Trigeminal Neuralgia TRPM8 Mutation
Enhanced Activation, Basal [Ca^{2+}]_i and Menthol Response

Roberta Gualdani, PhD,* Jun-Hui Yuan, MD, PhD,* Philip R. Effraim, MD, PhD, Giulia Di Stefano, MD, Andrea Truini, MD, Giorgio Cruccu, MD, PhD, Sulayman D. Dib-Hajji, PhD, Philippe Gailly, MD, PhD, and Stephen G. Waxman, MD, PhD


Abstract

Objective
To assess the functional effects of a variant, c.89 G > A (p.Arg30Gln), in the transient receptor potential melastatin 8 (TRPM8) cold-sensing, nonselective cation channel, which we have previously identified in a patient with familial trigeminal neuralgia.

Methods
We carried out Ca^{2+} imaging and whole-cell patch-clamp recording.

Results
The TRPM8 mutation enhances channel activation, increases basal current amplitude and intracellular [Ca^{2+}] in cells carrying the mutant channel, and enhances the response to menthol.

Conclusions
We propose that Arg30Gln confers gain-of-function attributes on TRPM8, which contribute to pathogenesis of trigeminal neuralgia in patients carrying this mutation.

*Contributed equally.

From the Institute of Neuroscience (R.G., P.G.), Université catholique de Louvain, B-1200 Brussels, Belgium; Department of Neurology (J.-H.Y., S.D.D.-H., S.G.W.), Yale School of Medicine, New Haven, CT and Center for Neuroscience and Regeneration Research, Veterans Affairs Connecticut Healthcare, West Haven, CT; Department of Anaesthesiology (P.R.E.), Yale School of Medicine New Haven, CT and Center for Neuroscience and Regeneration Research, Veterans Affairs Connecticut Healthcare, West Haven, CT; and Department of Human Neuroscience (G.D.S., A.T., G.C.), Sapienza University, Rome, Italy.

Go to Neurology.org/NG for full disclosures. Funding information is provided at the end of the article.

The Article Processing Charge was funded by the authors.

This is an open access article distributed under the terms of the Creative Commons Attribution-NonCommercial-NoDerivatives License 4.0 (CC BY-NC-ND), which permits downloading and sharing the work provided it is properly cited. The work cannot be changed in any way or used commercially without permission from the journal.
Trigeminal neuralgia (TN) is a unique pain disorder in which affected individuals suffer from paroxysms of severe pain in the distribution of the trigeminal nerve, i.e., over the face, usually with onset in older adults. The disorder is often unilateral, and in many cases, although not all, it is associated with neurovascular compression of the trigeminal root. Although most cases of trigeminal neuralgia are sporadic, familial occurrence has been reported. Di Stefano et al. recently reported a positive family history for 12 patients with trigeminal neuralgia within a series of 88 patients enrolled consecutively for assessment at a specialized neuropathic pain center. The occurrence of familial TN suggests a genetic contribution to this disorder. Whole-exome sequencing of genes within the electrogenosome known to be expressed within trigeminal ganglion revealed multiple variants of ion channels including sodium channels, potassium channels, chloride channels, calcium channels, and transient receptor potential (TRP) channels in the genomes of these familial patients with TN, but the possible contribution of these variants to TN must be empirically assessed. Here, we use Ca$^{2+}$ imaging and whole-cell patch clamp to assess a variant in the transient receptor potential melastatin 8 (TRPM8) cold-sensing, nonselctive cation channel, c.89 G > A (p.Arg30Gln), in a patient with familial trigeminal neuralgia. We demonstrate that this TRPM8 mutation displays multiple gain-of-function characteristics including enhanced channel activation, increased basal current amplitude and intracellular [Ca$^{2+}$] in cells carrying the mutant channel, and enhanced response to menthol. We suggest a model in which these gain-of-function attributes contribute to the pathogenesis of trigeminal neuralgia.

**Methods**

**Patient**

The patient was a 61-year-old woman with severe attacks of unilateral facial pain, beginning at the age of 60, triggered by chewing, talking, and light touch of the nasal wing (case 1 in DiStefano et al., 2020). Two brothers also had a history of trigeminal neuralgia. MRI revealed neurovascular compression at atrophy and dislocation of the trigeminal root. Oxcarbazepine provided pain relief. The study was approved by the Ethnic Committee of Sapienza University (reference 649/17). Written informed consent was obtained from the participant.

DNA analysis revealed that this patient and her older brother, also carrying a diagnosis of trigeminal neuralgia, share a 9-bp duplication (c.642_650dup; 27/190,394 on gnomAD v2) in potassium channel gene KCNC3 and a synonymous splice site variant (c.1467 G > A; 3/250,442 on gnomAD v2) in KCND2 predicted to reduce splicing efficiency using Alternative Splice Site Predictor (wangcomputing.com/assp/) and Human Splicing Finder (umd.be/HSF/). The patient carried a novel missense variant c.1489 G > A (p.Gly497Ser) in KCNH2 and a rare missense variant c.6032 G > A (p.Arg2011Gln; 4/282,448 on gnomAD v2) in CACNA1D. Notably, the patient was heterozygous for a variant, c.89 G > A (p.Arg30Gln; 9/282,688 on gnomAD v2), in TRPM8. Given the expression of TRPM8 within trigeminal ganglia and reported association of TRPM8 with pain, we asked whether this variant might produce any functional changes in the TRPM8 channel or within cells expressing it.

**Plasmid, Cell Culture, and Transfection**

Patch-clamp studies were carried out in human embryonic kidney (HEK) 293 cells transiently transfected with vectors that encode human TRPM8 or the Arg30Gln variant (referred to in the single letter amino acid code R30Q hereinafter). The wild-type TRPM8 plasmid was obtained from GenScript, in which the insert was cloned in-frame with 2A-GFP at the C-terminus of the channel in the vector pcDNA3.1. The single TRPM8-2AGFP transcript is translated into the separate channel protein and the transfection marker GFP protein. The R30Q mutation was introduced into the TRPM8 channel using QuikChange Lightning site-directed mutagenesis (Agilent Technologies). For heterologous expression, cells were plated in 6-well cell culture dishes with 2-mL growth medium (DMEM, 10% FBS, 2 mM L-glutamine, 2 U/mL penicillin, and 2 mg/mL streptomycin) 24 hours before transfection. Transfection was performed using the Lipofectamine LTX Reagent (Invitrogen) as previously described.

**Electrophysiology**

Patch-clamp recordings of HEK293 cells were carried out at room temperature using an EPC-9 amplifier (HEKA Elektronik, Lambrecht, Germany) controlled by PatchMaster software (HEKA Elektronik, Lambrecht, Germany). Patch-clamp electrodes were pulled and fire polished to 4.0 ± 0.5 MΩ resistance when filled with intracellular solution using a DMZ-Universal Puller (Zeitz Instruments, Munich, Germany). An AgCl wire was used as a reference electrode. To elicit I-V whole-cell currents, repetitive 400 ms voltage ramps (at 2 seconds intervals) from −100 to +100 mV were applied, from a holding potential of 0 mV. Currents were sampled at 20 kHz and digitally filtered at 2.9 kHz. For statistical analysis, the currents were normalized to cell capacitance. Solutions were applied to the cells via a custom-built gravity-fed...
perfusion system, connected to a 5-way manifold, to a RC25
perfusion chamber (Warner Instruments, Hamden, CT). For
whole cell recordings, the standard extracellular solution had
the following composition (in mM): 150 NaCl, 5 KCl, 1
MgCl₂, 1.8 CaCl₂, 10 glucose, and 10 HEPES, buffered at pH
7.4 with NaOH. The osmolality of this solution was 320 ± 5
mOsm/kgH₂O. The intracellular solution had the following
composition (in mM): 140 CsCl, 0.3 CaCl₂, 2 MgATP, 10
EGTA and 10 HEPES, adjusted to pH 7.2 with CsOH (290 ±
5 mOsm).

To analyze the effects of the mutation R30Q on the voltage
dependence of TRPM8, we applied voltage step protocols
from -100 to +200 mV. At each data point, we calculated G/
Gmax, where Gmax is the maximal steady state conductance
in the presence of 1 mM menthol. Therefore, we fit the G/
Gmax values using a Boltzmann function of the form:

$$G = \frac{G_{MAX}}{1 + \exp \left( \frac{V - V_{1/2}}{k_B T} \right)}$$

where z is the gating charge, V is voltage, V₁/₂ is the voltage for
half-maximal activation, kₜ is the Boltzmann constant, and T
is the absolute temperature. For simplicity, we assume for our
calculations a two-state model of channel gating.¹⁶

Ca²⁺ Imaging
Loading of the HEK293 cells with Fura2-AM was achieved at
room temperature in a Krebs medium with 5 μM fura-2-AM
and 0.01% pluronic acid for 1 hours [Ca²⁺], was measured in
10–15 individual cells for each experiment using an alternative
excitation of Fura2-AM (0.5 Hz) at 340 and 380 nm using a
Lambda DG-4 Ultra High Speed Wavelength Switcher (Sut-
ter Instrument, Novato, CA). Images were acquired with a
Zeiss AxioCam camera coupled to a 510-nm emission
filter and analyzed with Axiovision software. Ca²⁺ imaging exper-
iments were performed in a Krebs solution (NaCl 135 mM,
KCl 5.9 mM, MgCl₂ 1.2 mM, CaCl₂ 1.8 mM, HEPES
11.6 mM, glucose 11.5 mM, pH 7.35).

Data Availability
The raw data are available upon request.

Results
To investigate the effect of the R30Q variant on TRPM8
activity, wild-type and mutant human TRPM8 were expressed
in HEK293 cells. In cultured cells transiently transfected with
either wild-type or R30Q TRPM8 channels, exposure to
100 μM menthol, added in the bath at the time indicated by
the arrow and continuously perfused until the end of the
experiment, evoked a robust increase in intracellular calcium,
measured as a change in the Fura-2 fluorescence signal ratio
(figure 1, A and B). In comparison with cells expressing wild-
type TRPM8, the R30Q mutation yielded a larger Ca²⁺ in-
crease in response to menthol (figure 1C). Moreover, the
initial Fura-2 fluorescence signal ratio, which reflects the basal
intracellular [Ca²⁺], was slightly but significantly higher in
cells expressing R30Q compared with the wild-type TRPM8
(figure 1D). In vitro cotransfections of mutant and wild-type
to model in vivo heterozygosity revealed a slight increase of
Ca²⁺ uptake in response to menthol (Supplementary figure
1A, links.lww.com/NXG/A359) and a significant increase of
basal Ca²⁺ (Supplementary figure 1B, links.lww.com/NXG/
A359) compared to wild-type TRPM8 transfected cells.

To further characterize the R30Q mutation under basal
conditions, we recorded steady-state evoked currents during
voltage steps from -100 mV to +200 mV from a holding
potential of 0 mV (figure 2A). The basal current amplitude of
the R30Q mutant was significantly higher compared with
TRPM8 wild-type at potentials greater than 150 mV (figure
2B). Normalized conductance for each cell, referred to as G/
Gmax, was plotted for the given voltages and fitted with a
Boltzmann function (figure 2C), Gmax being the maximal
steady state conductance in the presence of 1 mM menthol.
We found that the R30Q mutation left-shifted the activation
curve toward physiologic voltages, from 153 ± 8 mV to 115 ±
5 mV (**p < 0.01, unpaired Student’s t test). Because saturating
effects in the presence of 1 mM menthol could not be
obtained for the R30Q mutant, values for V₁/₂ and slope
factor should be considered as approximates.

Finally, because menthol is well-known to be an agonist for
TRPM8, we tested menthol-induced responses of mutant and
wild-type TRPM8 channels using patch clamp recordings.
Menthol at nonsaturating concentration of 100 μM evoked
robust currents with strong outward rectification in cells
expressing both R30Q and wild-type TRPM8 (figure 2, D and
E). The current is characterized by a reversal potential near
0 mV, suggesting that TRPM8 is a nonselective Ca²⁺ channel.
In particular, when activated, it allows the entry of Na⁺ and
Ca²⁺ ions into the cell. At positive membrane potential (+80
mV) the amplitude of the current in response to 100 μM
menthol was significantly higher in R30Q transfected cells
compared with wild-type TRPM8 expressing cells (figure 2F).

Discussion
Trigeminal neuralgia is characterized by recurrent excruciat-
ing pain in affected individuals, which is thought to arise from
episodes of hyperactivity of primary trigeminal afferents.¹⁷–¹⁹
Despite intense study, the pathophysiologic basis of this tri-
geminal ganglion neuron hyperexcitability remains incom-
pletely understood. Trigeminal neuralgia is unilateral, and
in many cases, although not all cases, it is associated with
neurovascular compression of the trigeminal root.²

Although trigeminal neuralgia tends to occur in a sporadic
manner, familial occurrence has been reported.³–⁵ Together
with the occurrence of trigeminal neuralgia in the absence of
neurovascular compression, the occurrence of familial
trigeminal neuralgia supports a genetic contribution to this pain disorder. Here, we build on a study which demonstrated positive family history for 12 subjects, within a series of 88 patients with trigeminal neuralgia. Whole-exome sequencing of genes within the electrogenome of trigeminal ganglion neurons demonstrated variants of ion channels including sodium channels, potassium channels, chloride channels, calcium channels, and TRP channels.

In addition, to a mutation of TRPM8, the patient described in this report carried a rare missense variant c.6032 G > A (p.Arg2011Gln) in CACNA1D. Gain-of-function mutations of CACNA1D, encoding pore-forming Cav1.3 α1-subunit, have been reported to contribute to primary aldosteronism, seizures, and multiple psychiatric and neurologic syndromes. The current patient also carried rare variants in 3 potassium channel genes, consisting of a 9-bp duplication (c.642_650dup) in KCNC3, an synonymous splice site variant (c.1467 G > A) in KCND2 predicted to reduce splicing efficiency, and a novel missense variant p.1489 G > A (p.Gly497Ser) in KCNH2. Among these potassium channel genes, KCND2 encodes Kv4.2, a rapidly inactivating voltage-gated potassium channel, and Kv4.2 knockout mice display increased neuronal excitability and hypersensitivity to nociceptive stimuli. Missense variants of KCND2 gene have been found possibly associated with infantile-onset severe refractory epilepsy and persistent breast pain after breast cancer surgery. Loss-of-function mutations of KCNC3, encoding Kv3.3, have been linked to cerebellar ataxia, accompanied with hyperreflexia, deep sensory loss, seizures, involuntary movement, and cognitive impairment, whereas loss-of-function mutations of KCNH2, encoding Kv11.1, are found in approximately 30% of patients with Long QT syndrome. Within transcriptome data sets of mouse or human trigeminal/dorsal root ganglion, RNA expression levels of KCNC3, KCND2, and CACNA1D were higher than for KCND2, but no linkage or association between the former 3 genes and pain had been described (Supplementary table 1). Nevertheless, we cannot exclude a contribution of these variants to trigeminal neuralgia.

In this study, we focused on a mutation in TRPM8, a gene of which is known to be expressed in the trigeminal and
dorsal root ganglia.\textsuperscript{7,9} TRPM8 is an outwardly rectifying nonselective Ca\textsuperscript{2+} channel, first characterized as a neuronal detector of cold.\textsuperscript{32,33} It is activated by cold temperatures (<26°C) and compounds that produce a cooling sensation such as menthol, icilin, and eucalyptol.\textsuperscript{33,34} TRPM8 is also activated by voltage. In the absence of chemical agonists or high temperature, the channel is gated by strong depolarizations. In contrast, cold temperature or agonist application shift the steady-state activation curve of TRPM8 to more negative potentials, increasing the open...
Although the role of TRPM8 as cold transducer is well-established, its role in pain sensation is not clear. In fact it is still under debate whether TRPM8 amplifies and/or reduces pain sensation. A number of studies suggest that the activation of TRPM8 exaggerates pain sensation.\(^{11,12}\) In support of these results, selective TRPM8 antagonists reduced chronic pain and allodynia in animal models.\(^{10,37}\) In contrast, other studies report that TRPM8 agonists have analgesic properties.\(^{38,39}\) A possible explanation for these apparently disparate results, where the activation of TRPM8 seems to cause cold pain after injury while simultaneously reducing mechanical and heat pain, could be that TRPM8-expressing afferent fibers have the ability to either enhance or attenuate pain, but the outcome is determined by context.\(^{40}\)

We studied the effect of the substitution of the Arg30, located in the N-terminus, by glutamine on the biophysical properties of TRPM8. Our results indicate that the R30Q mutation in TRPM8 enhances channel activation, increases basal current amplitude and intracellular [Ca\(^{2+}\)] in cells carrying the mutant channel, and enhances the channel response to menthol. Interestingly, coexpression of mutant and wild type to model in vivo heterozygosity revealed a significant increase of basal Ca\(^{2+}\) suggesting a dominant effect of the R30Q mutation. It was previously observed that deletion and substitution of the first 40 residues yielded TRPM8 channels with augmented responses to cold and menthol.\(^{41}\) In particular, electrophysiologic analysis of the S26P and S27P mutants revealed that the enhanced sensitivity to agonists was related to a strong leftward shift in the voltage dependence of activation. Because this phenotype occurs only when a proline is introduced, without a significant effect of alanine or aspartate mutations, the authors suggest that the augmented response of the channels is because of the alteration of the structure of TRPM8. They speculated that in wild-type TRPM8 channels, the interaction of the N-terminal region with other TRPM8 domains or with a partner protein reduces channel activity. As a consequence, specific deletion or substitution of the first 40 amino acids of TRPM8 could compromise the interaction with modulatory proteins or internal regions that constitutively inhibits TRPM8 function. In the light of our findings, we can hypothesize that a mutation of the Arg30, which is close to Ser26 and Ser27 residues, could alter the stability of the N-terminus of TRPM8, similarly, to the effect observed for S26P and S27P mutations, leading to its gain-of-function enhancement.

Small persistent Na\(^+\) currents\(^{42}\) are known to trigger reverse Na\(^+\)/Ca\(^{2+}\) exchange that can lead to increased intracellular Ca\(^{2+}\), which can produce axonal injury;\(^{43}\) gain-of-function mutations of Na\(^+\) channels impair axonal integrity and ionic homeostasis in vitro;\(^{44,45}\) and have been linked to painful human neuropathies.\(^{36,47}\) Although the predicted increase in intracellular Ca\(^{2+}\) resulting from the R30Q mutation at physiologic membrane potentials would be expected to be small, there is evidence that the cumulative effects of small increases in intracellular Ca\(^{2+}\) can predispose axons to time-dependent injury, i.e., adult-onset axonal disease.\(^{48}\) Consistent with the view that mutations underlie a slow influx of Na\(^+\) or Ca\(^{2+}\) can predispose trigeminal axons to mechanical injury, a gain-of-function mutation of sodium channel Nav1.6 has been described in a patient with adult onset trigeminal neuralgia and ipsilateral neurovascular compression of the trigeminal root.\(^{49}\) Alternatively, it is possible that vascular compression causes demyelination of the trigeminal root. Electrophysiologic recordings and computer simulations show that focally demyelinated axons can become hyperexcitable\(^{50,51}\) and the enhanced TRPM8 channel activation increased basal current amplitude and intracellular [Ca\(^{2+}\)] in cells carrying the mutant channel could summate with this hyperexcitability to produce ectopic activity.

The familial occurrence of trigeminal neuralgia in some patients, together with the presence of unilateral pain and adult onset, suggest a multihit model in which genetic factors can contribute to pathophysiology. Our results provide support for the idea that genetic factors can predispose axons to injury in response to mechanical insults and specifically for the hypothesis that variants of ion channels within the trigeminal neuron electrogenicome can predispose to the development of trigeminal neuralgia in response to microvascular compression.

**Acknowledgment**

The authors thank Fadia B. Dib-Hajj for technical assistance.

**Study Funding**

This work was supported in part by Merit Award (I01 RX003201) from the Rehabilitation Research and Development Service, U.S. Department of Veterans Affairs; by the Queen Elisabeth Medical Foundation (Belgium); and the Concerted Research Action from the General Direction of Scientific Research of the French Community of Belgium (ARC17/22-083).

**Disclosure**

R. Gualdani, J.H. Yuan, P.R. Effram, G. Di Stefano, A. Truini, G. Cruccu, and S.D. Dib-Hajj report no disclosures relevant to the manuscript. P. Gailly was funded by the Queen Elisabeth Medical Foundation (Belgium) and the Concerted Research Action from the General Direction of Scientific Research of the French Community of Belgium (ARC17/22-083). S.G. Waxman was funded by Merit Award (I01 RX003201) from the Rehabilitation Research and Development Service, U.S. Department of Veterans Affairs. Go to Neurology.org/NG for full disclosures.

**Publication History**

Received by Neurology: Genetics August 11, 2020. Accepted in final form November 9, 2020.
### Appendix Authors

<table>
<thead>
<tr>
<th>Name</th>
<th>Location</th>
<th>Contribution</th>
</tr>
</thead>
<tbody>
<tr>
<td>Roberta Gualdani  PhD</td>
<td>Université catholique de Louvain, Brussels, Belgium</td>
<td>Carried out electrophysiological experiments and data analysis, preparation of the first draft of the study, and finalization of the manuscript</td>
</tr>
<tr>
<td>Jun-Hui Yuan  MD, PhD</td>
<td>Yale School of Medicine (New Haven) and Veterans Affairs Connecticut Health care (West Haven), Connecticut</td>
<td>Carried out genetic analysis, preparation of the first draft of the study, and finalization of the manuscript</td>
</tr>
<tr>
<td>Philip R. Effraim, MD, PhD</td>
<td>Yale School of Medicine (New Haven) and Veterans Affairs Connecticut Health care (West Haven), Connecticut</td>
<td>Data analysis and interpretation and finalization of the manuscript</td>
</tr>
<tr>
<td>Andrea Truini, MD</td>
<td>Sapienza University, Rome, Italy</td>
<td>Clinical assessment of patients, analysis of kindred, and finalization of the manuscript</td>
</tr>
<tr>
<td>Giorgio Crucchi, MD, PhD</td>
<td>Sapienza University, Rome, Italy</td>
<td>Clinical assessment of patients, analysis of kindred, and finalization of the manuscript</td>
</tr>
<tr>
<td>Sulayman D. Dib-Hajj, PhD</td>
<td>Yale School of Medicine (New Haven) and Veterans Affairs Connecticut Health care (West Haven), Connecticut</td>
<td>Data analysis and interpretation and finalization of the manuscript</td>
</tr>
<tr>
<td>Philippe Gally, MD, PhD</td>
<td>Université catholique de Louvain, Brussels, Belgium</td>
<td>Supervised electrophysiology, data analysis and interpretation, and finalization of the manuscript</td>
</tr>
<tr>
<td>Stephen G. Waxman, MD, PhD</td>
<td>Yale School of Medicine (New Haven) and Veterans Affairs Connecticut Health care (West Haven), Connecticut</td>
<td>Overall supervision of the project, data analysis and interpretation, preparation of the first draft of the study, and finalization of the manuscript</td>
</tr>
</tbody>
</table>

### References

Trigeminal Neuralgia TRPM8 Mutation: Enhanced Activation, Basal \([\text{Ca}^{2+}]_i\) and Menthol Response  
*Neurol Genet* 2021;7;  
DOI 10.1212/NXG.0000000000000550

This information is current as of January 11, 2021

| Updated Information & Services | including high resolution figures, can be found at:  
http://ng.neurology.org/content/7/1/e550.full.html |
|-------------------------------|---------------------------------------------------|
| References                    | This article cites 51 articles, 8 of which you can access for free at:  
http://ng.neurology.org/content/7/1/e550.full.html##ref-list-1 |
| Subspecialty Collections      | This article, along with others on similar topics, appears in the following collection(s):  
**Ion channel gene defects**  
http://ng.neurology.org/cgi/collection/ion_channel_gene_defects  
**Neuropathic pain**  
http://ng.neurology.org/cgi/collection/neuropathic_pain  
**Trigeminal neuralgia**  
http://ng.neurology.org/cgi/collection/trigeminal_neuralgia |
| Permissions & Licensing       | Information about reproducing this article in parts (figures, tables) or in its entirety can be found online at:  
http://ng.neurology.org/misc/about.xhtml#permissions |
| Reprints                      | Information about ordering reprints can be found online:  
http://ng.neurology.org/misc/addir.xhtml#reprintsus |

*Neurol Genet* is an official journal of the American Academy of Neurology. Published since April 2015, it is an open-access, online-only, continuous publication journal. Copyright Copyright © 2021 The Author(s). Published by Wolters Kluwer Health, Inc. on behalf of the American Academy of Neurology.. All rights reserved. Online ISSN: 2376-7839.