The Journal of Headache and Pain

RESEARCH

Open Access

$Ca_V3.2$ T-type calcium channels contribute to CGRP- induced allodynia in a rodent model of experimental migraine

Darciane F. Baggio[1], Eder Gambeta[2], Ivana A. Souza[2], Sun Huang[2], Gerald W. Zamponi[2] and Juliana G. Chichorro[1*]

Abstract

Background Migraine is a painful neurological syndrome characterized by attacks of throbbing headache, of moderate to severe intensity, which is associated with photo- and phono- sensitivity as well as nausea and vomiting. It affects about 15% of the world's population being 2–3 times more prevalent in females. The calcitonin gene-related peptide (CGRP) is a key mediator in the pathophysiology of migraine, and a significant advance in the field has been the development of anti-CGRP therapies. The trigeminal ganglion (TG) is thought to be an important site of action for these drugs. Moreover, experimental migraine can be induced by CGRP injection in the TG. The signaling pathway induced by CGRP in the TG is not fully understood, but studies suggest that voltage-gated calcium channels contribute to CGRP effects relevant to migraine.

Objective We hypothesised that CGRP injection in the TG enhances $Ca_V3.2$ T-type calcium channel currents to contribute to the development of periorbital mechanical allodynia.

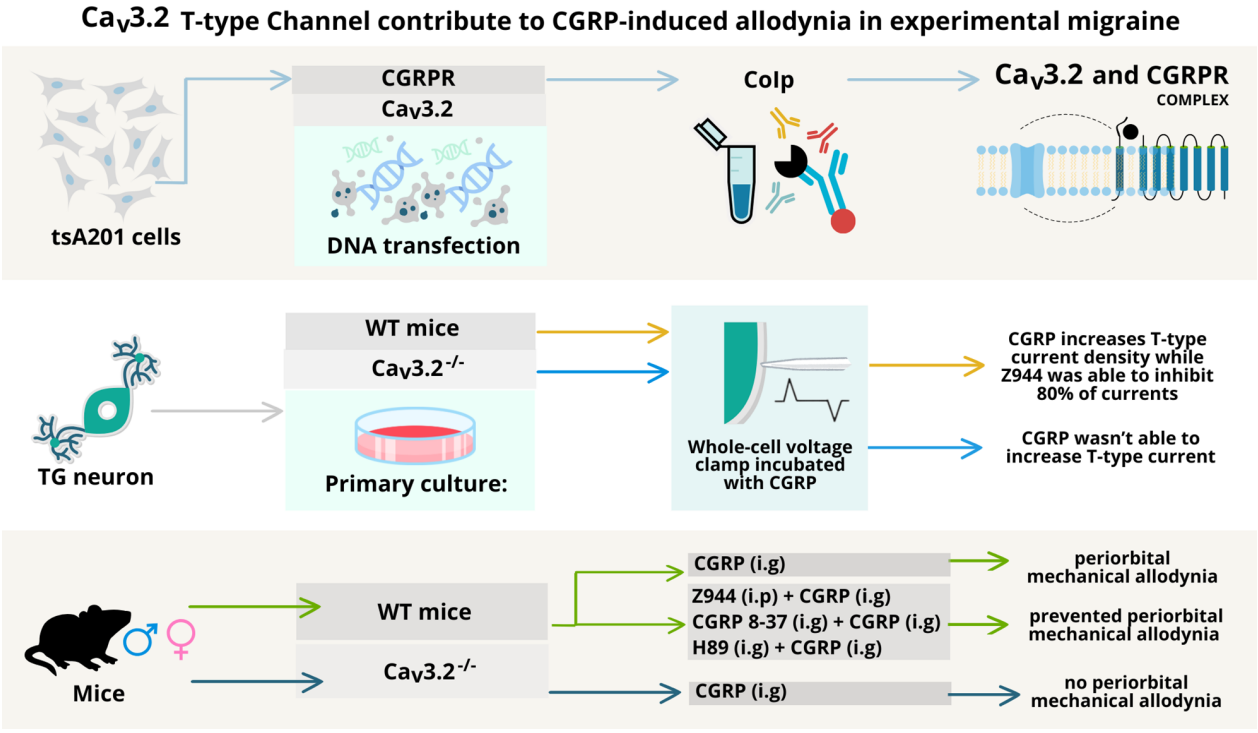
Results A Co-Immunoprecipitation assay in tsA-201 cells revealed that $Ca_V3.2$ channels form a complex with RAMP-1, a component of the CGRP receptor. Constitutive CGRPR activity was able to inhibit $Ca_V3.2$ channels and induce a depolarizing shift in both activation and inactivation curves. Incubation of TG neurons with CGRP increased T-type current density by ~ 3.6 fold, an effect that was not observed in TG neurons from $Ca_V3.2$ knockout mice. Incubation of TG neurons with Z944, a pan T-type channel blocker, resulted in an approximately 80% inhibition of T-type currents. In vivo, this treatment abolished the development of periorbital mechanical allodynia induced by CGRP in male and female mice. Likewise, $Ca_V3.2$ knockout mice did not develop periorbital mechanical allodynia after intraganglionic CGRP injection. Finally, we demonstrated that the CGRP effect depends on the activation of its canonical GPCR, followed by protein kinase A activation.

Conclusion The present study suggests that CGRP modulates $Ca_V3.2$ in the TG, an effect possibly mediated by the canonical CGRP receptor and PKA activation. The increase in T-type currents in the TG may represent a contributing factor for the initiation and maintenance of the headache pain during migraine.

*Correspondence:
Juliana G. Chichorro
juliana.chichorro@ufpr.br

Full list of author information is available at the end of the article

Graphical abstract



Keywords Voltage-gated calcium channels, Calcitonin gene-related peptide, RAMP-1, Periorbital mechanical allodynia, Mice, Trigeminal ganglion, Electrophysiology

Introduction

Migraine is a complex neurological disorder which significantly impacts the patient's quality of life, daily activities, productivity, and is associated with substantial healthcare costs [1]. A migraine crisis is typically characterized by recurring attacks of severe headache, nausea, vomiting, high-sensitivity to light and sound, and other variable physical, mental and psychological signs and symptoms, lasting 4–72 h [2]. The cumulative lifetime risk of migraine in the general population is about 33% in women and 18% in men [3]. Besides the higher prevalence, women also present more debilitating symptoms, longer attacks and worse response to treatments [4]. In fact, migraine is the number one cause of disability in women younger than 50 years [5].

Migraine pathophysiology is still not fully understood and involves multiple mediators and events in the trigeminovascular system and in the central nervous system. The calcitonin gene related peptide (CGRP) is considered a key mediator in migraine pathophysiology [6]. For instance, CGRP infusion resulted in delayed migraine-like headache in migraine patients [7], and is released into the cranial circulation in patients with prolonged, severe migraine [8, 9]. In the periphery it causes potent vasodilation of cerebral arteries and neurogenic inflammation [10]. In the trigeminal ganglia (TG), CGRP is the most prominent neuropeptide being expressed by around 40–50% of the TG neurons [11, 12]. It is released from the cell bodies of stimulated C-fibers and can cause excitation of Aδ neurons and satellite glial cells, promoting peripheral sensitization and pain maintenance during a migraine crisis [13, 14].

The CGRP receptor (CGRPR) is a $G\alpha_s$ linked G-protein coupled receptor formed by the calcitonin-like receptor (CLR), a small transmembrane protein called receptor activity modifying protein 1 (RAMP-1) and a cytoplasmic protein, receptor component protein, which enhances receptor coupling to the G-protein signalling machinery. The CGRPR complex is widely distributed in the trigeminovascular system in addition to the terminals of second order neurons in the medullary dorsal horn, where its activation contributes to the facilitation of pain transmission from the periphery to the central nervous system [15, 16].

The cumulative knowledge about CGRP structure, function and signalling mechanisms has led to the development of effective and highly selective anti-CGRP therapies for migraine. However, these therapies are neither

accessible nor effective for all migraine sufferers [17]. Thus, a better understanding of migraine mechanisms and the identification of potential targets are still clearly warranted.

Voltage-gated calcium channels (VGCCs) are divided into two major groups: high voltage-activated (HVA) and low voltage-activated (LVA). The latter, known as T-type calcium channels, encompasses $Ca_V3.1$, 3.2 and 3.3. There is growing evidence that $Ca_V3.2$ is the major isoform expressed in nociceptive neurons and its activity and/or expression is significantly increased in the spinal dorsal horn and in the dorsal root ganglia (DRG) in different inflammatory and neuropathic pain models [18]. In the DRG, $Ca_V3.2$ channels contribute to increased neuronal excitability, spontaneous activity and mechanical allodynia in models of inflammatory and neuropathic pain. Thus, its is likely that these channels also modulate TG neurons excitability. Here, we hypothesised that CGRP injection in the TG may modulate $Ca_V3.2$ channels, thus leading to enhanced T-type currents as a contributing mechanism to the development of periorbital mechanical allodynia.

Methods

Animals

Male and female C57BL mice (7–9 weeks old) were purchased from Jackson laboratories and *Cacna1h* knockout ($Ca_V3.2^{-/-}$) mice were produced in house from breeding pairs originally obtained from Jackson Laboratories (strain #013770). Experimental procedures were performed during the light cycle, between 7 a.m. and 7 p.m., with at last 48 h for the acclimatization of the animals to the environment. Animals were kept in groups of 5 per cage (30×20×15 cm) in a climate-controlled room (22±2 ºC) on a 12-hour light/dark cycle with chow and water *ad libitum*. All procedures were performed in accordance with animal care regulations and policies of the Canadian Council on Animal Care, ARRIVE guideline and the guideline of the Federal University of Parana, and were approved by the Universities' Ethics Committees (AC21-0088; CEUA/BIO-UFPR; #1532). All efforts were made to optimize the number of animals used and to reduce their stress and suffering, respecting the 3 R's statement. Sample size was determined based on the GPower 3.1 software, defining a large standardized effect size of F=0.5; power of 0.8; and α=0.05, and estimated 10 mice per group in the different experimental designs.

Cell culture and cDNA transfection

Human embryonic kidney tsA-201 cells were cultured in standard Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum (FBS) (heat inactivated) and 50 U/ml penicillin and 50 µg/ml streptomycin. Cells were kept at 37 °C in a humidified incubator with 5% CO_2. For the biochemistry experiments, cells were co-transfected with 3 µg of $hCa_V3.2$, 3 µg of hCLR-FLAG (Origene, NM_005795) and 3 µg of hRAMP-1-FLAG (Origene, NM_005855) cDNA using the calcium phosphate method [19]. For the electrophysiology experiments, cells were transfected with either 3 µg $hCa_V3.2$ or co-transfected with 3 µg of hCLR and 3 µg of hRAMP-1, and 0.5 µg of eGFP (cDNA generously donated by Dr. Debbie Hay, University of Otago, NZ).

TG of 5- to 7-week-old female mice were collected and cut into small pieces and incubated with 4 mg/mL of collagenase and 40 µL/mL of papain in DMEM supplemented with 10% heat-inactivated FBS and 1% penicillin/streptomycin at 37˚C for 30 min and then followed by 3 washes and mechanical trituration, as previously described [20]. TG neurons were plated on glass coverslips pretreated with poly-D-lysine and laminin and kept at 37˚C in a 5% CO_2 incubator. Cells were washed with DMEM containing 5% FBS, 10 ng/mL of nerve growth factor and 10 ng/mL of glial cell line–derived neurotrophic factor. TG neurons were recorded between 16 and 24 h post plating on small to medium sized cells.

Compounds

CGRP (rat and human) was purchased from Sigma Aldrich (Cat. No. C0292, C0167, respectively) and Z944 was generously donated by Dr. Terrance Snutch (University of British Columbia, CA). CGRP was dissolved in distilled water at a stock concentration of 100 µM and Z944 was dissolved in 100% dimethyl sulfoxide (DMSO) at a stock concentration of 238 mM and stored at -20ºC. Fresh solutions were made before experiments, for in vitro experiments CGRP was diluted to a final concentration of 1 µM and Z944 of 10 µM. Cells were treated overnight with either hCGRP or water and the final concentrations were kept during recordings. For in vivo experiments CGRP was diluted in distilled water and administered to animals at 380 ng/5 µl (i.g.) and Z944 was diluted in 0.5% carboxymethylcellulose (Sigma Aldrich, USA) and administered to animals at 10 mg/kg (i.p.). CGRP8-37 and H-89 (Sigma Aldrich, USA) were diluted in sterile saline solution. Doses were selected based on previous studies [21–26].

Co-immunoprecipitation assay

tsA-201 cells were washed once with ice-cold Hepes-based saline solution and then lysed in a modified RIPA buffer (50-mM Tris, 150-mM NaCl, 0.25% Triton X-100, pH 7.5) containing 1x complete protease inhibitor cocktail. Lysates were centrifuged at 13.000 rpm for 20 min at 4ºC to remove cell debris. One milligram of cell lysates was used to immunoprecipitate $Ca_V3.2$ channels with a specific $Ca_V3.2$ polyclonal antibody (2 µg per immunoprecipitation; Novus) both were incubated in RIPA buffer

overnight at 4ºC with rotation, followed by immunoprecipitation for 1.5 h using protein A or G Sepharose beads, respectively. Immunoprecipitates were washed 3x in RIPA buffer and eluted with 2x Laemmli sample buffer. Proteins were resolved using SDS-PAGE, transferred to polyvinylidene fluoride membranes immunoblotted with one of the following antibody anti-FLAG (1:1000; Sigma) overnight at 4ºC. Membranes were then incubated with horseradish peroxidase-linked anti-mouse or anti-rabbit antibodies and developed using SuperSignal West Dura substrate (Thermo Scientific) and a C-DiGit blot scanner (LI-COR).

Electrophysiology recordings

Whole-cell voltage-clamp recordings in tsA-201 cells were performed 72 h post transfection at room temperature (22–24 °C). Currents were recorded using an Axopatch 200B amplifier linked to a computer equipped with pClamp 11.2 software. The external solution contained (in mM): 10 BaCl2, 125 CsCl, 1 MgCl2, 10 HEPES, and 10 glucose (pH 7.4 adjusted with CsOH). The linear leak component of the current was corrected using a P/4 subtraction protocol and current traces were digitized at 10 kHz and filtered at 2 kHz. Glass patch pipettes were pulled, and fire polished to a resistance of between 3 and 5 MΩ and filled with internal solution containing (in mM): 130 CsCl, 2.5 MgCl2, 10 HEPES, 5 EGTA, 0.5 Mg-GTP, and 3 Na-ATP (pH 7.4 adjusted with CsOH).

Current density-voltage (IV) relation curves were obtained from the peak current of 140 ms depolarizing pulses between −80 and +40 mV in 5 mV increments from a holding potential of −110 mV. The IV relationship was obtained by dividing the peak current at each voltage by the cell capacitance. Data from individual cells were fitted using the following Boltzmann equation: $I = ((V_m - V_{rev}) \times G_{max})/(1+\exp((V_m - V_{1/2,act})/k))$ where I is the peak current, V_m is the membrane potential, V_{rev} is the reversal potential, G_{max} is the maximum conductance, $V_{1/2,act}$ is the voltage for half activation, and *k* is the slope factor. Conductance curves were obtained from the IV curves by dividing the peak current at each depolarizing step by the driving force according to the equation: $G=I/(V_m - V_{rev})$ and normalized against the maximum conductance (G_{max}).

Steady-state inactivation (SSI) curves were obtained by applying 1-s conditioning pre-pulses from −100 to −15 mV in 5 mV increments followed by a 50-ms test pulse to − 30 mV. Curves were fitted with the equation: $I_{normalized} = 1/(1+\exp(-(V_m - V_{1/2,inact})/k))$ where $V_{1/2,inact}$ is the potential for half inactivation and *k* is the slope factor.

For recordings of TG neurons, recordings were performed as described previously [22]. The external solution contained (in mM): 40 tetraethylammonium chloride, 20 BaCl2, 65 CsCl, 1 MgCl2, 10 HEPES and 10 glucose, in addition to 300 nM tetrodotoxin to block voltage-gated sodium channels. The pH was adjusted to 7.4 with CsOH. The internal solution contained the following (in mM): 140 CsCl, 2.5 CaCl2, 1 MgCl2, 5 EGTA, 10 HEPES, 2 Na-ATP and 0.3 Na-GTP, pH adjusted to 7.3. Patch clamp recordings were performed by an EPC 10 amplifier linked to a computer equipped with Pulse (V8.65) software (HEKA Elektronik, Bellmore, USA). To isolate T-type channels current, the stimulation protocol was altered slightly. TG neurons were held at −90 mV, with a 300 ms depolarization to a test potential of −30 mV. All other conditions remained the same between these experiments.

Intraganglionic injection

Animals were anesthetized with isoflurane and placed on a ventral position. The head of the mice was restrained using one hand, and with the other hand a sterile short 30-G needle was introduced into the zygomatic process through the infraorbital foramen. The needle was connected to a Hamilton 0.5-mL syringe and positioned at an 10º angle relative to the midline of the head. The needle was inserted through the infraorbital canal until its tip passed the foramen rotundum and reached the TG [21]. Only the right TG was injected with CGRP (0.1 nmol) or vehicle and the injection volume was 5 µL. After the injection, animals were monitored until complete recovery from anesthesia (around 5 min).

Assessment of periorbital mechanical allodynia

Mice was placed in a clear Plexiglas box and left to habituate for 2 h, before the assessment of periorbital cutaneous allodynia by successive applications (10 consecutive times at 30 s-intervals) of von Frey filament (Semmes–Weinstein Monofilaments, Stoelting, IL, USA) to the forehead (force 0.04 g). Attack/escape or head withdrawal reactions will be considered positive responses to mechanical periorbital stimulation [27, 28]. Only mice showing mean basal positive response frequencies up to 25% to the 10 applications was used in the subsequent experiments. After CGRP injection, animals were tested hourly, up to 4 h.

Statistical analysis

Data from the electrophysiology recordings was analyzed by two-tailed Student's *t* test while two-way analysis of variance with repeated measures followed by the Bonferroni post hoc test was used to periorbital mechanical allodynia test. All data were expressed as mean ± SEM, and results were considered statistically significant if $p<0.05$. GraphPad Prism version 9 for Windows was used for statistical analysis data (GraphPad Software, San Diego, CA).

Results

$Ca_V3.2$ forms a complex with the canonical CGRPR

Co-immunoprecipitation assays were performed to determine whether the receptor forms a complex with $Ca_V3.2$ channels (Fig. 1). tsA-201 cells were transfected with hCLR-FLAG, hRAMP-1-FLAG and $hCa_V3.2$ and FLAG was immunoprecipitated from the cell lysate. Blotting against $Ca_V3.2$ revealed a band in the 250 kDa range, indicative of a protein complex formed by $Ca_V3.2$ and the CLR+RAMP-1-FLAG (Fig. 1A). Whole cell lysate showing the expression of Ca V 3.2 channels (Fig. 1C). The reverse process was also done, such that tsA-201 cells were transfected with both RAMP-1-FLAG and $Ca_V3.2$ and $Ca_V3.2$ was immunoprecipitated from cell lysate. Blotting against RAMP-1-FLAG revealed a band in the 15 kDa range, that confirms the indicative of a protein complex formed by $Ca_V3.2$ and the RAMP-1-FLAG (Fig. 1B). Whole cell lysate showing the expression of RAMP1-FLAG (Fig. 1D).

Biophysical properties of $Ca_V3.2$ channels co-expressed with CGRPR

To assess if the CGRPR modulates $Ca_V3.2$ channels, we co-transfected tsA-201 cells with either the $Ca_V3.2$ channel alone or in combination with hCLR and hRAMP-1. Whole-cell voltage clamp was performed, and currents were recorded using Ba^{2+} as main ion carrier. IV relation curves were fitted using a modified Boltzmann equation and shows that when the $Ca_V3.2$ channel is co-expressed with CGRPR, there is a reduction in current-density as well as a depolarizing shift in the IV curve (Fig. 2A). Figure 2B shows the peak current density in each condition, with a significant reduction when CGRPR is co-expressed. A depolarizing shift can be observed in the conductance curve, leading to a depolarizing voltage for half activation when CGRPR is present (Fig. 2C and D). A similar depolarizing shift can be observed in the SSI curve and in the voltage for half inactivation (Fig. 2E and F). This depolarizing shift in both activation and inactivation curves reflects a shift in the window current, with an approximately 6 mV shift at the intersection ($Ca_V3.2$ = -58.4 mV, $Ca_V3.2$+CGRP receptor: -52.1 mV, Suppl. Figure 1 A and 1B). When the area under the window current was analyzed, the $Ca_V3.2$+CGRPR shows around 20% increase in the area (Suppl. Figure 1 C). Overall, the functional effects of receptor co-expression are consistent with the presence of a protein complex.

CGRP increases T-type currents in TG neurons

We performed whole-cell voltage clamp recordings from acutely dissociated TG neurons from WT and $Ca_V3.2^{-/-}$ mice. To isolate T-type calcium channel currents, the cell membrane was maintained at – 90 mV, with voltage steps to – 30 mV. Chronic treatment with 1 µM rat CGRP increased T-type current density in ~3.6 fold (Vehicle=3.3 pA/pF and CGRP=11.9 pA/pF) in TG neurons from WT mice (Fig. 3A). In $Ca_V3.2^{-/-}$ mice, overall T-type calcium channel current was not significantly

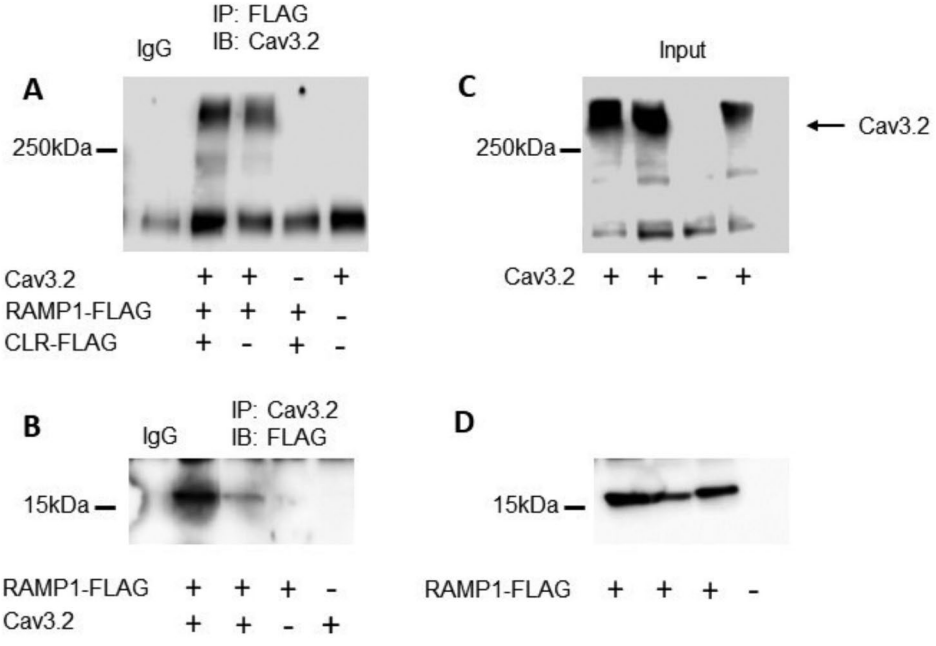


Fig. 1 $Ca_V3.2$ co-immunoprecipitates with a component of the CGRP receptor. (**A**) Protein from tsA-201 cells expressing $Ca_V3.2$ and CLR + RAMP-1-FLAG was immunoprecipitated using a FLAG antibody followed by western blot analysis with a $Ca_V3.2$ antibody. (**B**) Input: Expression of $Ca_V3.2$ at lysates. (**C**) Protein from tsA-201 cells expressing $Ca_V3.2$ and RAMP-1-FLAG was immunoprecipitated using a $Ca_V3.2$ antibody followed by western blot analysis with a FLAG antibody. (**D**) Input: Expression of RAMP-1-FLAG at lysates. These experiments were repeated 3 times with identical results

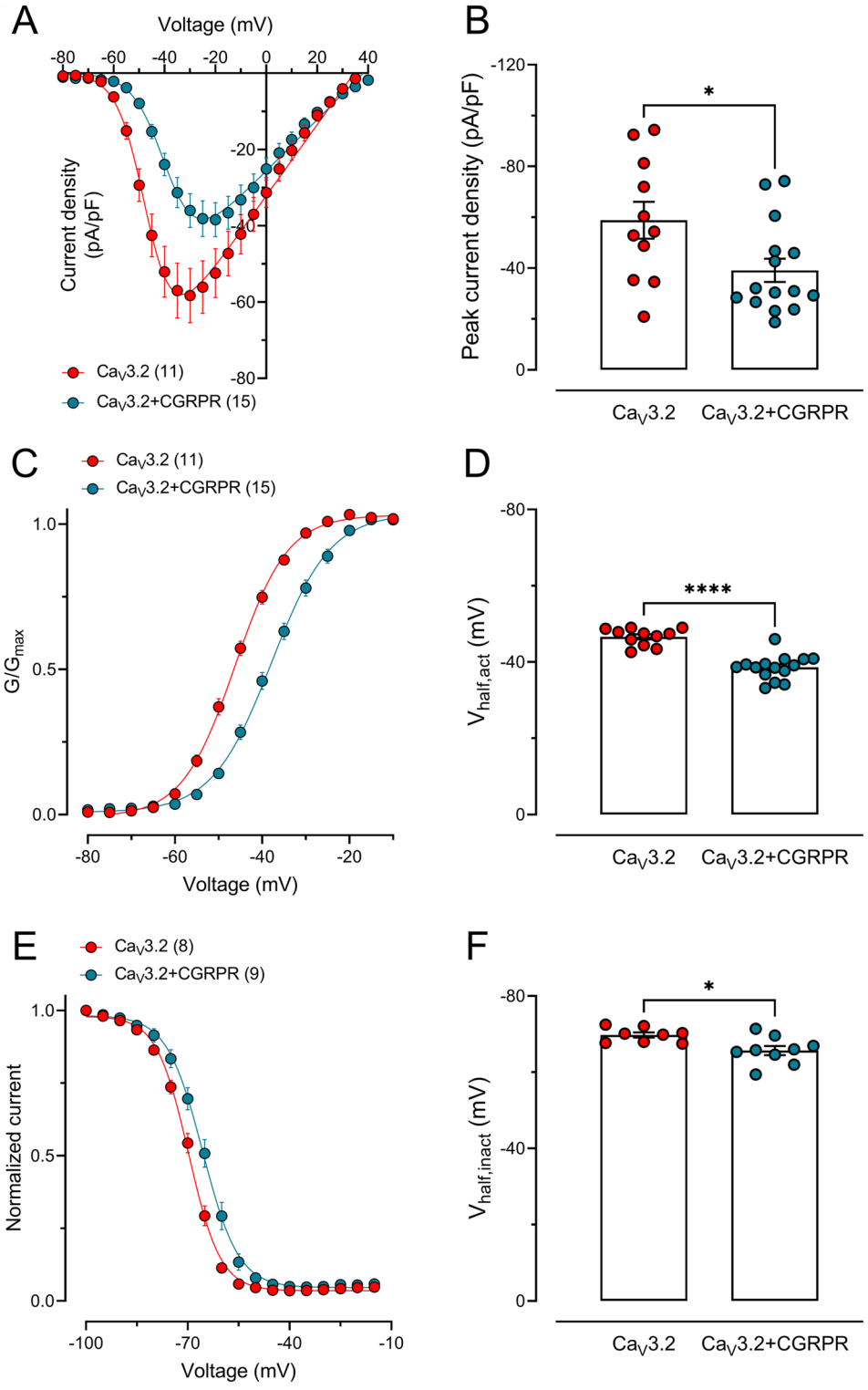


Fig. 2 Effect of CGRPR in the $Ca_V3.2$ channel biophysical properties. (**A**) Current density-voltage (IV) relation curves of cells expressing $hCa_V3.2$ or co-expressed with hCGRPR. (**B**) Average peak current density from cells cells expressing $hCa_V3.2$ or co-expressed with hCGRPR. (**C**) Voltage dependence of activation curves from cells expressing $hCa_V3.2$ or co-expressed with hCGRPR. Activation curves were constructed based on the parameters obtained from the IV curves. (**D**) Average voltage for half activation from cells expressing $hCa_V3.2$ or co-expressed with hCGRPR. (**E**) Steady-state inactivation curves from cells expressing $hCa_V3.2$ or co-expressed with hCGRPR. (**F**) Average voltage for half inactivation from cells expressing $hCa_V3.2$ or co-expressed with hCGRPR. Data is expressed as mean ± SEM, two-tailed Student's *t* test $^*p < 0.05$ and $^{****}p < 0.0001$ vs. $Ca_V3.2$

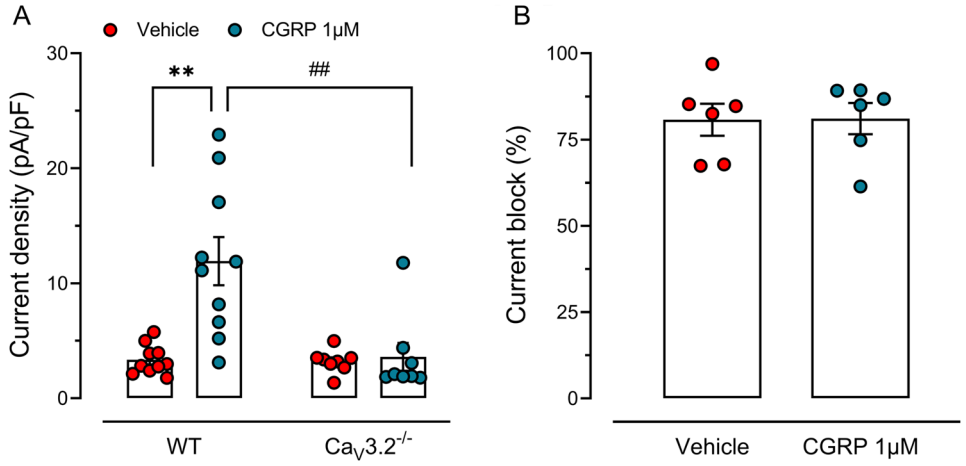


Fig. 3 CGRP receptor modulates T-type currents in trigeminal ganglion neurons. (**A**) T-type calcium channels current density in trigeminal ganglion neurons from wild-type (WT) and $Ca_V3.2^{-/-}$ mice treated with vehicle (water) or 1μM rat CGRP. (**B**) Acute treatment with 10 μM Z944 on T-type calcium channels current in TG neurons treated with vehicle (water) or 1 μM rat CGRP. Data is expressed as mean ± SEM, two-tailed Student's *t* test. $^{**}p < 0.01$ vs. Vehicle, $^{\#\#}p < 0.01$ vs. WT

different from WT when the TG neurons were incubated with vehicle, which could indicate a compensatory mechanism. However, the increase in T-type channel current induced by CGRP treatment was lost in neurons from $Ca_V3.2^{-/-}$ mice (Fig. 3A). Moreover, acute treatment with 10 μM of Z944, a pan T-type blocker, was able to inhibit approximately 80% of the T-type calcium channel current in both TG neurons treated with vehicle or 1 μM rat CGRP (Fig. 3B). Altogether, these results suggest that CGRP modulates T-type calcium channels in TG neurons.

Z944 blocks the development of periorbital mechanical allodynia

Male and female WT mice received an intraperitoneal injection of Z944 (10 mg/kg) fifteen minutes before the intraganglionic injection of CGRP (0.1 nmol/5 μL). Z944 abolished the nociceptive responses induced by CGRP in male and female mice, without causing any change in the mechanical threshold of vehicle-injected animals (Fig. 4A and B, respectively). This result indicates that T-type calcium channels contribute to mechanical allodynia induced by CGRP in both sexes.

CGRP induces periorbital mechanical allodynia in WT but not in $Ca_V3.2$-/- mice

Figure 5 shows that WT male and female mice treated with CGRP (0.1 nmol/5 μL.) exhibited an increase in the frequency of response up to 2 h after the intraganglionar injection, compared with the corresponding vehicle-injected mice (Fig. 5A and B, respectively). The nociceptive responses induced by CGRP were abolished in $Ca_V3.2^{-/-}$ male and female mice (Fig. 5A and B, respectively), suggesting that $Ca_V3.2$ channels play a significant role in the development of periorbital mechanical allodynia induced by CGRP.

CLR/PKA pathway participates in periorbital allodynia induced by CGRP

Figure 6A shows the effect of the pharmacological inhibition of the canonical CGRP receptor in female WT mice through the injection of CGRP 8–37 (10 nmol /5 μl) fifteen minutes before the intraganglionic injection of CGRP (0.1 nmol/5 μL). Likewise, Fig. 6B shows the effect of the pharmacological inhibition of protein kinase A (PKA) in female WT mice through the injection of H89 (2.5 μg/5 μl) fifteen minutes before the intraganglionic injection of CGRP (0.1 nmol/5 μL). Both treatments prevented the development of periorbital mechanical allodynia in female mice, indicating that it depends on the activation of the canonical CGRP receptor followed by PKA signalling.

Discussion

In the present study we investigated the contribution of T-type calcium channels for CGRP-induced periorbital mechanical allodynia and signaling mechanisms in the TG. We demonstrated that $Ca_V3.2$ channels form a complex with CGRPR which could modulate the channel activity. Moreover, CGRP was able to increase T-type channel currents in TG neurons, and this effect was lost in $Ca_V3.2^{-/-}$ mice. Intraganglionar injection of CGRP induce periorbital hypersensitivity, which was reversed by Z944, a pan T-type channel blocker. Finally, we showed that CGRP induced mechanical hypersensitivity might be via PKA signalling.

The canonical CGRPR is formed by a complex including the CLR and the receptor activity modifying protein 1 (RAMP-1) [29]. We observed that RAMP-1

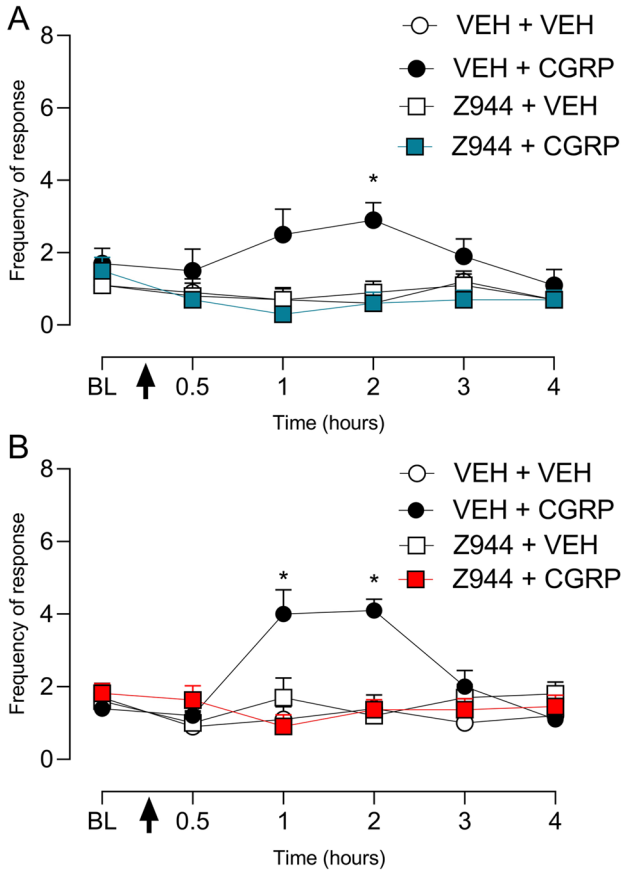


Fig. 4 Z944 blocks periorbital mechanical allodynia induced by CGRP in male and female mice. Mice were treated with Z944 (10 mg/kg; i.p.) and fifteen minutes later received an intraganglionar injection of CGRP (0.1 nmol/5 µL; i.g.). Control groups received the corresponding vehicles. Periorbital mechanical allodynia was assessed in male mice (**A**) and in female mice (**B**), before the treatments (BL) and after CGRP injection. Data is expressed as mean ± SEM ($n = 10$). Two-way ANOVA with repeated measures followed by the Bonferroni post hoc test. *$p < 0.05$, compared to Vehicle (VEH + VEH). The arrow indicates CGRP injection

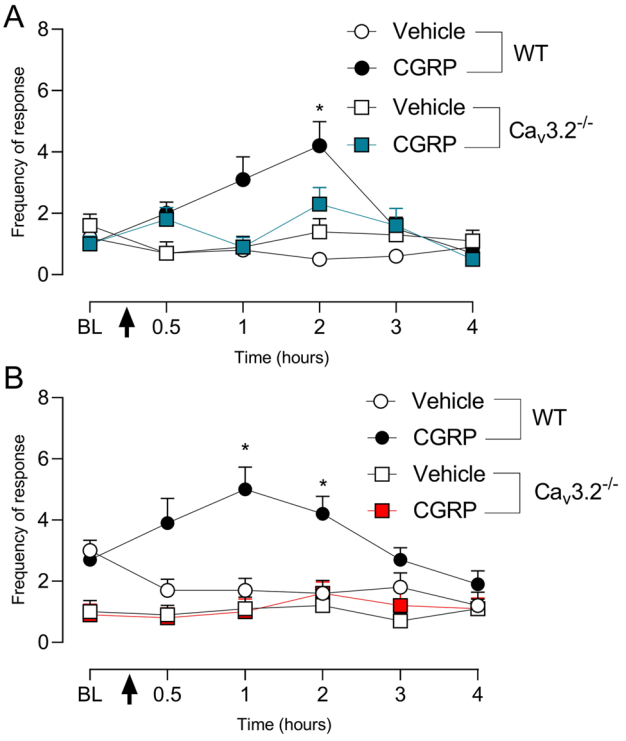


Fig. 5 $Ca_V3.2$ knockout mice does not develop periorbital mechanical allodynia after CGRP injection. CGRP (0.1 nmol/5 µL) was injected in the TG of wild-type (WT) and $Ca_V3.2^{-/-}$ mice. Periorbital mechanical allodynia was assessed in male (**A**) and in female (**B**) mice before the treatments (BL) and after CGRP injection. Data is expressed as mean ± SEM ($n = 10$). Two-way ANOVA with repeated measures followed by the Bonferroni post hoc test. *$p < 0.05$, compared to the respective Vehicle. The arrow indicates CGRP injection

co-immunoprecipitated with the $Ca_V3.2$ channel indicating that the channel is in a complex with the CGRP receptor. The complex between RAMP-1 and $Ca_V3.2$ suggests that the canonical CGRP receptor might be well positioned to modulate $Ca_V3.2$ channel activity. In fact, it is widely accepted that VGCCs are subject to robust modulation by a broad range of heterotrimeric G protein-coupled receptors (GPCRs) [30]. The modulation may be mediated by either Gα-GTP or Gβγ subunits, which are dissociated upon the GPCR activation. Modulation may involve downstream intracellular mediators, such as protein kinases, involved in the GPCR signalling pathway, but also by direct biding of the Gβγ dimer to neuronal VGCCs [30]. Regarding T-type channels, it has been reported that $G\alpha_s$ activation leads to enhancement of T-Type currents via increased levels of cAMP and consequent stimulation of PKA [31]. However, inhibition of T-type currents after GPCR activation has also been reported [31–34]. The differential regulation of T-type channels by GPCRs may depend on several factors, including the biological system, presence or absence of accessory proteins (e.g. PKA-anchoring proteins), the balance between excitatory and inhibitory effects triggered by Gα-GTP and Gβγ subunits, among others [35].

Considering that the CGRP canonical receptor and $Ca_V3.2$ form a complex, we next evaluated if the presence of the CGRPR would modulate $Ca_V3.2$ channel activity. Interestingly, we found that the presence of the receptor was able to produce a depolarizing shift in both activation and inactivation curves, and a reduction in the peak current-density. A similar result was found when cells were co-transfected with dopamine receptors D1 and D5 [36]. Collectively, these results demonstrate that $G\alpha_s$ GPCRs could modulate the basal activity of $Ca_V3.2$ calcium channel. Despite this constitutive inhibition in heterologous system, we failed to see modulation of Cav3.2 channels in tsA-201 cells after acute application or overnight incubation with CGRP (data not shown). One possibility for this lack of effect is that overnight incubation of tsA-201 cells with CGRP caused receptor internalization, which has been reported to occur rapidly after CGRP exposure

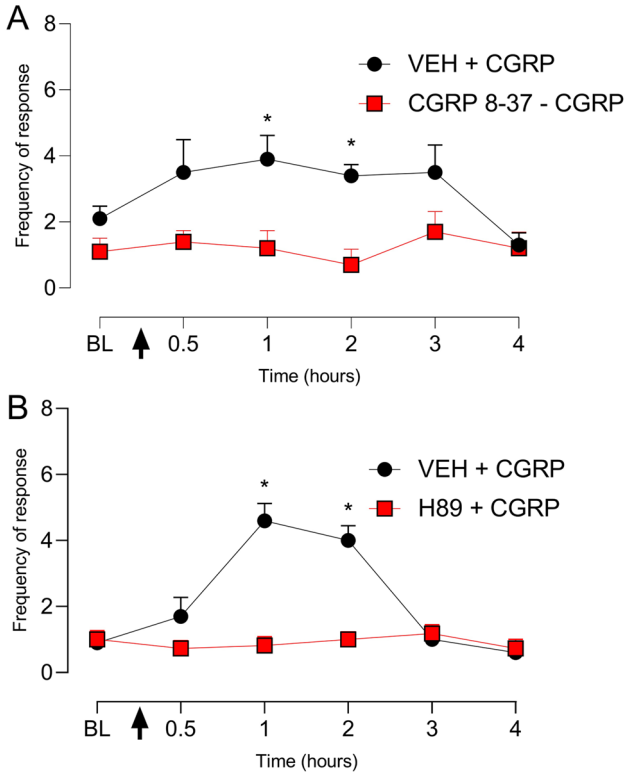


Fig. 6 Periorbital mechanical allodynia induced by CGRP involves the activation of the canonical CGRP receptor and PKA. Female mice were treated with (**A**) CGRP 8–37 (10 nmol/5 µl; i.g.) or (**B**) H89 (2.5 µg/5 µl; i.g.). and 15 min later received an intraganglionar injection of CGRP (0.1 nmol/ 5 µL; i.g.). Control groups received the corresponding vehicles. Periorbital mechanical allodynia was assessed in female mice before the treatments (BL) and after CGRP injection. Data is expressed as mean ± SEM ($n = 10$). Two-way ANOVA with repeated measures followed by the Bonferroni post hoc test *$p < 0.05$, compared to CGRP (VEH + CGRP). The arrow indicates CGRP injection

[37]. Therefore, we next decide to investigate the ability of CGRP to modulate $Ca_V3.2$ in a native tissue from wild type and $Ca_V3.2^{-/-}$ mice. In TG neurons from wild type mice acute treatment with CGRP did not cause any effect (data not shown), however chronic treatment with CGRP caused a significant increase in the T-type current density. Previous findings shows that activation of a GPRC coupled to $G\alpha_s$ protein increase T-type currents in different type of cells, including in TG neurons [36, 38–42]. The reason for the discrepancy in relation to tsA-201 cells remains to be explored, but it is possible that overnight treatment with CGRP does not cause the internalization of the CGRP receptors in the TG in the same magnitude as it does in the heterologous system. Moreover, it has been reported that TG neurons express other receptors of the calcitonin family that may be targeted by CGRP, which could explain the lack of effect in the heterologous system [43]. The contribution of these receptors to CGRP effects and migraine physiopathology remains to be determined [43]. The increase in T-type currents was abolished in TG neurons from $Ca_V3.2^{-/-}$ mice, suggesting that T-type calcium channel sensitization induced by CGRP is via $Ca_V3.2$ channels. In addition, TG neurons treated with Z944, a pan T-type calcium channel blocker, inhibited around 80% of T-type currents irrespective of CGRP receptor activation. Altogether, these results indicate that CGRPR activation by CGRP increases T-type currents in TG neurons and this effect is mainly through $Ca_V3.2$ channels.

The contribution of $Ca_V3.2$ channels to CGRP-induced responses was next evaluated in vivo by using an experimental migraine model that consists in CGRP injection in the TG, followed by evaluation of periorbital mechanical allodynia [21]. Systemic treatment with Z944, before CGRP intraganglionic injection, abolished the development of periorbital mechanical allodynia in male and female mice. This result agrees with the in vitro data and indicates that T-type channels play a significant role in CGRP-induced responses potentially related to migraine. In addition, it reinforces previous reports that Z944 potently reverses pain hypersensitivity across sexes in inflammatory acute and chronic models [22], including in in a model of trigeminal neuropathic pain [20]. Likewise, intraganglionic injection of CGRP in WT male and female mice induced periorbital allodynia, but this effect was not observed when CGRP was injected in the TG of $Ca_V3.2^{-/-}$ male and female mice. Silencing of the $Ca_V3.2$ T-type calcium channel has been used to demonstrate its contribution to chronic pain in different experimental models [23, 24]. Indeed, it has been reported that deletion of $Ca_V3.2$ results in robust long-lasting and reversible mechanical and thermal antinociceptive effects in healthy mice, and elicited a marked anti-hyperalgesic effect in a model of neuropathic pain [44]. Altogether, these results provide in vivo evidence that T-type channels contribute to mechanical allodynia induced by CGRP, which may have implication for the treatment of migraine pain.

Our in vitro and in vivo data also provide evidence for the $Ca_V3.2$ channel modulation by CGRPR. As already discussed, activation of GPCRs may modulate T-type channels via a PKA intracellular signaling pathway. To suggest a potential mechanism of modulation of the $Ca_V3.2$ channel by CGRPR, we investigated whether the periorbital mechanical allodynia induced by CGRP involves the activation of canonical CGRP receptor and PKA activation. The present results demonstrated that this pathway contributes to CGRP effects, suggesting a potential mechanism for $Ca_V3.2$ channels modulation.

Conclusion

In the TG of male and female rodents, CGRP induces a signalling pathway that culminates in periorbital mechanical allodynia, a well-established measure of pain related to experimental migraine. The present study

demonstrated that $Ca_V3.2$ channel contributes to CGRP effects. It is possible that CGRP modulates the $Ca_V3.2$ channel through the activation of its canonical GPCR, followed by PKA activation. The increase in T-type currents in the TG may represent a contributing factor for the initiation and maintenance of the headache pain during migraine.

Abbreviations

CLR	Calcitonin-like receptor
CGRP	Calcitonin-gene-related peptide
CGRPR	Calcitonin-gene-related peptide receptor
DMSO	Dimethyl sulfoxide
DMEM	Dulbecco's modified eagle medium
DRG	Dorsal root ganglia
FBS	Fetal bovine serum
GPCR	G protein-coupled receptor
IV	Current density-voltage
LVA	Low voltage-activated
PKA	Protein kinase A
RAMP-1	Receptor activity-modifying protein-1
SSI	Steady-state inactivation
TG	Trigeminal ganglion
VGCCs	Voltage-gated calcium channels
WT	Wild-type

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s10194-024-01921-0.

Supplementary Material 1

Acknowledgements

The authors are grateful to Lina Chen for her technical support, also to Vanessa Bordenowsky Pereira Lejeune for the Graphical Abstract Illustration. We are grateful for Dr. Terrance Snutch (University of British Columbia) for providing the Z944 and for Dr. Debbie Hay (University of Otago) for providing the hCLR and hRAMP-1 constructs.

Author contributions

DFB: Methodology, Formal Analysis, Investigation, Writing. EG: Methodology, Formal Analysis, Investigation, Writing. IAS: Investigation. SH: Investigation. GWZ: Conceptualization, Resources, Supervision, Funding acquisition, Writing - Review & Editing. JCG: Conceptualization, Resources, Supervision, Funding acquisition, Writing - Review & Editing.

Funding

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the research paper. All authors contributed to the work and approved its submission. This work was supported by grants from CNPq (call 26/2021, grant # 400616/2022-0) and the Coordination for the Improvement of Higher Education Personnel (CAPES, Brazil, finance code 001). DFB is recipient of CAPES PhD scholarship; JGC is the recipient of a CNPq Research Productivity scholarship (#303781/2020-3); and GWZ is a Canada Research Chair.

Data availability

No datasets were generated or analysed during the current study.

Declarations

Competing interests

The authors declare no competing interests.

Author details

[1]Department of Pharmacology, Biological Sciences Sector, Federal University of Parana, Curitiba, PR, Brazil
[2]Department of Clinical Neuroscience, Hotchkiss Brain Institute and Alberta Children's Hospital Research Institute, Cumming School of Medicine, University of Calgary, Calgary, Alberta, Canada

Received: 29 October 2024 / Accepted: 18 November 2024
Published online: 18 December 2024

References

1. Lipton RB, Bigal ME, Diamond M, Freitag F, Reed ML, Stewart WF (2007) Migraine prevalence, disease burden, and the need for preventive therapy. Neurology 68(5):343–349
2. Headache Classification Committee of the International Headache Society (IHS) (2018) The International classification of Headache disorders, 3rd edition. Cephalalgia 38(1):1–211
3. Stewart WF, Wood C, Reed ML, Roy J, Lipton RB (2008) Cumulative lifetime migraine incidence in women and men. Cephalalgia 28(11):1170–1178
4. Vanood A, Rangel IC, Starling AJ (2023) Migraine and the gender divide. Neurol Clin 41(2):231–247
5. Global burden (2020) Of 369 diseases and injuries in 204 countries and territories, 1990–2019: a systematic analysis for the global burden of Disease Study 2019. Lancet 396(10258):1204–1222
6. Edvinsson L, Haanes KA, Warfvinge K, Krause DN (2018) CGRP as the target of new migraine therapies - successful translation from bench to clinic. Nat Rev Neurol 14(6):338–350
7. Lassen LH, Haderslev PA, Jacobsen VB, Iversen HK, Sperling B, Olesen J (2002) CGRP may play a causative role in migraine. Cephalalgia 22(1):54–61
8. Goadsby PJ, Edvinsson L, Ekman R (1990) Vasoactive peptide release in the extracerebral circulation of humans during migraine headache. Ann Neurol 28(2):183–187
9. Goadsby PJ, Edvinsson L (1993) The trigeminovascular system and migraine: studies characterizing cerebrovascular and neuropeptide changes seen in humans and cats. Ann Neurol 33(1):48–56
10. Edvinsson L, Fredholm BB, Hamel E, Jansen I, Verrecchia C (1985) Perivascular peptides relax cerebral arteries concomitant with stimulation of cyclic adenosine monophosphate accumulation or release of an endothelium-derived relaxing factor in the cat. Neurosci Lett 58(2):213–217
11. Eftekhari S, Salvatore CA, Calamari A, Kane SA, Tajti J, Edvinsson L (2010) Differential distribution of calcitonin gene-related peptide and its receptor components in the human trigeminal ganglion. Neuroscience 169(2):683–696
12. Lennerz JK, Rühle V, Ceppa EP, Neuhuber WL, Bunnett NW, Grady EF et al (2008) Calcitonin receptor-like receptor (CLR), receptor activity-modifying protein 1 (RAMP1), and calcitonin gene-related peptide (CGRP) immunoreactivity in the rat trigeminovascular system: differences between peripheral and central CGRP receptor distribution. J Comp Neurol 507(3):1277–1299
13. Thalakoti S, Patil VV, Damodaram S, Vause CV, Langford LE, Freeman SE et al (2007) Neuron-glia signaling in trigeminal ganglion: implications for migraine pathology. Headache 47(7):1008–1023 discussion 24 – 5
14. Dodick D, Silberstein S (2006) Central sensitization theory of migraine: clinical implications. Headache 46(Suppl 4):S182–S191
15. Melo-Carrillo A, Strassman AM, Nir RR, Schain AJ, Noseda R, Stratton J et al (2017) Fremanezumab-A Humanized Monoclonal Anti-CGRP antibody-inhibits thinly myelinated (Aδ) but not unmyelinated (C) meningeal nociceptors. J Neurosci 37(44):10587–10596
16. Melo-Carrillo A, Noseda R, Nir RR, Schain AJ, Stratton J, Strassman AM et al (2017) Selective inhibition of Trigeminovascular neurons by Fremanezumab: a humanized monoclonal Anti-CGRP antibody. J Neurosci 37(30):7149–7163
17. Labastida-Ramírez A, Caronna E, Gollion C, Stanyer E, Dapkute A, Braniste D et al (2023) Mode and site of action of therapies targeting CGRP signaling. J Headache Pain 24(1):125
18. Cai S, Gomez K, Moutal A, Khanna R (2021) Targeting T-type/CaV3.2 channels for chronic pain. Transl Res 234:20–30
19. Weiss N, Black SA, Bladen C, Chen L, Zamponi GW (2013) Surface expression and function of Cav3.2 T-type calcium channels are controlled by asparagine-linked glycosylation. Pflugers Arch 465(8):1159–1170
20. Gambeta E, Gandini MA, Souza IA, Zamponi GW (2022) Ca V 3.2 calcium channels contribute to trigeminal neuralgia. Pain 163(12):2315–2325

21. Araya EI, Turnes JM, Barroso AR, Chichorro JG (2020) Contribution of intraganglionic CGRP to migraine-like responses in male and female rats. Cephalalgia 40(7):689–700
22. Harding EK, Dedek A, Bonin RP, Salter MW, Snutch TP, Hildebrand ME (2021) The T-type calcium channel antagonist, Z944, reduces spinal excitability and pain hypersensitivity. Br J Pharmacol 178(17):3517–3532
23. Antunes FTT, Huang S, Chen L, Zamponi GW (2024) Effect of ABT-639 on Cav3.2 channel activity and its analgesic actions in mouse models of inflammatory and neuropathic pain. Eur J Pharmacol 967:176416
24. Antunes FTT, Gandini MA, Gadotti VM, Quintão NLM, Santin JR, Souza IA et al (2024) Contribution of T-type calcium channel isoforms to cold and mechanical sensitivity in naïve and oxaliplatin-treated mice of both sexes. Br J Pharmacol
25. Zhang FF, Wang H, Zhou YM, Yu HY, Zhang M, Du X et al (2022) Inhibition of phosphodiesterase-4 in the spinal dorsal horn ameliorates neuropathic pain via cAMP-cytokine-Cx43 signaling in mice. CNS Neurosci Ther 28(5):749–760
26. Marquez de Prado B, Hammond DL, Russo AF (2009) Genetic enhancement of calcitonin gene-related peptide-induced central sensitization to mechanical stimuli in mice. J Pain 10(9):992–1000
27. Luiz AP, Schroeder SD, Rae GA, Calixto JB, Chichorro JG (2015) Contribution and interaction of kinin receptors and dynorphin A in a model of trigeminal neuropathic pain in mice. Neuroscience 300:189–200
28. Lopes RV, Baggio DF, Ferraz CR, Bertozzi MM, Saraiva-Santos T, Verri Junior WA et al (2023) Maresin-2 inhibits inflammatory and neuropathic trigeminal pain and reduces neuronal activation in the trigeminal ganglion. Curr Res Neurobiol 4:100093
29. Poyner DR, Sexton PM, Marshall I, Smith DM, Quirion R, Born W et al (2002) International Union of Pharmacology. XXXII. The mammalian calcitonin gene-related peptides, adrenomedullin, amylin, and calcitonin receptors. Pharmacol Rev 54(2):233–246
30. Weiss N, Zamponi GW (2024) The T-type calcium channelosome. Pflugers Arch 476(2):163–177
31. Kim JA, Park JY, Kang HW, Huh SU, Jeong SW, Lee JH (2006) Augmentation of Cav3.2 T-type calcium channel activity by cAMP-dependent protein kinase A. J Pharmacol Exp Ther 318(1):230–237
32. Hu C, Depuy SD, Yao J, McIntire WE, Barrett PQ (2009) Protein kinase A activity controls the regulation of T-type CaV3.2 channels by Gbetagamma dimers. J Biol Chem 284(12):7465–7473
33. Zhang Y, Ji H, Wang J, Sun Y, Qian Z, Jiang X et al (2018) Melatonin-mediated inhibition of Cav3.2 T-type ca(2+) channels induces sensory neuronal hypoexcitability through the novel protein kinase C-eta isoform. J Pineal Res 64(4):e12476
34. Jackson JL, Kuriyama A, Kuwatsuka Y, Nickoloff S, Storch D, Jackson W et al (2019) Beta-blockers for the prevention of headache in adults, a systematic review and meta-analysis. PLoS ONE 14(3):e0212785
35. Zhang Y, Jiang X, Snutch TP, Tao J (2013) Modulation of low-voltage-activated T-type Ca^{2+} channels. Biochim Biophys Acta 1828(7):1550–1559
36. Mustafá ER, McCarthy CI, Portales AE, Cordisco Gonzalez S, Rodríguez SS, Raingo J (2023) Constitutive activity of the dopamine (D(5)) receptor, highly expressed in CA1 hippocampal neurons, selectively reduces ca(V) 3.2 and ca(V) 3.3 currents. Br J Pharmacol 180(9):1210–1231
37. Gingell JJ, Rees TA, Hendrikse ER, Siow A, Rennison D, Scotter J et al (2020) Distinct patterns of internalization of different calcitonin gene-related peptide receptors. ACS Pharmacol Transl Sci 3(2):296–304
38. Yue J, Zhang Y, Li X, Gong S, Tao J, Jiang X (2014) 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. Pharmazie 69(11):804–808
39. Chen C, Xu R, Clarke IJ, Ruan M, Loneragan K, Roh SG (2000) Diverse intracellular signalling systems used by growth hormone-releasing hormone in regulating voltage-gated Ca2 + or K channels in pituitary somatotropes. Immunol Cell Biol 78(4):356–368
40. Lenglet S, Louiset E, Delarue C, Vaudry H, Contesse V (2002) Activation of 5-HT(7) receptor in rat glomerulosa cells is associated with an increase in adenylyl cyclase activity and calcium influx through T-type calcium channels. Endocrinology 143(5):1748–1760
41. Contesse V, Hamel C, Lefebvre H, Dumuis A, Vaudry H, Delarue C (1996) Activation of 5-hydroxytryptamine4 receptors causes calcium influx in adrenocortical cells: involvement of calcium in 5-hydroxytryptamine-induced steroid secretion. Mol Pharmacol 49(3):481–493
42. Sekiguchi F, Aoki Y, Nakagawa M, Kanaoka D, Nishimoto Y, Tsubota-Matsunami M et al (2013) AKAP-dependent sensitization of ca(v) 3.2 channels via the EP(4) receptor/cAMP pathway mediates PGE(2) -induced mechanical hyperalgesia. Br J Pharmacol 168(3):734–745
43. Rees TA, Hendrikse ER, Hay DL, Walker CS, Beyond CGRP (2022) The calcitonin peptide family as targets for migraine and pain. Br J Pharmacol 179(3):381–399
44. Bourinet E, Alloui A, Monteil A, Barrère C, Couette B, Poirot O et al (2005) Silencing of the Cav3.2 T-type calcium channel gene in sensory neurons demonstrates its major role in nociception. Embo j 24(2):315–324

Publisher's note