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Activation of G-protein-coupled receptor 30 increases T-type calcium currents in trigeminal ganglion neurons *via* the cholera toxin-sensitive protein kinase A pathway

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G protein-coupled receptor 30 (GPR30) is a seven transmembrane domain G protein coupled receptor. In our study, GPR30 expression was found in trigeminal ganglia (TG) in mice, detected by RT-PCR and western blotting. We examined the effects of GPR30 activation on T-type calcium channels using GPR30-specific compound 1 (G-1), a GPR30-selective agonist, in TG neurons and demonstrated that G-1 induced an increase in T-type calcium channel currents (T-currents) in TGs. Intracellular infusion of GDP-β-S and pretreatment of the neurons with cholera toxin (CTX) blocked the effects of G-1, suggesting that the G_s-protein was involved. Intracellular application of the protein kinase A (PKA) inhibitor PKI 6-22 or pretreatment of the neurons with H89 abolished G-1-induced enhancement of T-currents in TG neurons. However, incubation with PKC inhibitor elicited no such effects. In conclusion, our study shows that activation of GPR30 by G-1 increases T-currents *via* the CTX-sensitive and PKA-dependent pathway.

1. Introduction

Estrogens have many different molecular targets, including two well-characterized members of the nuclear receptor superfamily, the estrogen receptors (ER) α and β (Dahlman-Wright et al. 2006). ERα and/or ERβ are widely considered to mediate the effects of estrogen. Recently, a novel estrogen receptor was identified, G protein-coupled receptor 30 (GPR30) (Bologa et al. 2006). GPR30, also called G protein-coupled estrogen receptor 1, is a seven transmembrane domain G protein coupled receptor. Expression of GPR30 has been identified in heart, skeletal muscle, kidney, lung, liver, prostate and ovarian tissue and also in discrete areas of the brain (Carmeci et al. 1997; O'Dowd et al. 1998; Owman et al. 1996). In 2006, the first GPER-selective agonist, GPR30-specific compound 1 (G-1), was identified (Bologa et al. 2006), and G15, another GPER-selective antagonist, was identified in 2009 (Dennis et al. 2009). G-1 has not only very high affinity for GPR30 but also great selectivity toward GPR30 as compared with ERα and ERβ (Bologa et al. 2006).

T-type calcium channels display rapid gating kinetics and a small single channel amplitude (Iftinca and Zamponi 2009). In addition, they require much smaller membrane depolarizations for opening (Iftinca and Zamponi 2009). As a result of this voltage sensitivity, T-type calcium channels are well suited for regulating cellular excitability. Inappropriate calcium entry through T-type calcium channels has been associated to some pathophysiologies like epilepsy and pain (Nelson et al. 2006; Todorovic and Jevtovic-Todorovic 2007). Moreover, T-currents in trigeminal ganglion (TG) neurons have been detected in electrophysiological recordings (Borgland et al. 2001; Ikeda and Matsumoto 2003). The expression of T-type calcium channels in TGs suggests that they may be involved in pain-sensing processes.

In the present study, we investigated the effects and the related mechanisms of GPR30 activation on T-currents using G-1, a GPR30-selective agonist, in TG neurons in mice.

2. Investigations and results

2.1. *GPR30 expressed in mouse TGs*

To determine whether GPR30 is expressed in TGs in mice, RT-PCR and western blotting were used to characterize mRNA and protein expression, respectively. RT-PCR analysis demonstrated that GPR30 mRNA (the predicted size of the specific transcripts was 434 bp) was detected in TGs (Fig. 1A). Negative controls did not yield a detectable band. We then performed western blotting on homogenates of TGs using a GPR30-specific antibody, and a single band of approximately 38kDa was detected (Fig. 1B). As a positive control, mouse uterus which is known to express GPR30, produced a band of approximately 38kDa (Fig. 1B).

2.2. *G-1 increases T-currents in TG neurons*

In this study, small-size (<30 μm soma diameter) TG neurons were selected for recording, because these cells are typically involved in nociceptive processing (Todorovic and Lingle 1998; Todorovic et al. 2001). To isolate T-currents, we recorded the barium currents elicited by a 40-ms long depolarizing step pulse with a holding potential of -110 mV to -40 mV. The addition of Ni^{2+} (100 μM) (Lee et al. 1999), a specific T-type calcium channel blocker, inhibited inward currents by approximately 89.2% ($p < 0.001$, $n = 8$) (Figs. 2A and B), further confirming the identification of T-currents. The effect of G-1 (100 nM) was tested on T-currents in TG neurons. The traces presented in Fig. 2C and

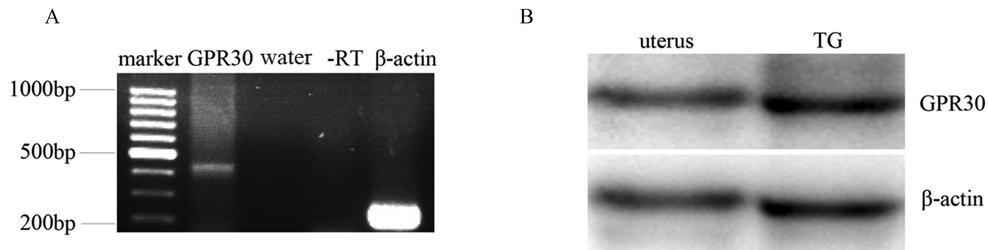

Fig. 1: (A) Detection of GPR30 mRNA in TGs by RT-PCR. The reactions without cDNA template (water) or without reverse transcriptase (-RT) were served as negative control. (B) Western blotting analysis of GPR30 (~38kDa) protein expression in TGs. Mouse uterus expression of GPR30 (~38kDa) is used as a positive control.

the temporal record depicted in Fig. 2E show a representative TG neuron, for which the application of 100 nM G-1 in external solution reversibly increased the peak T-currents by approximately 26.8% ($n = 9$, $p < 0.001$). The increase in T-currents induced by short exposures to G-1 (approximately 1-2 min) was reversible during washout (Fig. 2D). We then analyzed the sensitivity of the T-currents increase by testing different concentrations of G-1 in different TG neurons (Fig. 2F).

2.3. *The G-1 mediated increase in T-currents is sensitive to cholera toxin*

As GPR30 is a G protein coupled receptor, we initially examined whether heterotrimeric G-proteins are involved in the G-1-mediated increase in T-currents. We injected the neurons with guanosine-5′-*O*-(2-thiodiphosphate) (GDP-β-S, 1 mM), which is a non-hydrolysable GDP analog. Fig. 3A shows that GDP-β-S completely abolished the increase in T-currents induced by 100 nM G-1 ($1.5 \pm 0.5\%$, $n = 6$), suggesting that G-1 action requires G-protein activation.

We then tested which form of G_α was involved in the increase. First, we examined the effects of G-1 on T-currents in the presence of pertussis toxin (PTX, 0.2 μg/ml), which catalyzes the ADP-ribosylation of $G_{i/o}\alpha$, G-1 still increased the T-currents under these conditions ($23.2 \pm 1.9\%$, $n = 6$, Fig. 3B), indicating that the increase in T-currents was $G_{i/o}\alpha$-independent. We then pretreated the cells with cholera toxin (CTX, 0.5 μg/ml), which inactivates $G_S\alpha$ by ADP-ribosylation. Notably, the enhancing effect of G-1 was abolished ($3.4 \pm 0.8\%$, $n = 8$, Fig. 3C). The increase in T-currents induced by G-1 was sensitive to CTX, but not PTX, suggesting that $G_S\alpha$, but not $G_{i/o}\alpha$, was involved in the increase.

2.4. *Protein kinase A is involved in G-1-mediated T-currents increase*

As G-1 appeared to increase T-currents *via* the indirect action of the $G_S\alpha$-protein, we then investigated the downstream intracellular signaling molecules involved in this process. It has been previously reported that T-type calcium channels can be regulated by the PKC pathway (Chemin et al. 2007; Park et al. 2006). Therefore, we determined whether the PKC dependent pathway mediated the effects of G-1. Pretreatment of mouse TG neurons

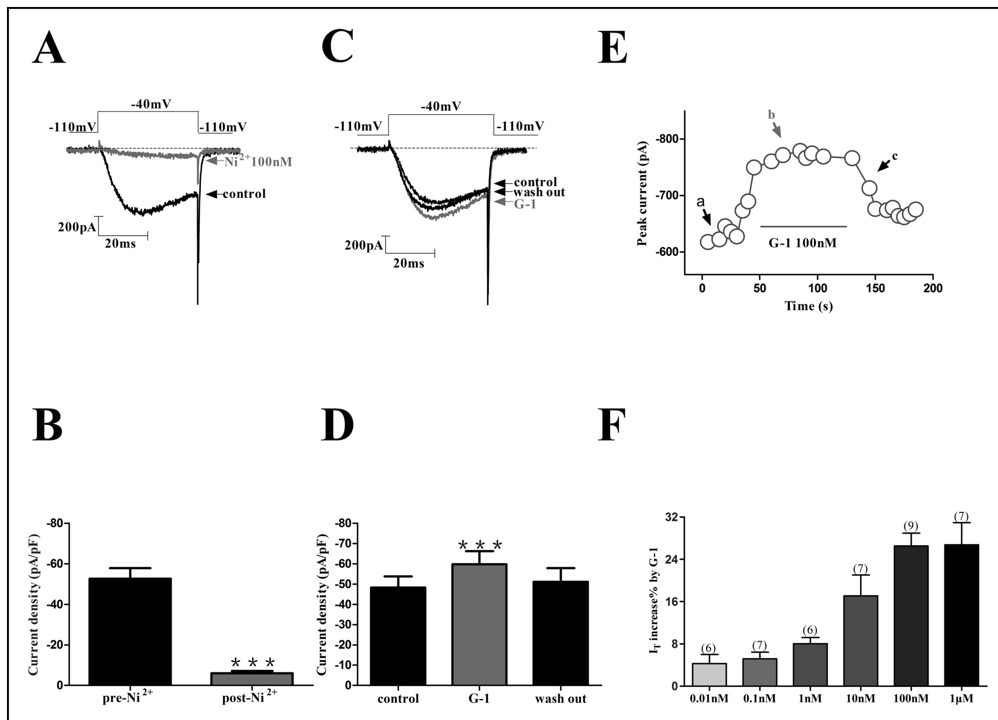

Fig. 2: (A) The effect of 100 μM Ni^{2+} on T-currents. The dotted line above current traces represents the level of zero current. (B) Summary data showing the effect of 100 μM Ni^{2+} on T-currents density. (C) Traces of T-currents in a representative TG neuron before, after and during exposure to 100 nM G-1 (gray trace). (D) Summary data showed T-currents density under control conditions, in the presence of G-1 (100 nM) and during washout. (E) A temporal record of T-currents recorded under the conditions presented in panel C. (F) The concentration–response relationship for a G-1-induced increase in T-currents. Numbers in brackets indicate the number of experiments for each concentration of G-1.

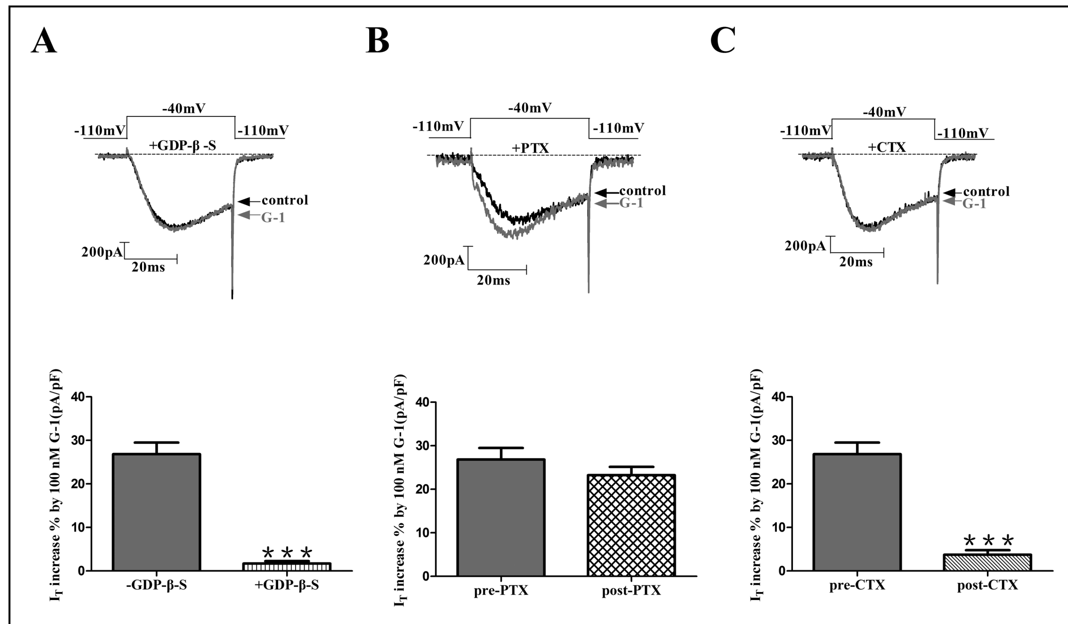

Fig. 3: (A-C) Exemplary traces and summary data showed the effects of G-1 (100 nM) on T-currents in the presence of GDP-β-S (1 mM, A), PTX (0.2 μg/ml, B) and CTX (0.5 μg/ml, C). $^{***}p < 0.001$ *versus* control.

with the PKC inhibitors GF109203X (1 μM, 24.5 ± 2.5%, n = 6, Figs. 4A) or Ro 31–8220 (2 μM, 25.2 ± 3.1%, n = 6, Figs. 4B) did not significantly affect the T-currents increase induced by G-1. Previous studies have shown that $G\alpha_s$ stimulates protein kinase A (PKA) activity (Chemin et al. 2007; Kim et al. 2006). Therefore, we determined whether PKA is involved in the G-1-induced increase in T-currents. We pretreated the cells with a PKA inhibitor, H89 (1 μM), which entirely abolished the G-1-induced increase in T-currents (1.9 ± 0.3%, n = 6, Fig. 4C). In order to further confirm the PKA mediated T-currents increase, a pipette internal solution containing another PKA inhibitor, PKI 6-22, was dialyzed into the neurons. Our results indicated that intracellular application of PKI 6-22 (1 μM) blocked the G-1-induced increase in T-currents (2.8 ± 0.7%, n = 8, Figs. 4D). To further reinforce these observations, we investigated whether 8-Br-cAMP, a PKA activator, would occlude the G-1-mediated increase in T-currents. In fact, application of 100 μM 8-Br-cAMP to TG neurons mimicked the G-1-induced increase in T-currents (20.0 ± 1.6%, n = 7, Figs. 5A and B).

3. Discussion

Our results indicated that activation of GPR30 by G-1 enhanced T-currents in a concentration-dependent manner *via* the CTX-sensitive PKA pathway. These results suggest that GPR30 may play a role in primary sensory neurons in estrogen-modified pain.

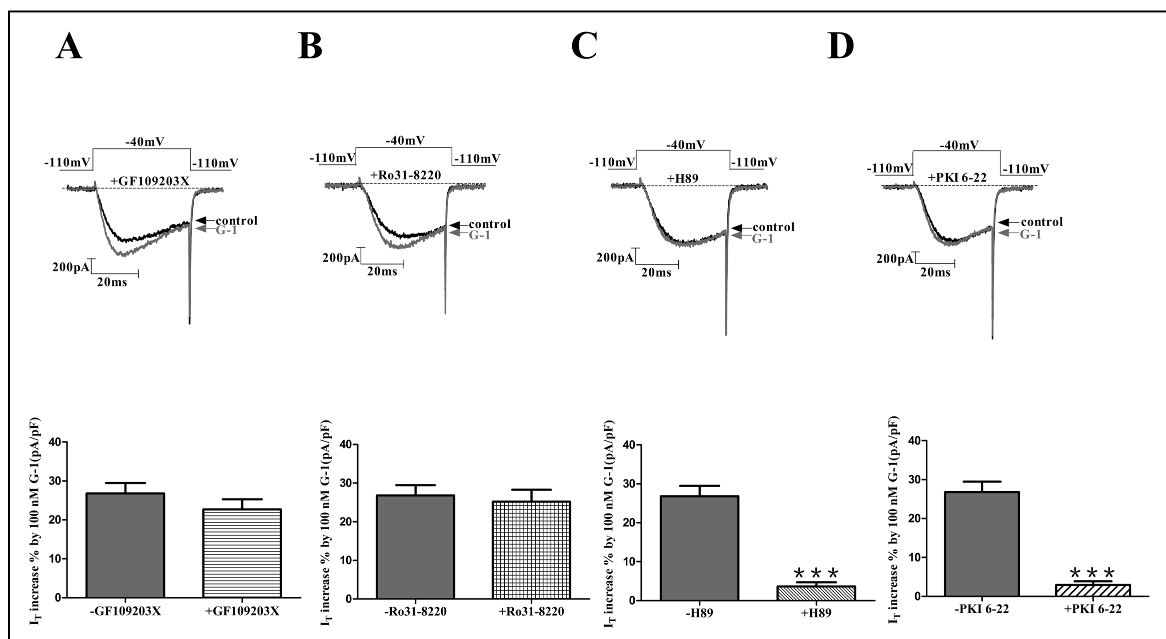

Fig. 4: Exemplary traces and summary data showed an increase in T-currents induced by G-1 (100 nM) in the presence of GF109203X (1 μM, A), Ro 31-8220 (2 μM, B), H89 (1 μM, C) and PKI 6-22 (1 μM, D). $^{***}p < 0.001$ *versus* control.

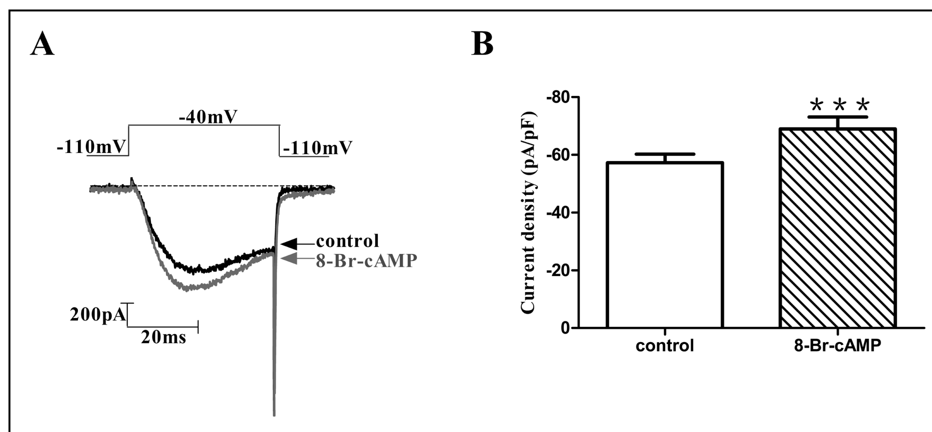

Fig. 5: (A) Effect of 100 μM 8-Br-cAMP on T-currents. (B) Summary data showed the effect of 100 μM 8-Br-cAMP on T-currents density. (C) Effect of treatment with G-1 (100 nM) or G-1 (100 nM) in combination with H89 (1 μM) on cAMP production. ***$p < 0.001$ *versus* control.

3.1. Indirect G_s- mediated increase in T-currents

GPR30 is a seven transmembrane-domain G-protein-coupled receptor. G proteins are αβγ heterotrimers, and members of this family include the PTX-sensitive (G_i and G_o) and CTX-sensitive (G_s) G proteins. In this study, the effects of G-1 on T-currents in TG neurons were CTX-sensitive, and the effect was abolished by the nonselective G-protein inhibitor GDP-β-S. These results suggest that the G-1-mediated increase in T-currents was coupled with $G_{s.}$

3.2. PKA is an intracellular mediator downstream of GPR30

Some reports have shown that PKC activation can modulate T-currents (Chemin et al. 2006). In this study, we found that the effects of G-1 on T-currents were independent of PKC, which suggests that some other mechanisms were involved in the G-1-mediated effects. Our data indicated that the preincubation of TG neurons with H89 or intracellular application of PKI 6-22 could remarkably block the effects of G-1 on T-currents in TG neurons, which indicated that the PKA pathway was involved in the G-1-mediated increase in T-currents. Our current results are supported by previous studies that demonstrate that T-currents are increased by 5-HT in rat glomerulosa cells via a PKA-dependent mechanism (Lenglet et al. 2002).

3.3. An increase in T-currents caused by GPR30 may participate in estrogen-modified pain

Molecular studies have shown that T-type calcium channels are encoded by three different genes ($Ca_v3.1$, $Ca_v3.2$, and $Ca_v3.3$), and electrophysiological characterization has demonstrated that the activation thresholds of these three isoforms are close to the resting membrane potential. Therefore, they could play an important role in initiating depolarizing signals at the resting membrane potential that contribute to the formation of action potentials in the coding of pain signals. Indeed, previous studies have shown that T-type calcium channels are involved in pain (Bourinet et al. 2005; Kim et al. 2003).

Some pain may be estrogen dependent. For example, estrogen supplementation in transsexuals has resulted in strong pain phenotypes (Aloisi et al. 2007). Moreover, some studies have shown that GPR30 agonists induce mechanical hyperalgesia in the rat (Kuhn et al. 2008) and increase orofacial sensitivity (Liverman et al. 2009). In our study, we have demonstrated that activation of GPR30 by G-1 enhanced T-currents in TGs. This process may be a novel pathway through which estrogen modulates the activity of sensory neurons and thus may be related to the effects of hormonal fluctuations in pain.

Overall, the result of this study shows a novel action of GPR30 activation on T-currents in TGs. We firstly demonstrate a CTX-sensitive PKA pathway by which G-1increased T-currents and thus provide a new insight into the complex relationship between estrogen and pain. Nevertheless, further study is required to understand the details of this cascade.

4. Experimental

In accordance with the *Guide for Care and Use of Laboratory Animals* published by the US National Institutes of Health, all efforts were made to minimize the number and suffering of animals used in this study.

4.1. Pharmacological agents

All drugs were purchased from Sigma (USA) unless otherwise indicated. Stock solutions of GDP-β-S, cholera toxin (CTX), pertussis toxin (PTX), PKI 6-22, Ro 31-8220 and H-89 were prepared in ultra-pure water. Stock solutions of GF109203X was prepared in dimethyl sulfoxide (DMSO). The final concentration of DMSO in the bath solution is less than 0.01% and had no functional effects on T-type calcium channels (Tao et al. 2009). Stock solutions of G-1 (Cayman Chemical), 8-Br-cAMP (Sigma-Aldrich) were prepared in 100% ethanol.

4.2. Acutely isolated TG neurons

TGs were isolated from female adult mice (ICR, 4-6 weeks) and transferred to dissecting solution, containing (in mM) 5 KCl, 1.5 $CaCl_2$, 130 NaCl, 2 KH_2PO_4, 6 $MgSO_4 \bullet 7H_2O$, 10 HEPES, and 10 glucose, pH 7.2

Table: Primers used for RT-PCR analysis of GPR30

Gene	Primers for RT-PCR	Accession number (GenBank)
GPR30	5'-ACCTGTCGAAGCTCATCCAGGTGAG-3' 5'-GGTGGAGATCTACCTAGGTCCCGTG-3'	NM_029771.3
β-Actin	5'-TCAGGTCATCACTATCGGCAAT-3' 5'-AAAGAAAGGGTGTAAAACGCA-3'	NM_011526

(osmolarity: 305 mOsm). The TGs were cleared of connective tissue and incubated in dissecting solution containing collagenase IV (1.5 mg/mL, Sigma) for 40 min. The tissues were then incubated with collagenase IV (1.5 mg/mL, Sigma) and trypsin (1 mg/mL, Sigma) for another 30 min at 34.5 °C. Next, TGs were washed with dissecting solution. Finally, a single-cell suspension was obtained by gentle trituration using a Pasteur pipette and cells were plated onto glass coverslips.

4.3. *Reverse transcription-PCR (RT-PCR)*

Total RNA was extracted from mouse TGs as described previously (Tao et al. 2008). Synthesis of first-strand cDNA was conducted with SuperScript II (Invitrogen). The primer sequences used in this study are shown in the Table. PCR reactions were performed using the following conditions: 94 °C for 5 min; followed by 35 cycles of 94 °C for 30 s, 60 °C for 30 s and 72 °C for 30 s; and a final extension for 10 min at 72 °C. PCR was performed at least twice on each sample to confirm reproducibility.

4.4. *Western blotting*

Western blotting analysis was performed following the procedure as described previously (Tao et al. 2008). For antibody detection, membranes were incubated in 5% non-fat milk in TBST at room temperature for 1 h. Then, membranes were incubated with a rabbit anti-GPR30 antibody (1:400, Santa Cruz) overnight at 4 °C. After 12 h, the membranes were washed in TBST three times and incubated at room temperature for 1 h with secondary antibody (1:2,000, Bioworld). A CCD camera system (Bio-Rad) was used to detect the specific binding of the primary antibody.

4.5. *Whole-cell patch-clamp recording*

Whole cell current-clamp recordings were made using a MultiClamp 700B amplifier (Molecular Devices). A Digidata 1440A interfaced with a Clampex 10.2 (Molecular Devices) was used to control the digitization of membrane voltages and currents. Currents were lowpass filtered at 2-5 kHz. Series resistance compensation was employed to improve voltage-clamp control (>75%). Recording electrodes were pulled from borosilicate glass micro-capillary tubes (World Precision Instruments). The external solution for voltage-clamp experiments contained (in mM), 140 TEA-Cl, 5 $BaCl_2$, 25 glucose, 20 CsCl, and 10 HEPES, adjusted to pH 7.35 with TEA-hydroxide (OH). To permit the study of isolated, clamped T-currents in acutely isolated TG neurons, we used the following internal solution (in mM): 10 EGTA, 110 CsCl, 4 MgATP, 0.3 Na_2-GTP and 25 HEPES. The pH was adjusted to 7.4 with CsOH.

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