the expression of T-type Ca^{2+} channels is most abundant in the heart[16]. Sympathetic fibers, which innervate the heart, contain a large amount of NPY[17]. These findings imply a functional relevance of NPY to the T-type Ca^{2+} channel.

NG108-15 is a hybridoma cell line of neuroblastoma and glioblastoma origin, and is known to differentiate to neuron-like cells by dibutyric cAMP-treatment[18]. Under undifferentiated conditions, NG108-15 cells exhibit a T-type Ca^{2+} channel current, without expressing other Ca^{2+} channel currents[19]. Moreover, undifferentiated NG108-15 cells can express at least the Y_1 subtype of the NPY receptor family[20].

In this study, we examined the effect of NPY on the T-type Ca^{2+} channel current expressed in undifferentiated NG108-15 cells using Ba^{2+} as the charge carrier, and found that NPY augments the Ba^{2+} current. The role of NPY in regulating excitability through the augmentation of T-type Ca^{2+} channels is also discussed.

MATERIALS AND METHODS

Cell culture

Cultivation of NG108-15 cells, a neuroblastoma/glioma hybrid cell line, was carried out according to previous methods[18]. The cells were maintained at 37°C in Dulbecco's modified Eagles medium (high glucose) supplemented with 10% fetal calf serum (FCS), 100 μM hypoxanthine, 0.4 μM aminopterin, and 16 μM thymidine in a humidified atmosphere of 90% air-10% CO_2.

Electrophysiology

The whole cell recordings were performed according to the previous method[21,22] with minor modifications. For electrophysiological recordings, 35-mm dishes were transferred onto the stage of a microscope. The superfusing external solution had the following composition (in mM): $BaCl_2$ 55, NaCl 77, CsCl 5.5, TEA-Cl 11 Glucose 28, HEPES 11, TTX 0.001, Nifedipine 0.001 (pH 7.3). A high concentration of Ba^{2+} was used as the charge carrier in order to increase the amplitudes of Ca channel activity. This resulted in a positive shift in the I-V relationship (see Fig. 3B). The external solution was superfused at 1 ml/min. Cells located between the inlet and outlet tubes were selected for recordings. The patch pipettes (3-5 MΩ, Hilgenberg, Malsfeld, Germany) were filled with an internal solution having the following composition (in mM): CsCl 140, HEPES 10, EGTA 1, ATP 2, $MgCl_2$ 1 (pH 7.3). After whole cell recordings were made, voltage-dependent Ba^{2+} currents were elicited by rectangular pulses as indicated in the figures. Recordings were made at room temperature (23-26°C) using an Axopatch 200B amplifier (Axon Instruments, Foster City, CA, USA). To avoid the nonspecific binding of NPY to the perfusion tube, NPY (1×10^{-7} M) was dissolved in an external solution containing 0.01% fatty acid-free BSA (referred to as "Vehicle"). Currents were filtered at 5 kHz, digitized every 100 μsec, and analyzed by Clampex 8.0 and Clampfit 8.0 (Axon Instruments). Series resistance (Rs) and membrane resistance (Rm) were frequently monitored. Data from cells showing a change (>20%) in the Rs value and those from cells showing a Rm value below 700 MΩ were rejected. Rs compensation was performed at 75%. Data are presented as mean±SE. To avoid a wash-out effect on the T-type current, vehicle applications were started at least 5 min after rupture of the cell membrane patch[23]. Current density was calculated as the ratio of current to membrane capacitance. Data are shown as averages of eight traces recorded every 19s. Statistical significance was evaluated by the two-tailed t-test. A P value of 0.05 was considered significant.

The half activation voltage ($V_{1/2}$) estimated by fitting the I-V curves with the modified Boltzmann equation, IBa=Gmax*(Vm−Veq)/[1+exp((V1/2−Vm)/k)], where Gmax, Vm, Veq, V1/2, and k represents maximum Ba^{2+} conductance of currents, membrane potential, equilibrium potential of Ba^{2+} current, half activating voltage and slope factor respectively[24].

RT-PCR

Total RNA was extracted from undifferentiated NG108-15 cells using Trizol (Gibco BRL, Rockville, MD, USA), and then the RNA was treated with RQ1 RNase-Free DNase (Promega, Madison, WI, USA) at 37°C for 30 min. For measurement of NPY Y_1 and Y_5 receptors, a mRNAs sample was subjected to the RT-PCR analysis using Access RT-PCR (Promega). The RT-PCR program consisted of a 45 min reverse transcription at 48°C followed by 2 min heat denaturation at 94°C, 40 cycles of amplification (94°C, 30 sec; 60°C, 1 min; 68°C, 2 min), and 5 min final elongation at 68°C, according to the manufacture's instruction. Primers used for the PCRs of Y_1 and Y_5 receptors were GCAGAATTCCTTGCTGGTCGCAGTCATGT/GCAGAATTCGAAGAGTCGTGTAAGACAGCC and AAAGCGGCCGCCACCATGGAGTTTAAGCCT/AAAGAATTCTCATGACATGTGTAGGCAGTG, respectively. For the analysis of mRNAs

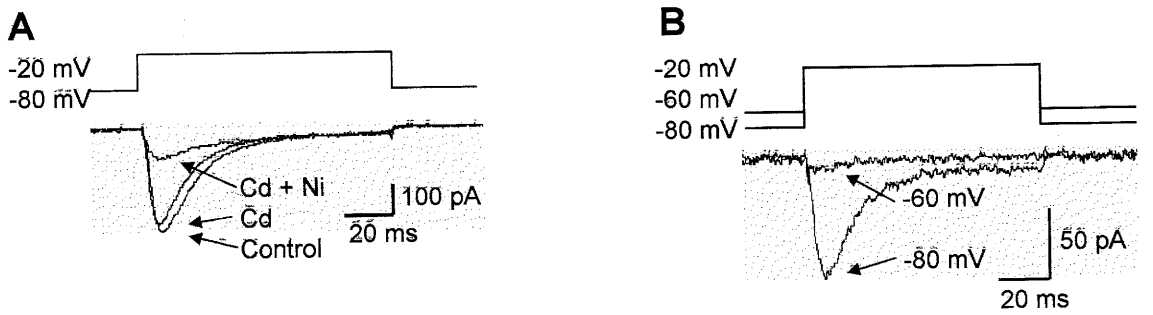

Fig. 1. Expression of T-type Ca^{2+} channel current in undifferentiated NG108-15 cells. **A.** Sensitivities to divalent cations. After formation of a stable whole cell patch clamp, step pulses from −80 mV to −20 mV elicited transient inward currents. Representative traces in the absence (Control), and in the presence of 50 μM $CdCl_2$ (Cd), or 50 μM $CdCl_2$+200 μM $NiCl_2$ (Cd+Ni) are indicated. **B.** Steady state inactivation of T-type Ca^{2+} channel current. An NG108-15 cell was initially voltage clamped at −80, and then a voltage-dependent current was evoked by a step pulse to −20 mV. In contrast, when the cell was held at −60 mV, the evoked current was almost negligible.

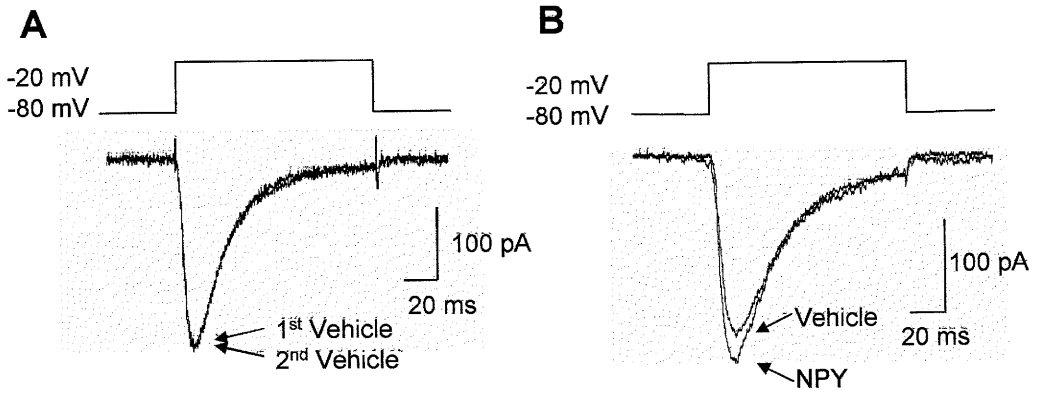

Fig. 2. Effect of NPY on T-type Ca^{2+} current. After formation of a stable whole cell patch clamp, the external solution containing 0.01% BSA was applied for 3 min as vehicle control, and then a second round of the vehicle **A** or NPY (1×10^{-7} M) **B** was applied for 3 min. Averaged traces during the preceding vehicle treatment and later ones are overlaid.

coding the Y_2 receptor, total RNAs were initially subjected to the RT-PCRs using Access PCR as described above. Primers used for the amplifications of the Y_2 receptor gene were AAAGCGGCCGCCAC-CATGGGCCCATTAGGT and AAGAATTCTTAC-ACGTTGGTGGCCTCTGA. Because this RT-PCR program non-specifically amplified unidentified genes, one μl of the PCR products was further subjected to the nested PCRs, which consisted of a 5 min initial denaturation at 95°C followed by 25 cycles of amplification (94°C, 45 sec; 55°C, 45 sec; 72°C, 45 sec), and 5 min final elongation at 72°C, using Taq DNA polymerase (Promega). Primers used for the nested PCR of Y_2 receptor gene were AAGAATTCTAGG-TGCAGAGGCAGATGAGAA and AAGAATTCTG-TACTCCTTCAGGTCCAGGAC. Validity of the PCR assays was confirmed by subcloning and sequencing of the PCR products using rat brain RNA as a template (Yamaguchi T., et al., unpublished data). The PCR products were then subjected to agarose gel electrophoresis and ethidium bromide staining.

RESULTS

Ca^{2+} channel current in undifferentiated NG108-15 cells

Ca^{2+} channel currents in undifferentiated NG108-15 cells were recorded using 55 mM Ba^{2+} as the charge carrier in a whole-cell configuration. Cells were

voltage-clamped at −80 mV, and voltage-dependent currents were elicited by rectangular test pulses to −20 mV for 100 ms. A transient inward current, which was inactivated within 50 ms, was observed in most undifferentiated cells (Fig. 1A). The decay time constant of the current evoked by the step pulse from −80 mV to −20 mV was 20.0±3.5 ms (n=5). This decay time constant was comparable to that of the T-type Ca^{2+} channel currents reported previously[21]. The peak amplitudes ranged from 30 pA to 300 pA. To investigate the I-V relationship of this inward current, test pulses from −80 mV to +60 mV were applied at every 10 mV. The inward current was elicited from −40 mV, and had a peak at −10 mV (Fig. 3B), in agreement with a previous report on T-type Ca^{2+} channels[19].

T-type Ca^{2+} channels are sensitive to Ni^{2+} but not to Cd^{2+}. Conversely, L-, N-, and P/Q-type Ca^{2+} channels are resistant to Ni^{2+} but sensitive to Cd^{2+} [2,21]. To characterize the current property, 50 μM $CdCl_2$ and subsequently 200 μM $NiCl_2$ were added to the external solution (Fig. 1A). While the current through undifferentiated NG108-15 cells was virtually unchanged by Cd^{2+}, the current was almost completely suppressed by the addition of Ni^{2+}. Furthermore, the inward current was greatly suppressed when the cells were voltage-clamped at −60 mV (Fig.1B), indicating a steady-state inactivation[21]. Therefore, the undifferentiated NG108-15 cells nearly exclusively expressed T-type Ca^{2+} channels, as reported previously[19]. In a small number of undifferentiated NG108-15 cells, however, high voltage-activated and long-lasting currents were also observed. These currents were sensitive to 50 μM Cd^{2+}, but insensitive to 200 μM Ni^{2+} (data not shown). In the following experiments, the I-V relationship and Ni^{2+} sensitivity were checked at the beginning and the end of each recording. NG108-15 cells, which were confirmed to express the T-type Ca^{2+} channel current by a I-V curve, fast kinetics, and Ni^{2+} sensitivity were selected for the experiments.

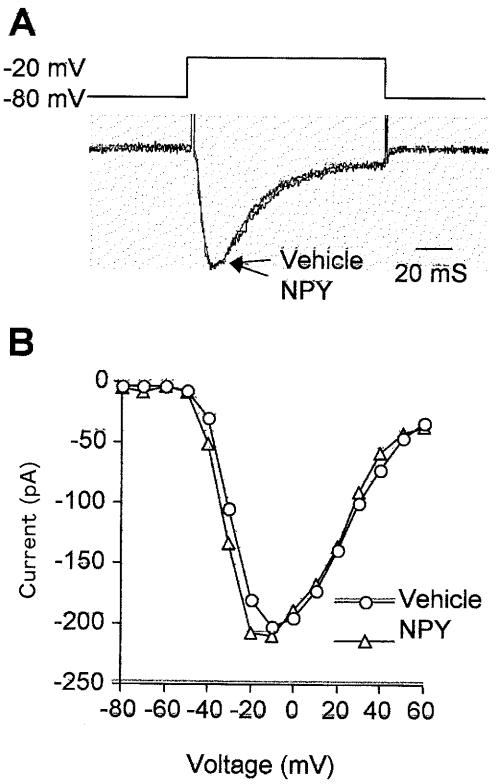

Fig. 3. Effect of NPY on kinetics and I-V relationship of T-type Ca^{2+} channel current. **A.** Lack of effect of NPY on the kinetics of T-type Ca^{2+} channel current. When traces during vehicle and NPY applications were normalized and overlaid, no essential difference was observed in the time courses of rise or decay. **B.** I-V relationship between the vehicle and NPY treatment. Test pulses from −80 to +60 mV were applied to an NG108-15 cell every 10 mV. Peak amplitudes during vehicle and NPY treatments are plotted as a function of voltage.

Effect of NPY on the T-type Ca^{2+} channel current

To examine the effect of NPY on T-type Ca^{2+} channels, 5 min after the formation of a whole cell patch clamp, NG108-15 cells were superfused with the vehicle for 3 min, followed by NPY (1×10^{-7} M) superfusion for 3 min. As shown in Fig. 2B, the averaged amplitude of evoked Ba^{2+} current (at −20 mV) during NPY application was significantly larger than that during the preceding vehicle application (109.8±1.8%, n=6, $P<0.01$, paired t-test). The alterations in the Ba^{2+} current were not attributable to changes in the quality of recordings because Rs and Rm remained stable in the recordings selected for analysis (see Materials and Methods). In addition, a small number of undifferentiated NG108-15 cells expressed high voltage-activated and long-lasting inward currents, but those were unaffected or reduced by NPY (data not shown).

To rule out the possibility of a wash-out effect, NG108-15 cells were treated with the vehicle twice. As shown in Fig. 2A and Fig. 4B, the peak amplitude increased to a lesser extent (2.7±2.5%), albeit the increase was not significant in comparison with the preceding vehicle treatment ($P=0.36$, n=7, paired t-test).

When traces during vehicle treatment and those of NPY treatment were normalized and overlaid, little change in the kinetics of rise or inactivation was

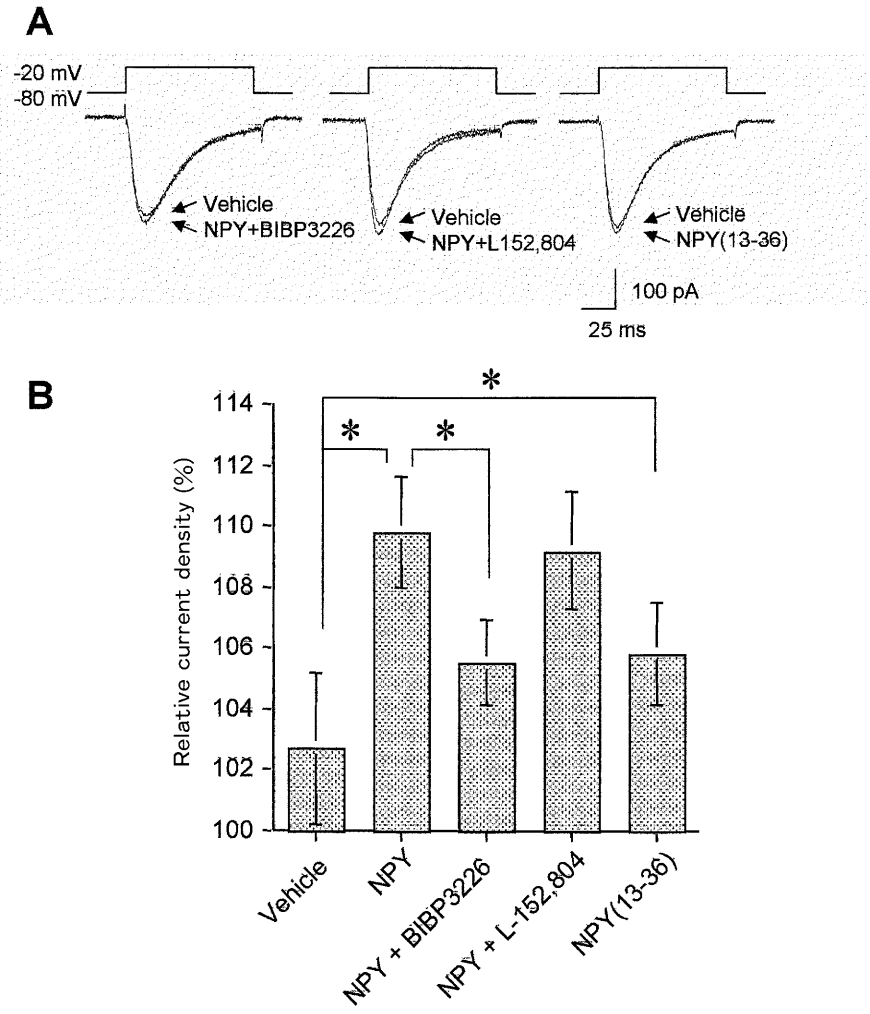

Fig. 4. NPY receptor subtypes involved in the augmentation.

After formation of a stable whole cell patch clamp, the vehicle was applied for 3 min prior to drug application, and then a second round of the vehicle (n=7), NPY alone (n=6), NPY+BIBP3226 (n=8), NPY+L-152,804 (n=6), or NPY(13-36) (n=7) was applied for 3 min. **A.** Traces of the vehicle and those of NPY+BIBP3226, NPY+L152,804, or NPY(13-36) were overlaid, respectively. **B.** Ordinate indicates mean and SE of relative values, which were calculated with evoked current densities during drug applications to those during the preceding vehicle application. *, $P<0.05$.

observed (Fig. 3A). The sensitivity to Ni^{2+} was also unaffected (data not shown), indicating the increased component to be a T-type Ca^{2+} channel current. No substantial difference was observed between the effects of 3×10^{-8} and 1×10^{-7} M NPY (data not shown). Since EC_{50} values of NPY to inhibit cAMP formation by Y_1, Y_2, and Y_5 receptors expressed in 293 cells were 0.14 nM, 1.2 nM and 0.96 nM, respectively[25], it is unlikely that higher doses of NPY have a larger effect on the Ba^{2+} current.

When the I-V relationship during vehicle-treatment and that of NPY were compared, the curve was shifted negatively (Fig. 3B). The half activation voltage was significantly lowered by NPY treatment (from -29.0 ± 1.5 mV to -32.9 ± 1.8 mV, n=6, $P<0.005$, paired t-test). This negative shift resulted in relatively higher extents of augmentation at lower voltages: $53.2\pm4.2\%$, at -40 mV; $18.1\pm3.1\%$, at -30 mV; $9.8\pm1.8\%$, at -20 mV (n=6).

NPY receptor subtypes involved in the augmentation

To determine which subtype of the NPY receptor

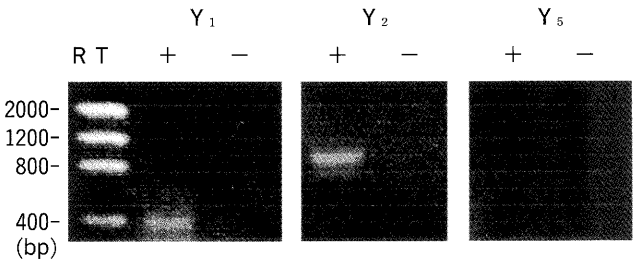

Fig. 5. Expression of NPY receptor subtypes in undifferentiated NG108-15 cells.
Total RNA prepared from undifferentiated NG108-15 cells was subjected to reverse transcription (RT)-PCR for Y_1, Y_2 and Y_5 receptors. The RT (-) reactions served as a negative control for the lack of contamination of genomic DNA. Mobility of size markers is shown at the left.

mediates the augmentation, subtype-specific antagonists were added to the external solution with NPY. As shown in Fig. 4, NPY (1×10^{-7} M) augmented the Ba^{2+} current in the presence of a Y_5-specific antagonist, L-152,804 (1×10^{-6} M). However, a Y_1-specific antagonist, BIBP3226 (1×10^{-6} M), suppressed the augmentation to 39.4% of that by NPY alone. Likewise, NPY (13-36) (1×10^{-7} M), a Y_2-receptor selective agonist, significantly increased the amplitude, but the effect was partial (43.7%). Thus, it can be suggested that both Y_1 and Y_2 receptor subtypes are additively involved in this augmentation, and their relative contributions are approximately 60 and 40%, respectively.

We next examined mRNA expression for Y_1, Y_2, and Y_5 receptor subtypes in undifferentiated NG108-15 cells with RT-PCR. PCR products for Y_1 and Y_2 receptor genes were detected at the predicted sizes (404 bp and 918 bp, respectively). In contrast, our PCR assay for the Y_5 receptor failed to detect any product at the predicted size (1362 bp).

DISCUSSION

T-type Ca^{2+} channels play many physiologically important roles in the central and peripheral nervous systems. Nevertheless, their modification has been poorly understood. Expressions of NPY and its receptors colocalize with those of T-type Ca^{2+} channels, implying a functional interaction between them. In this report, the expression of the T-type Ca^{2+} channel current in undifferentiated NG108-15 cells was confirmed by sensitivities to divalent cations and kinetic properties with the whole-cell patch-clamp technique. NPY treatment significantly augmented the T-type Ca^{2+} channel current without affecting its kinetics. The I-V relationship was shifted negatively by this treatment. Thus, NPY is suggested to augment the T-type Ca^{2+} channel current, and thereby may play a role in regulating the excitability of neuronal and non-neuronal cells.

A selective Y_1 antagonist, BIBP3226, partly reduced the augmentation of the T-type Ba^{2+} current while a Y_5 antagonist, L-152, 804, did not. Similarly a Y_2 agonist, NPY (13-36), partially increased the amplitude, suggesting that both Y_1 and Y_2 receptor subtypes mediate this effect additively. Consistently, RT-PCR assays detected the expression of mRNAs coding Y_1 and Y_2 receptors, but not that for the Y_5 receptor. These receptors couple to pertusis toxin-sensitive G-proteins, Gi and Go, and thereby inhibit adenylyl cyclase and mobilize intaracellular Ca^{2+}. These intracellular signaling pathways might be involved in the augmentation of the Ba^{2+} current in undifferentiated NG108-15 cells. Indeed, Ca^{2+}- and calmodulin-dependent protein kinase II was shown to modulate the amplitudes of the T-type Ca^{2+} channel[26,27].

Our present results are inconsistent with a previous report[9], which found that NPY treatment reduced N- and P/Q-type Ca^{2+} channel currents but did not affect those of T-type in thalamic slice preparations. This might be simply attributable to the extent of augmentation being so small that this effect was not mentioned. Alternatively, this discrepancy might be attributable to a difference in the effect of NPY depending on the cell type. In fact, differential effects of NPY on L-type Ca^{2+} channel have been reported in PC12, and sympathetic and vascular smooth muscle cells[6,10,28,29]. Similarly, angiotensin II stimulated T-type Ca^{2+} channels in adrenal glomerulosa cells[30], but

inhibited those in NG108-15 cells[31]. Moreover, a recent study showed that the regulation of T-type Ca^{2+} currents by $\beta\gamma$ subunits of trimer G-protein depends not only on subtypes of T-type Ca^{2+} channel per se, but also on those of β and γ subunits[32].

T-type is the sole voltage-gated Ca^{2+} channels that can be activated at a subthreshold potential for the Na^{+} channel. Therefore, the augmentation by NPY might play significant roles in both regulating neuronal excitability and in the Ca^{2+}-dependent signaling pathway of neuronal and non-neuronal cells. NPY has differential effects on the hypothalamic neuronal activities; NPY signaling, which originates from neurons in the arcuate nucleus, stimulates neuronal activity in the pareventricular nucleus, but inhibits that in the lateral hypothalamic area[33]. Augmentation of the T-type Ca^{2+} channel current might be involved in the differential effects of NPY on neuronal excitability together with the inhibitory effect of NPY on the other Ca^{2+} channels. In this study we showed the augmentation by NPY on T-type Ca^{2+} channel current in undifferentiated NG108-15 cells in spite of the small effect. This might be due to a methodological problem. The whole cell cramp technique used in this paper might interfere with the signaling pathways of NPY because intracellular concentrations of second messengers such as cAMP and Ca^{2+} could be strongly affected by this technique. Further study is necessary to reveal the functional interaction between the T-type Ca^{2+} channel and NPY using slice preparations of the hypothalamus, thalamus, hippocampus, and sinoatrial node, where both proteins are expressed abundantly.

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