sub>3</sub>, 10 mM glucose, 20 mM HEPES (pH 7.4), 0.1% BSA (fraction V, Sigma-Aldrich Corp., St. Louis, MO), and 0.3 μM TTX (Seikagaku Corp.), and the osmolality was adjusted to 300 mosmol. Pipettes were fabricated with borosilicate glass capillaries and had a resistance of 7–9 MΩ. The pipettes were targeted to GnRH neurons in the extracellular solution without BSA. After touching the cell, slight negative pressure was applied to the pipette, which made a seal resistance of 5–10 GΩ. Perforation with amphotericin B was achieved within 5–10 min after giga-seal formation. Currents were filtered at 2.3 kHz, digitized at 10 kHz, and recorded. Series resistance was 70% electronically compensated. Data were taken when the series resistance was stable and less than 30 MΩ. Capacitive and leak currents were subtracted by the p/4 protocol, and the liquid junction potential was not compensated. Cell capacitances were 9.2 ± 2.2 pF (n = 46) in males and 9.8 ± 2.4 pF (n = 34) in females in neonates, and 12.8 ± 2.6 pF (n = 13) in males and 10.8 ± 2.7 pF (n = 11) in females around puberty. The input resistance of the cells ranged from 1–5 GΩ. Cells with a peak Ca<sup>2+</sup> current less than –100 pA were excluded from the analysis, because it is difficult to obtain a reliable subtracted current with such small currents. To confirm the perforated patch configuration, we examined the capacitive current and its change by rupturing the patch membrane at the end of the recording. Data are expressed as the mean ± SD unless otherwise stated. The Kruskal-Wallis test and paired *t* test were used for statistical analysis. The significance level was set at *P* < 0.05.

### Chemicals

Nimodipine and nifedipine were obtained from Wako Junyaku (Osaka, Japan). ω-Conotoxin GVIA (GVIA), ω-conotoxin MVIIC (MVIIC), ω-agatoxin IVA (Aga-IVA), and SNX-482 were purchased from Peptide Institute, Inc. (Osaka, Japan).

## Results

In the transgenic rats, EGFP fluorescence was observed only in GnRH-immunoreactive neurons, approximately one

third of which had strong EGFP fluorescence (Fig. 1). The fluorescence was observed not only in soma, but also in processes including axons in the median eminence (data not shown). GnRH neurons were also identified with EGFP in a dissociated culture (Fig. 1, D–F).

### Ca<sup>2+</sup> currents in neonatal GnRH neurons

In neonatal GnRH neurons Ca<sup>2+</sup> currents were activated by 100-msec voltage steps from –60 to 60 mV in 10-mV increments from a holding potential of –80 mV at 0.2 Hz (Fig. 2). The maximum amplitudes were –57.8 ± 20.7 pA/pF (n = 13) in males and –46.6 ± 12.2 pA/pF (n = 14) in females. The maximum current was activated at 0–20 mV and showed a rapid activation and a relatively slow inactivation.

The effects of several Ca<sup>2+</sup> channel blockers on the maximum currents are shown in Fig. 3. The maximum currents were elicited by 100-msec voltage pulses to 0 or 10 mV from the holding potential of –80 mV at 0.2 Hz. After the control currents were recorded, 10 μM nimodipine, 1 μM GVIA, 200 nM Aga-IVA, and 100 nM SNX-482 were successively applied (Fig. 3, A and B). The initial peak currents and late sustained currents were examined. In the initial peak currents, nimodipine and GVIA each attenuated the currents by approximately 20%, and SNX-482 reduced the currents by about 55% in both sexes. A similar inhibition by SNX-482 (60 ± 7%; n = 4) was observed when SNX-482 was applied without prior application of the other Ca<sup>2+</sup> channel blockers. Inhibition by Aga-IVA was small and negligible in both sexes. Aga-IVA exerted 7.5 ± 3.3% inhibition in 4 of 11 male cells examined and 3% inhibition in 1 of 14 female cells examined. No inhibition was observed in other cells. After combined application of all the above drugs, 6–7% of the control current remained. To examine the presence of T-type Ca<sup>2+</sup> current, the membrane potential was held at –100 mV, and the voltage steps to –70, –60, and –50 mV were given at 0.2 Hz. In this voltage protocol the current density of –1 pA/pF was activated at –50 mV in 2 cells among 10 male cells examined, and that of –1.4 pA/pF was activated at –50 mV in 3 cells among 9 female cells examined (Fig. 7C). No current was activated at –50 mV in the other cells. In the late sustained currents, the inhibition caused by GVIA was approximately 30%, and that by SNX-482 was 33% in males and 47% in females. The proportion of SNX-482-sensitive currents was smaller in the late sustained currents than in the initial peak currents. This is probably due to inactivation of the SNX-482-sensitive currents. The inhibition by each blocker was significant (*P* < 0.01), except for that by Aga-IVA.

The action potential waveform (APW) was used for activation of the Ca<sup>2+</sup> currents (Fig. 4). The half-amplitude width of APW was set at 2.5 msec, because that of the GnRH neuron ranged from 2.5–3 msec at room temperature (data not shown). In this voltage protocol, the inhibitory effect of nifedipine was small (9.4%), and the inhibition caused by GVIA and Aga-IVA was 29%, whereas that by SNX-482 was 45%, so that the contribution of the nifedipine-sensitive current was smaller in the APW than in the current activated by the square pulse.

The voltage-dependent activation of R-type current was studied by measuring tail currents at –80 mV after 10-msec

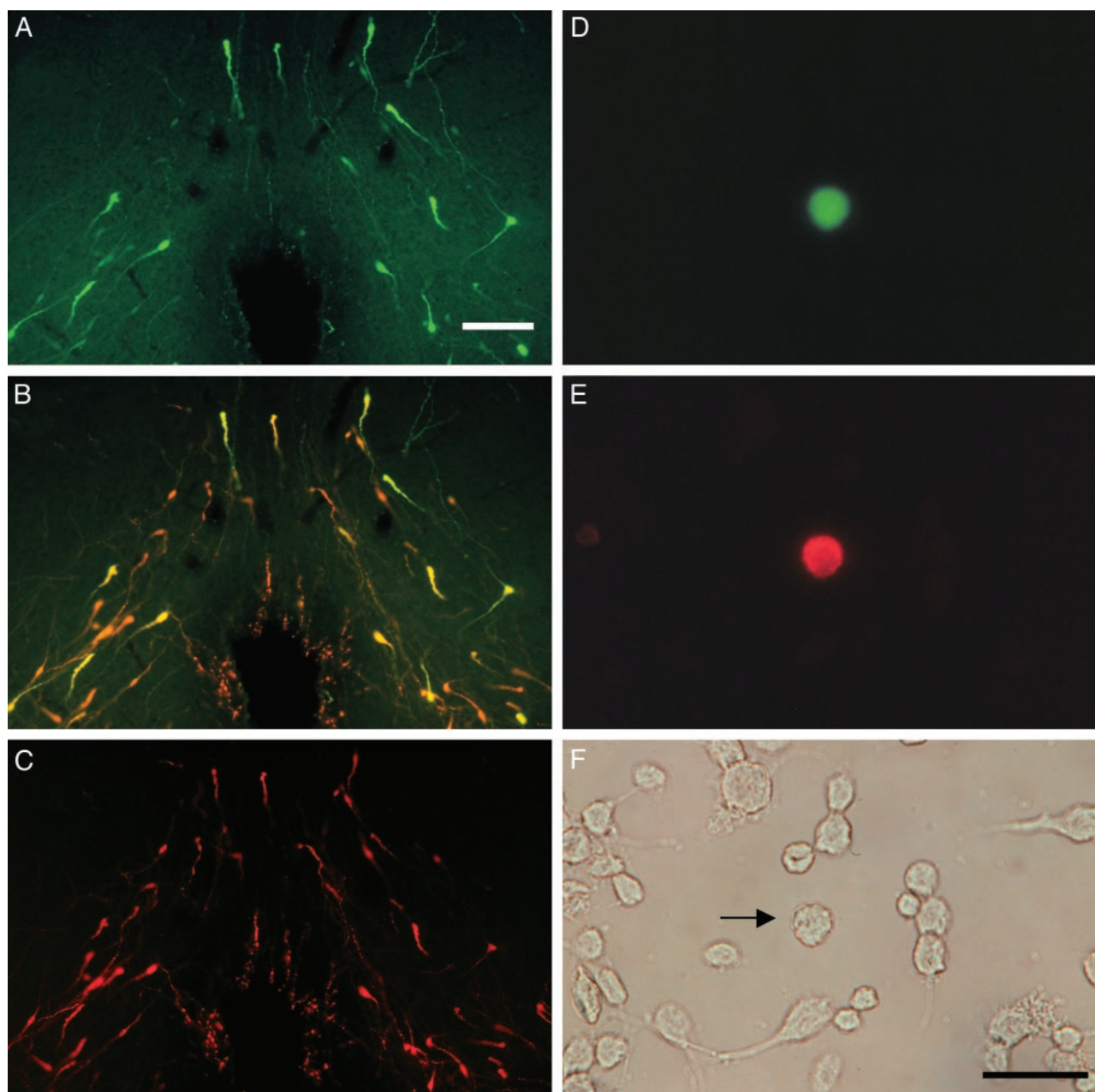


FIG. 1. EGFP fluorescence in GnRH neurons. A–C, Frontal section of the OVLT, diagonal band of Broca region. The dark area in the lower middle of each panel is the rostral part of the third ventricle. A, EGFP fluorescence. B, Double exposure for EGFP and Cy3 (GnRH). Neurons colored yellow are positive for both EGFP and GnRH. C, Cy3-labeled GnRH neurons. D–F, Dissociated cells, D, EGFP fluorescence. E, Cy-3-labeled GnRH neuron. F, Cells in brightfield. The cell indicated with an arrow is the EGFP-positive GnRH neuron. Scale bars, 100  $\mu\text{m}$  (A) and 25  $\mu\text{m}$  (F).

prepulses of  $-60$  to  $60$  mV in  $10$ -mV increments from the holding potential of  $-80$  mV at  $0.2$  Hz in the presence of nimodipine, GVIA, and Aga-IVA (Fig. 5). The activation started at a prepulse of  $-40$  mV and reached full activation at  $30$ – $40$  mV. The half-activation voltage was  $0$  mV (two male and two female neurons). Steady state inactivation was also studied in the R-type  $\text{Ca}^{2+}$  current (Fig. 5). The holding potential varied from  $-100$  to  $0$  mV in  $10$ -mV increments, and a  $100$ -ms test pulse was applied at  $0.2$  Hz. The inactivation started from the holding potential of  $-80$  mV and reached almost complete inactivation at  $0$  mV. The half-

inactivation voltage was  $-39$  mV (seven male and three female neurons).

#### $\text{Ca}^{2+}$ currents in GnRH neurons around puberty

$\text{Ca}^{2+}$  currents were activated by  $100$ -msec voltage steps from  $-60$  to  $60$  mV in  $10$ -mV increments from the holding potential of  $-80$  mV at  $0.2$  Hz. The activation started at  $-40$  mV and reached maximum amplitude around  $0$  mV. The maximum amplitudes were  $-49.5 \pm 15.1$  pA/pF ( $n = 13$ ) in males and  $-43.9 \pm 26.8$  pA/pF ( $n = 10$ ) in females. The

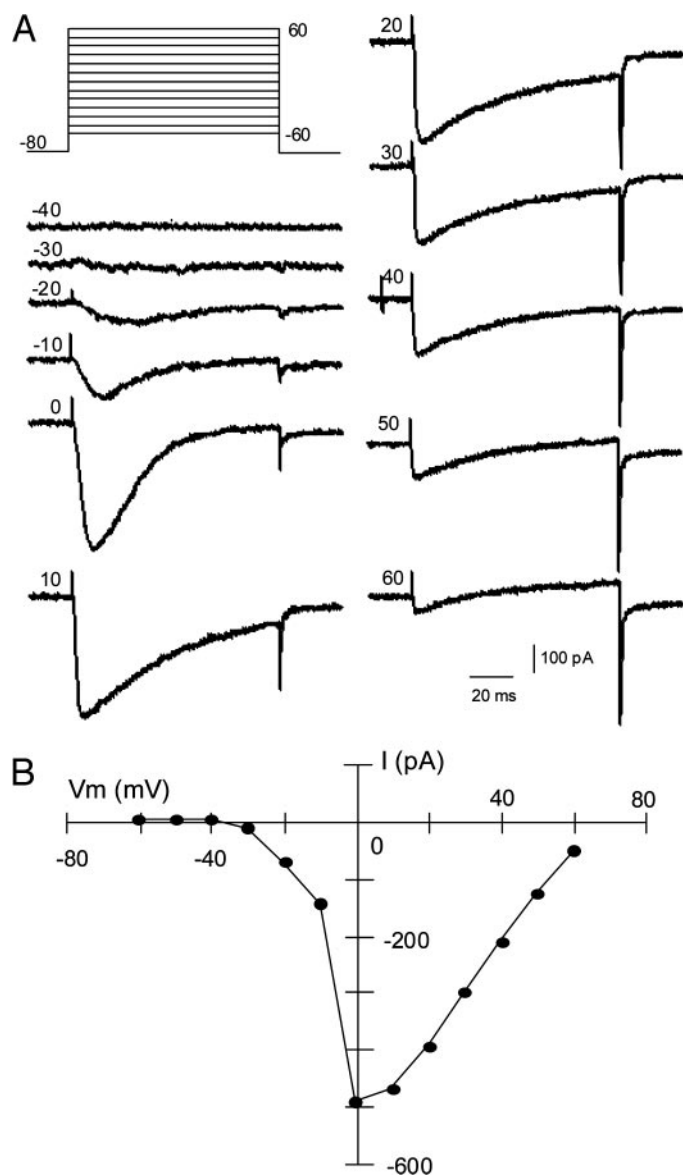


FIG. 2. Voltage-gated Ca<sup>2+</sup> currents in neonatal GnRH neurons. After perforation with amphotericin B, cells were clamped at  $-80$  mV and given 100-msec voltage pulses from  $-60$  to  $60$  mV in 10-mV steps from a holding potential of  $-80$  mV as shown at the upper left in A. A, Current traces elicited with voltage pulses as indicated at the upper left in each trace. Current traces at  $-60$  mV and  $-50$  mV are not shown. B, IV relationship of the peak currents with the same data as in A.

comparisons were made in four groups according to developmental stage and sex. There was no significant difference in the control maximum current densities among these four groups. Nifedipine, GVIA, and SNX-482 exerted similar inhibitory effects to that in the neonatal GnRH neurons (Fig. 6). The inhibitory effect of Aga-IVA was stronger and clearer than that in the neonatal GnRH neurons. The overall inhibitions were 8% in males and 5% in females. The number of cells in which Aga-IVA attenuated the peak current more than 5 pA was eight in 13 male cells and four in 10 female cells. A similar inhibition was exerted by 2  $\mu$ M MVIIC (P/Q-type Ca<sup>2+</sup> channel blocker) in four male and six female

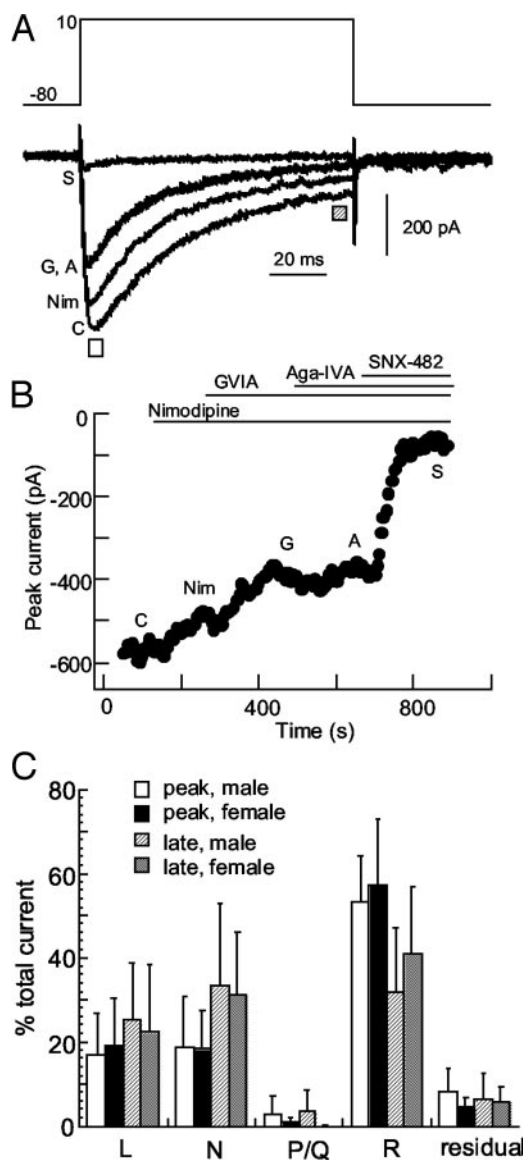


FIG. 3. Pharmacological characterization of the Ca<sup>2+</sup> currents in neonatal GnRH neurons. A, Voltage protocol and the representative current traces. Voltage pulses (10 mV in this cell) were applied from the holding potential of  $-80$  mV at 0.2 Hz. The control trace is labeled C. The current was attenuated by 10  $\mu$ M nimodipine (Nim), 1  $\mu$ M GVIA (G), and 100 nM SNX-482 (S). A concentration of 200 nM Aga-IVA (A) had no effect on the current in this cell. Therefore, traces labeled G and A are overlapped. These traces were taken from the same data for B at time points denoted by C, Nim, G, A, or S. B, Time course of the effect of drugs on the peak Ca<sup>2+</sup> current is shown. Drugs were applied as indicated with horizontal bars. C, The effects of drugs are collectively shown as a percentage of the total current ( $n = 11$  in males, 14 in females) for the initial peak current (peak) and the late sustained current (late). The late sustained current was measured as the mean current of the last 5 msec of the 100-msec pulse (hatched square in A). The L-type current indicates the current blocked by nimodipine. The N-type current (N) is the current blocked by GVIA. The P/Q-type current (P/Q) is the current blocked by Aga-IVA. The R-type current (R) is the current blocked by SNX-482. Residual indicates the current resistant to all these drugs.

neurons examined ( $6.6 \pm 9\%$ ). The proportions of the remaining currents after treatment with all of the above drugs were  $12.2 \pm 7.3\%$  ( $n = 13$ ) in males and  $16.5 \pm 18.9\%$  ( $n =$

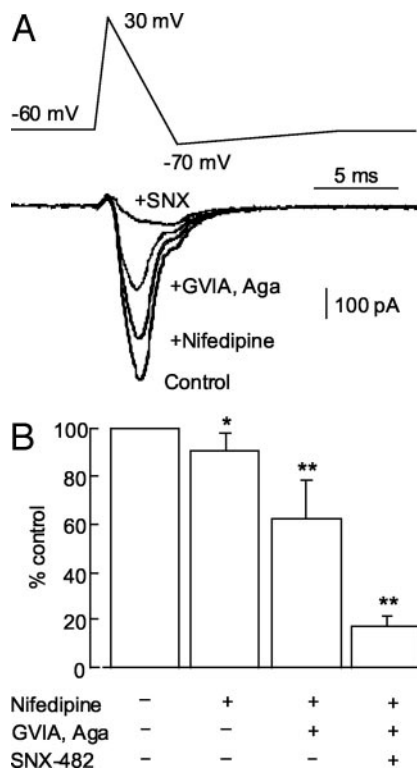


FIG. 4. The  $\text{Ca}^{2+}$  currents elicited by APW in neonatal GnRH neurons. The action potential waveform is composed of the 1-msec depolarization phase from  $-60$  mV to  $30$  mV, the 4-msec repolarization phase from  $30$  to  $-70$  mV, and the 10-msec afterhyperpolarization phase from  $-70$  mV to  $-60$  mV as shown in A. After control recording, drugs were successively applied as indicated. B, Collective presentation of the effects of drugs. The data for both sexes are combined ( $n = 8$ ). Aga, Aga-IVA; -, without drug application; +, with drug application. The inhibitory effect of each drug was evaluated by comparing the responses with and without the drug in a paired  $t$  test. \*,  $P < 0.05$ ; \*\*,  $P < 0.01$ .

10) in females. These remaining currents were further attenuated by  $50 \mu\text{M}$   $\text{Ni}^{2+}$  to  $6.5 \pm 4.5\%$  and  $3.8 \pm 4.7\%$ , respectively, which are comparable to the remaining currents in neonatal GnRH neurons without application of  $\text{Ni}^{2+}$  (Fig. 3). These  $\text{Ni}^{2+}$ -sensitive currents were clearly seen at  $-30$  mV in all cells examined (Fig. 7). The remaining current densities at  $-30$  mV were  $-3.2 \pm 2.2$  pA/pF ( $n = 8$ ) in males and  $-6.4 \pm 4.3$  pA/pF ( $n = 9$ ) in females. These currents were inhibited by about 90% with  $50 \mu\text{M}$   $\text{Ni}^{2+}$ . Similar currents were activated by a voltage step to  $-50$  mV from a holding potential of  $-100$  mV at  $0.2$  Hz in all cells examined (Fig. 7C). The values were  $-2.2 \pm 1.8$  pA/pF ( $n = 6$ ) in males and  $-4.4 \pm 2.5$  pA/pF ( $n = 6$ ) in females. The inhibition by each blocker was statistically significant, except for that by Aga-IVA in females.

### Discussion

In the present study we used isolated cells instead of cells in acute slice preparations because we could obtain much better and more reliable recordings of the  $\text{Ca}^{2+}$  currents in isolated cells. Moreover, the isolated cells may retain their original cellular characteristics to a certain extent even after overnight culture. It should be noted, however, that the cells

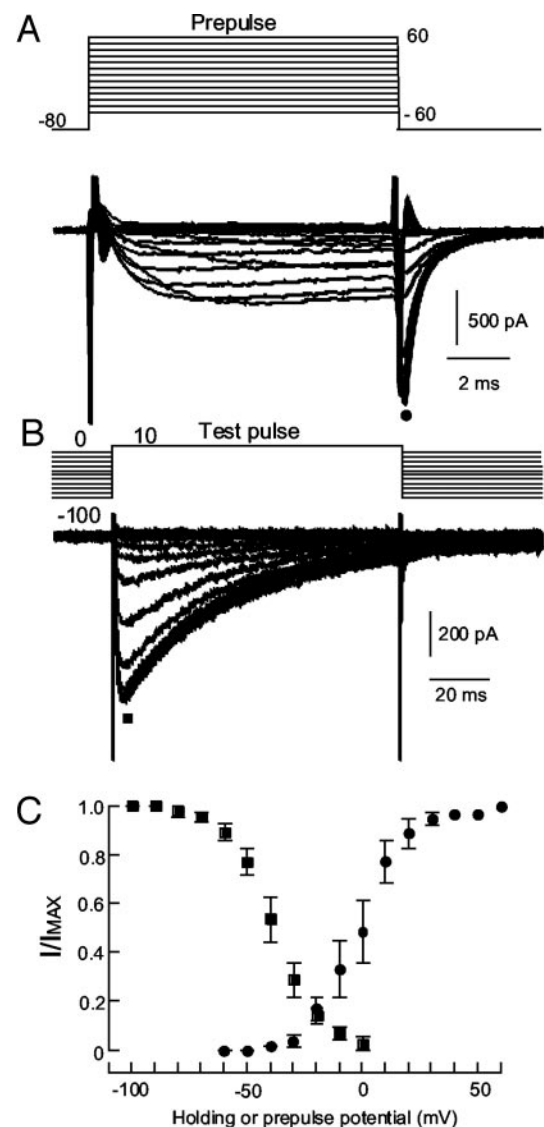


FIG. 5. Activation and steady-state inactivation of the R-type  $\text{Ca}^{2+}$  current in neonatal GnRH neurons. A, The upper panel shows the voltage protocol for the activation. The holding potential was  $-80$  mV. Ten-millisecond prepulses of  $-60$  to  $60$  mV were applied, and the tail currents at  $-80$  mV were measured as indicated ( $\bullet$ ). B, Holding potentials varied from  $-100$  to  $0$  mV, and the currents elicited by the test pulse ( $10$  mV) were measured as indicated ( $\blacksquare$ ). C, Activation ( $n = 4$ ) and steady state inactivation ( $n = 10$ ) are shown. The data for both sexes were combined. The half-activation voltage was  $0$  mV, and the half-inactivation voltage was  $-39$  mV.

used in the present experiments lacked both dendrites and axons, so that the currents originated in the cell soma.

We revealed an expression profile of the voltage-gated  $\text{Ca}^{2+}$  currents in GnRH neurons by using specific blockers for the voltage-gated  $\text{Ca}^{2+}$  currents. In neonatal GnRH neurons, L-, N-, and R-type  $\text{Ca}^{2+}$  currents were clearly observed in all cells examined, but P/Q- and T-type  $\text{Ca}^{2+}$  currents were small and were seen in less than 50% of the cells examined. In the GnRH neurons around puberty, besides L-, N-, and R-type  $\text{Ca}^{2+}$  currents, a P/Q-type  $\text{Ca}^{2+}$  current was observed in 62% of male cells examined and 40% of female cells, whereas a T-type  $\text{Ca}^{2+}$  current was clearly observed in all

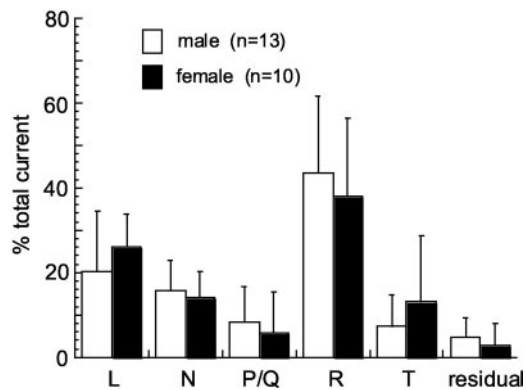


FIG. 6. Pharmacological characterization of the Ca<sup>2+</sup> currents in GnRH neurons around puberty. Effects of drugs are collectively shown as a percentage of the total current for the peak current. The L-type current (L) was the current blocked by 10  $\mu$ M nifedipine; the T-type current (T) was the current resistant to nifedipine, GVIA, Aga-IVA, and SNX-482 and blocked by 50  $\mu$ M Ni<sup>2+</sup>. Other currents are the same as in Fig. 3. The experimental procedure is the same as for the neonatal neurons.

cells examined, so that the expression of P/Q- and T-type Ca<sup>2+</sup> currents was developmentally regulated. There was no substantial sex difference in the profile of expression of the voltage-gated Ca<sup>2+</sup> currents in GnRH neurons either in neonates or around puberty. To date, the presence of L- and T-type Ca<sup>2+</sup> currents has been reported in mouse GnRH neurons in explant culture of olfactory pit (7) and GT1 cells (10, 11). No other types of Ca<sup>2+</sup> current were examined in these reports.

We identified an R-type current by two criteria. One was a current resistant to specific blockers for L-, N-, and P/Q-type Ca<sup>2+</sup> channels in high voltage-activated Ca<sup>2+</sup> currents (12–14). The other was a current that was blocked by 100 nM SNX-482 (15, 16). This concentration is specific to the R-type current, but does not block the SNX-482-resistant, R-type current (16). In the present results almost all of the remaining currents were blocked by 100 nM SNX-482, suggesting that rat GnRH neurons express no or a very small proportion of SNX-482-resistant, R-type current. Half-activation and half-inactivation voltages of R-type current were reported to be  $-14$  mV and approximately  $-70$  mV, respectively, in mouse hippocampal and neocortical neurons by Sochivko *et al.* (17). These values differ from ours mainly because they used 5 mM Ba<sup>2+</sup> without Ca<sup>2+</sup> as a charge carrier instead of the 10 mM Ca<sup>2+</sup> in our experiments.

It should be noted that the proportion of R-type current was surprisingly big both in neonates (55%) and around puberty ( $\sim$ 40%) compared with approximately 20% in magnocellular and unidentified hypothalamic neurons (18–21) and neocortical and neostriatal neurons (22, 23). This means that the R-type Ca<sup>2+</sup> current greatly contributes to intracellular Ca<sup>2+</sup> regulations in GnRH neurons in these developmental stages, but in adult GnRH neurons the proportion of R-type current was approximately 30% (our preliminary results). The half-inactivation voltage was  $-40$  mV in 10 mM Ca<sup>2+</sup> in the extracellular solution (Fig. 5). This value would be  $-50$  mV in a normal Ca<sup>2+</sup> concentration (2.5 mM). If we take  $-60$  mV as the resting potential value, the contribution of R-type Ca<sup>2+</sup> current would be more than 30% of the total

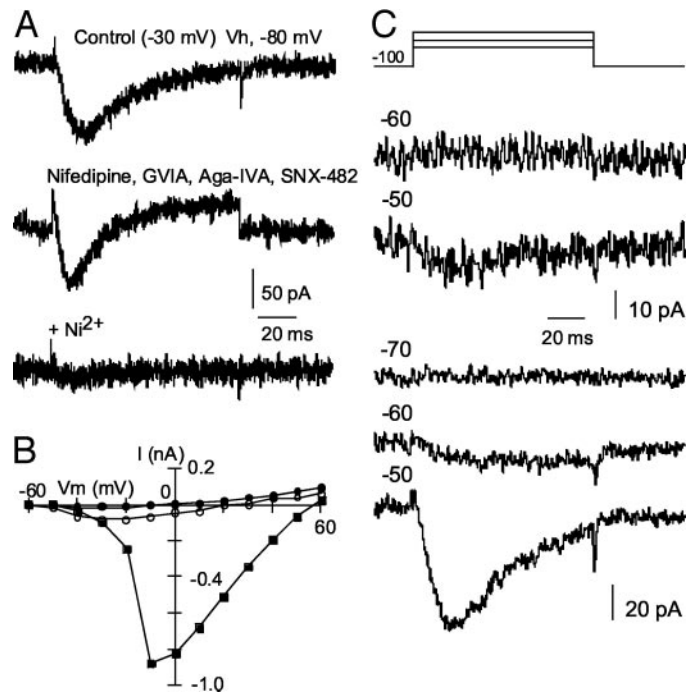


FIG. 7. T-Type current in GnRH neurons. A, The currents were elicited by a  $-30$  mV pulse from the holding potential of  $-80$  mV in neurons around puberty. Combined application of nifedipine (10  $\mu$ M), GVIA (1  $\mu$ M), Aga-IVA (200 nM), and SNX-482 (100 nM) slightly attenuated the peak amplitude of the current. This remaining current was almost completely blocked by 50  $\mu$ M Ni<sup>2+</sup> (lower trace). B, IV relationship of the peak current in the control (■); with nifedipine, GVIA, Aga-IVA, and SNX-482 (○); and with the addition of Ni<sup>2+</sup> (●). C, The voltage pulses to  $-70$ ,  $-60$ , and  $-50$  mV were given from the holding potential of  $-100$  mV at 0.2 Hz, as shown at the top. The upper two current traces are from the neonatal neuron (male, 3 d old). The current at  $-70$  mV is not shown. The lower three traces are from the neuron around puberty (female, 35 d old). The numbers at the upper left in each trace indicate the amplitude of voltage pulses. For clarity, the currents in C were filtered at 500 Hz.

Ca<sup>2+</sup> current activated by the action potential. In fact, the contribution of the R-type current was 45% in our APW experiment (Fig. 4). Cytochemistry revealed a wide distribution of the prime candidate of R-type channel  $\alpha$ 1E (17) in the brain in both mice (13) and rats (24), including the OVLT and medial preoptic area. These findings suggest that R-type Ca<sup>2+</sup> channels must be expressed at least in the somadendritic region of GnRH neurons and contribute to Ca<sup>2+</sup>-dependent regulation in GnRH neurons. The R-type Ca<sup>2+</sup> channels might be involved in GnRH release at nerve endings, because the R-type channels are reported to contribute transmitter release at a rat calyx synapse (25), oxytocin release from the nerve endings (26, 27), and exocytosis in mouse adrenal chromaffin cells (28).

We used the dihydropyridine antagonists nifedipine and nimodipine to block L-type current (29, 30). An L-type current was observed both in neonates and around puberty as approximately 20% of total Ca<sup>2+</sup> currents. Kusano *et al.* (7) reported a high voltage-activated Ca<sup>2+</sup> current sensitive to 100  $\mu$ M Cd<sup>2+</sup> and 1  $\mu$ M nifedipine expressed in mouse GnRH neurons in explant culture of the olfactory pit, suggesting the presence of an L-type current in these neurons. A similar type of current has been reported in GT1 cells (10, 11). The L-type

current is well known to contribute hormone release in a variety of neuroendocrine cells, including pancreatic  $\beta$  cells (31) and pituitary somatotrophs (32). In physiological conditions, an L-type current may be activated by slow depolarization, such as by an excitatory postsynaptic potential, rather than by an action potential (33). Moreover, L-type currents become prominent in slow depolarization because the inactivation process eliminates some other Ca<sup>2+</sup> currents, such as the R-type to a certain extent. Taken together with preferential expression of L-type Ca<sup>2+</sup> channels in the soma-dendritic region of central neurons (34), L-type currents may regulate Ca<sup>2+</sup>-dependent functions, such as protein phosphorylation (33), enzyme activity, and gene expression, in GnRH neurons in a different manner from that of the R-type current.

The peptide antagonist GVIA is widely used to identify the N-type Ca<sup>2+</sup> current in physiological studies (35, 36). We used 1  $\mu$ M GVIA and found that the proportion of N-type Ca<sup>2+</sup> current was 15–20% of the total Ca<sup>2+</sup> currents. N-Type Ca<sup>2+</sup> channels could be involved in GnRH release at nerve endings, because the N-type channel is known to be involved in vasopressin release (20), oxytocin release (26), and synaptic transmission in cultured hypothalamic neurons (37) and several central synapses (38). Immunostaining of N-type Ca<sup>2+</sup> channel subunit  $\alpha$ 1B revealed the presence of the N-type channel not only at nerve terminals, but also in the soma-dendritic region of central neurons (39), so that N-type channels in the GnRH neuron may play some roles in the soma-dendritic region besides at nerve terminals.

In the present study we did not separately identify P-type and Q-type Ca<sup>2+</sup> currents, but treated them as P/Q-type Ca<sup>2+</sup> currents by using a high concentration (200 nM) of Aga-IVA that does not distinguish between P- and Q-type channels (14). This was further confirmed with another P/Q-type channel blocker, MVIIC (2  $\mu$ M). The P/Q-type Ca<sup>2+</sup> current was small, but clearly observed around puberty in 40–62% of GnRH neurons examined. This developmental change in the expression of P/Q-type Ca<sup>2+</sup> current may have functional significance. For example, a P/Q-type channel might be involved in GnRH release from nerve terminals at the median eminence, which changes dramatically through puberty, thereby controlling gonadotropin release from the anterior pituitary. The P/Q-type Ca<sup>2+</sup> current is shown in various central neurons with different degrees of expression (40, 41). Q-type channels are present on a subset of the neurohypophysial terminals that release vasopressin (20). Developmental change in the contribution of P/Q-type Ca<sup>2+</sup> current is also demonstrated at several central synapses (38). Its contribution is greater on postnatal d 13–19 than on postnatal d 7–9.

Expression of T-type Ca<sup>2+</sup> current also showed a clear change in development. The T-type current is classified as a low voltage-activated current. Some R-type currents are also activated in a similar voltage range (13, 16, 17). Therefore, in the present study the T-type current was identified by its sensitivity to Ni<sup>2+</sup> and its insensitivity to SNX-482 (16) in addition to the low voltage activation. This type of current is demonstrated in mouse GnRH neurons in explant culture of the olfactory pit (7) and GT1 cells (10, 11). T-type Ca<sup>2+</sup> current in GnRH neurons possibly activates small conduc-

tance, Ca<sup>2+</sup>-activated K<sup>+</sup> channels (SK channels), such as in midbrain dopaminergic neurons (42), thereby controlling action potential firing. According to the several reports concerning the firing pattern of mouse GnRH neurons, irregular spontaneous firing of single action potentials and irregular bursting of spikes are observed in these neurons (1–6, 43). As the SK channel is responsible for sustained tonic firing of single spikes (42), the T-type Ca<sup>2+</sup> current may function as a regulator of SK channels in mouse and possibly rat GnRH neurons. The present results clearly demonstrate that the T-type current becomes active around the pubertal stage.

In conclusion, the present study revealed rat GnRH neurons functionally expressed L-, N-, and R-type Ca<sup>2+</sup> channels both in neonates and around puberty and expressed the P/Q- and T-type Ca<sup>2+</sup> channels around puberty. Cellular functions of these voltage-gated Ca<sup>2+</sup> channels remain to be analyzed in future experiments.

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