

Nerve injury induces a Gem-GTPase-dependent down-regulation of P/Q type
Ca²⁺ channels **contributing to neurite plasticity** in dorsal root ganglion neurons

Frédérique Scamps^{1,3*}, Sina Sangari¹, Melissa Bowerman^{1,3}, Mathieu Rousset^{2,3}, Michel
Bellis^{2,3}, Thierry Cens^{2,3}, Pierre Charnet^{2,3}

¹*Inserm U1051, Institut des Neurosciences, 80 rue Augustin Fliche, 34091 Montpellier, France*

²*CRBM, CNRS UMR5237, 1919 Route de Mende, 34293 Montpellier, France*

³*Universités de Montpellier I&II, 34293 Montpellier, France*

***Corresponding author:** Frederique Scamps;

Inserm U1051, Institut des Neurosciences, 80 rue Augustin Fliche, 34091 Montpellier, France
email: frederique.scamps@inserm.fr

Abstract

Small RGK GTPases, Rad, Gem, Rem1 and Rem2, are potent inhibitors of high voltage activated (HVA) Ca^{2+} channels expressed in heterologous expression systems. However, the role of this regulation has never been clearly demonstrated in the nervous system. Using transcriptional analysis we show that peripheral nerve injury specifically up-regulates *Gem* in mice dorsal root ganglia. Following nerve injury, protein expression of *Gem* was increased in ganglia and peripheral nerve, mostly under its phosphorylated form. This was confirmed *in situ* and *in vitro* in dorsal root ganglia sensory neurons. Knock-down of endogenous *Gem*, using specific siRNA, increased the HVA Ca^{2+} current only in the large somatic sized neurons. Combining pharmacological analysis of the HVA Ca^{2+} currents together with *Gem* siRNA-transfection of larger sensory neurons, we demonstrate that only the P/Q-type Ca^{2+} channels was enhanced. *In vitro* analysis of *Gem* affinity to various $\text{Cav}\beta\text{x-Cav}2.\text{x}$ complexes and immunocytochemical studies of *Gem* and $\text{Cav}\beta$ expression in sensory neurons suggest that the specific inhibition of the P/Q channels relies on both the regionalized up-regulation of *Gem* and the higher sensitivity of the endogenous $\text{CaV}2.1\text{-Cav}\beta4$ pair in a subset of sensory neurons including the proprioceptors. Finally, pharmacological inhibition of P/Q-type Ca^{2+} current reduces neurite branching of regenerating axotomized neurons. Taken together, the present results indicate that a *Gem*-dependent P/Q-type Ca^{2+} current inhibition may contribute to general homeostatic mechanisms following a peripheral nerve injury.

Key words: sensory neuron, high voltage activated Ca^{2+} current, RGK, proprioceptor, mechanoreceptor.

Abbreviations

1 Cacn β / Cav β , calcium channel β subunit; DRG, dorsal root ganglia; Gem, gene overexpressed in skeletal
2 muscle; HVA, high voltage-activated; LVA, low voltage-activated; Polr2j, Polymerase (RNA) II
3 polypeptide J.; siRNA, small-interfering RNA; Rad, ras related protein; Rem1, rad and gem related GTP
4 binding protein 1; Rem2, rad and gem related GTP binding protein 2.

5

6

Introduction

Following peripheral nerve **transection**, sensory neurons experience profound cellular remodeling aimed at promoting axonal regeneration [43]. Notably, nerve injury induces a complex regulation of the amplitude of high voltage-activated (HVA) Ca^{2+} currents [1, 4, 36]. Transcriptional down-regulation of the L-type pore forming subunit, Cav1.2, was shown to contribute to axon regrowth following injury [16]. In addition an increased amplitude of the N-type, Cav2.2, Ca^{2+} current that occurs in pain models of nerve injury [6, 27, 29] has been attributed to an up-regulation of the auxiliary Cav β and $\alpha 2/\delta$ subunits, two channel auxiliary subunits that play major roles in the functional expression and properties of Ca^{2+} currents [3]. Recently, the RGK subfamily of small GTP-binding proteins comprising Rem1, Rem2, Rad, and Gem (mouse homolog also referred to as Kir), has emerged as potential candidates for neuronal HVA Ca^{2+} current regulation. Indeed, these proteins are signaling molecules potentially involved in axonal plasticity and have been shown to control the activity of HVA Ca^{2+} channels (L, N and P/Q type) by interacting with their accessory β -subunits and/or with the $\alpha 1$ pore subunit [8, 18, 57]. Among the most unique features of RGK proteins is their ability to be transcriptionally regulated [31, 44]. Typically, RGK GTPases are found to be up-regulated in both developmental and disease processes [20, 40] as well as during adaptive responses to extracellular stimuli [14]. In addition RGK proteins possess multiple phosphorylation sites that regulate their nucleo-cytoplasmic localization and function [32]. However, most of these data on HVA Ca^{2+} channels have been established in vitro or following overexpression of exogenous Gem, and very few data are available concerning the endogenous expression and role of RGK proteins in the nervous system. Among the RGK family members, Rem2 is predominantly expressed in brain [19]. Exogenous Rem2 overexpression inhibits HVA Ca^{2+} currents in adult

1 sympathetic, dorsal root ganglion and embryonic hippocampal neurons [12, 50]. Rem2 also
2 reduces frequency of excitatory post synaptic currents in hippocampal neurons without effects
3 on HVA Ca^{2+} current and appears to be an important regulator of synapse development and
4 function [40, 50]. To the best of our knowledge, the dynamic regulation of neuronal HVA Ca^{2+}
5 channels by endogenous RGK proteins has never been demonstrated so far.

6 In this study we sought to determine the potential roles of the RGK GTPases following nerve
7 injury. We report that Gem is the only the RGK GTPase up-regulated in DRG and peripheral
8 nerve early after injury. The phosphorylated form of Gem is predominant in DRG and inhibits
9 the P/Q-type Ca^{2+} current expressed in axotomized sensory neurons. Further, using *in vitro*
10 analysis and immunochemistry we demonstrate that the specific inhibition of the P/Q channels
11 in large size DRG neurons is due to both expression of Gem in these neurons and the higher
12 sensitivity of the $\text{Cav}2.1\text{-Cav}\beta4$ pair. **Finally, we demonstrate that P/Q type Ca^{2+} current**
13 **regulates neurite branching of axotomized neurons. Overall our data suggest that Gem-**
14 **regulated P/Q-type current could participate to general homeostatic plasticity mechanisms**
15 **following peripheral nerve injury aimed to promote a successful regeneration.**

17 **Materials and Methods**

19 **Surgery and sensory neurons culture**

20 The protocols were validated by the Direction Départementale des Services Vétérinaires de
21 l'Hérault (Certificate of Animal Experimentation no. B 34-65, 17 August 2010). The care and
22 use of mice conformed to institutional policies and guidelines. Adult Swiss female mice (6-10
23 weeks old, CERJ, Le Genest St. Isle, France) were housed in cages with a 12 h light/dark cycle
24 and fed food and water *ad libitum*. For surgery, mice were deeply anesthetized by isoflurane
25 inhalation. The left sciatic nerve was exposed at the mid-thigh, sectioned, and a 3 - 5 mm

fragment removed. Six hours to one and three days after surgery, mice were killed by CO₂ inhalation followed by cervical dislocation and lumbar (L4-L5) dorsal root ganglia were removed and processed either for RNA or protein extraction. Neuronal cultures were established from L4-L5 lumbar dorsal root ganglia as previously reported [2]. The cells were maintained in culture at 37°C in an incubator with a humidified 95% air/ 5% CO₂.

Gene knockdown and overexpression experiments

Pooled non-targeting control small-interfering RNA (siRNA), achieved with a set of four non active siRNAs, or specific siRNA against mouse Gem, were designed via the on-target plus SMART pools from Dharmacon (Thermo Fisher Bioscience GmbH, Germany). Target sequences for Gem siRNA were n°1: UUGAUAACCUGCAGCGCAA; n°2: UUACUUAUGUGUCGUAGAA; n°3: CAACCAAGUGCACGGGCAA; n°4: GAAGAUACAUAUGAGCGUA. Gem siRNA n°4 recognizes a coding region in the human pGem used for our study. The pEGFP-Gem plasmid is described in [26].

Transfection

For *in vitro* transfection, neurons were individually electroporated two hours after plating [10]. Electrode tips were filled with 8 µl 145 mM KCl, 10 mM HEPES containing either dextran-rhodamine or fluorescein, to visualize transfected neurons, (3 mM)/siRNA (1µM) or plasmid DNA (0.1 µg/ml).

For validation of siRNA specificity, 1 µM control siRNA, or Gem siRNA were transfected into the N2A neuroblastoma cell line, known to express high level of Gem transcript (kindly provided by Dr F. Marmigère, U-1051 Montpellier). HEK cells were used for validation of anti-Gem antibody and siRNA specificity at the protein level. Lipofectamin was used for N2A neuroblastoma siRNA transfection and HEK cells plasmid and siRNA transfection.

Quantitative RT-PCR

Total mRNA was extracted from lumbar L4-L5 DRG or primary DRG culture of adult mice using the RNeasy Mini Kit (Qiagen, Courtaboeuf, France) in accordance with the manufacturer's instructions. Briefly, DRG were harvested in RNAlater stabilization buffer (Qiagen) and homogenized by passing the lysate 10 times through a 20-gauge needle. Lysates were then mixed with an equal volume of 70% ethanol, and total mRNA was separated from other cellular components using RNeasy minispin columns. The eluted mRNA was quantified using spectrophotometry (Nanodrop; Labtech, Paris, France). Following gDNA removal, reverse transcription was performed with 1 µg mRNA (lumbar) or 0.5 µg (culture) using the Quantitect RT kit (Qiagen). cDNA was then diluted to get a final concentration of 100 ng (lumbar) or 50 ng (culture) and stored at -20°C until further use. Primers were designed with Primers 3.0 software. Quantitative PCR was performed at least three times on duplicate samples of cDNA diluted to 1/10 (10 or 5 ng) using SYBR Green (Qiagen) for detection on the LightCycler system (Roche Diagnostics). After an initial activation step of 15 min at 95°C, 45 amplification cycles were performed consisting of 94°C for 15 sec, 60°C for 20 sec and 72°C for 35 sec. After PCR amplification, a melting curve analysis was generated to check the specificity of the PCR reaction. The identity of RT-PCR amplified products was confirmed by sequencing (Millegen, Labège, France). Polymerase (RNA) II polypeptide J (*Polr2J*) levels were used to normalize amounts of cDNA [41]. For gene comparison inside a same cDNA pool, C_t values were normalized to the most expressed gene (i.e. smallest C_t). The ΔC_t was calculated as the difference between the C_t values, determined using the equation $2^{-\Delta C_t}$. Forward (f) and reverse (r) primers used were as follows.

Gem (171 bp): (f) TCACAGACCGTGCAAGCTTCG,

(r) TGCAGTCGAACACCACAGCACA

1 Rad (199 bp): (f) ACGACGTGCCCATCATTCTCGT,
 2 (r) TCTTCTTTGCTGTCCCTGCGCA
 3 Rem1 (88 bp): (f) TGGATGAAAGCTGGTGCCAGGA,
 4 (r) TCAAAGCTGCTCGCATCCGCTA
 5 Rem2 (76 bp): (f) AAGCTAAGCGCTTCCTCGCCAA,
 6 (r) TCGTGACATGACCTGGAGCGTT
 7 Cacn β 1 (180 bp): (f) ACCAGTGGCTTTTGCTGTTCGGA,
 8 (r) ATGAAGCCAACCTCGCAGCCTT
 9 Cacn β 2 (98 bp): (f) AGCAGCAATGGCCACAGACACA,
 10 (r) TGCCGGCTTCGTTGTTTGATGC
 11 Cacn β 3 (81 bp): (f) AGCGCATATTCGAGCTGGCCAAA ,
 12 (r) TGGCAAGTTGTGCTGGGTGGTT
 13 Cacn β 4 (120bp): (f) TCTGTGTTTACGCAGGGGCCTT,
 14 (r) AACTCACAGCGATTTCCCCGCA
 15 Polr2j (160 bp): (f) ACCACACTCTGGGGAACATC,
 16 (r) CTCGCTGATGAGGTCTGTGA
 17

18 **Microarray analysis**

19 Raw Affymetrix microarray results (GSE26350 CEL files) generated in a study of transcription
 20 regulation induced by sciatic nerve crush on rat (Michaevski et al., 2010) were downloaded
 21 from Gene Expression Omnibus repository (<http://www.ncbi.nlm.nih.gov/geo/>) and were pre-
 22 processed with the robust multichip analysis algorithm (RMA, [9]). Resulting signals were
 23 analysed with Rank Difference Analysis of Microarray (RDAM) ([35],
 24 <https://github.com/mbellis/RDAMm>) which allows the identification of statistically significant
 25 signal variations between two biological conditions (specifically, between crushed sciatic nerve

24 hrs after injury and a sham control). Standardized variations (Z_{var}) with a False Discovery Rate (FDR) inferior to 10% were considered as significant (FDR estimates the fraction of selected genes that are likely to be invariant genes).

Whole-cell recording

Voltage-activated calcium currents in DRG neurons were recorded after 1 or 2 days *in vitro* (DIV). Electroporation was performed two hours after cell plating. Plasmid transfected sensory neurons were recorded at 1 DIV and siRNA transfected neurons recorded at 2 DIV. Whole-cell patch-clamp recordings were made at room temperature under conditions optimized for the isolation of calcium current separately from other voltage-activated currents. The bathing solution contained (in mM): 130 tetraethylammonium chloride, 2 CaCl_2 , 1.5 MgCl_2 , 10 HEPES and 10 glucose, and the pH was adjusted to 7.4 with CsOH. Recording pipettes were filled with the following solution (in mM): 145 CsCl, 10 HEPES, 2 Mg-ATP, 0.5 $\text{Na}_2\text{-GTP}$, and pH 7.35 adjusted with CsOH. All recordings were made at room temperature using an Axopatch 200B amplifier (Axon Inst., Dipsi Industrie, France).

Oocytes injection and electrophysiology

The following cDNAs were used: Cav2.1 (GenBank X57476), Cav2.2 (D14167), Cav α 2 δ 1 (M86621); Cav β 3 (NM_012828;), Cav β 4 (L02315) and Gem (from human expressed sequence tag IMAGE clones ID 4823985, see Leyris et al., 2009). These cDNAs were linearized and transcribed *in vitro* using SP6 or T7 polymerases (Mmessage-Mmachine; Ambion, Austin, TX, USA). Final RNA concentration was adjusted to 1 g/l. A mixture of Cav2.x, Cav α 2 δ 1 and Cav β x (stoichiometry 1:1:1) was made and diluted with either an equal volume of deionized water or Gem RNA at 1 g/l. Forty nanoliters of this mixture was then injected into freshly

isolated *Xenopus* oocytes. Whole-cell Ba^{2+} currents were recorded 2–5 d after injection under 2-electrode voltage-clamp using a GeneClamp 500 amplifier (Axon Instruments, Union City, CA, USA). Current and voltage electrodes ($<1\text{ M}\Omega$) were filled with 3 M KCl, pH 7.2, with KOH. Ba^{2+} current recordings were performed using the following external solution (in mM): 10 BaOH, 20 TEAOH, 50 NMDG, 2 CsOH, and 10 HEPES, pH 7.2 with methanesulfonic acid. Currents were filtered (500 Hz) and digitized (2 kHz) using a Digidata-1200 interface (Axon Instruments) and pClamp 7.01 software (Axon Instruments). Around 10–30 nl of BAPTA (in mM: 100 BAPTA-free acid, 10 CsOH, and 10 HEPES, pH 7.2 with CsOH) was injected into each oocyte (10 psi, 150 ms) at the beginning of the recording using a third electrode. Ba^{2+} currents were recorded during a 400-ms test pulse from -80 to +10 mV. Current amplitudes were measured at the peak of the current. Comparisons of averaged amplitudes between batches were always performed on currents measured on the same number of days following injection. Comparisons between experiments were made by normalizing all average amplitudes with respect to the control current amplitude set at 100%.

Immunostaining

Primary antibodies used were rabbit anti-Gem (Novus biologicus; 1:100–250), mouse anti- β III-Tubulin (Sigma; 1:500), mouse monoclonal anti-Cav β 4 (NeuroMab, clone N10/7; 1:1000), rabbit polyclonal anti-Cav β 3 (Sigma-Aldrich, C1978; 1:500), goat polyclonal anti-TrkC (Upstate biotechnology; 1:500). Secondary antibodies were Alexa Fluor-594 or Alexa Fluor-488 (Molecular Probes 1:500).

Neuronal cultures were fixed for 15 min in 4% paraformaldehyde in PBS, and incubated for 20 min in blocking buffer (10% donkey serum in 0.1 % triton PBS). They were then incubated overnight at 4°C with the primary antibodies in the blocking buffer diluted to 1:10. Cell cultures

were then incubated for 1h at room temperature with secondary antibody in the blocking buffer 1:10, followed by mounting in Mowiol. For immunohistochemistry, L4-L5 DRG were dissected in PBS, fixed for 2h in 4% paraformaldehyde, immersed overnight in 25% sucrose, and embedded in OCT compound. Fourteen- μ m frozen DRG sections were cut, and each was washed with PBS, incubated for 30 min in blocking buffer (10% donkey serum in 0.3 % triton PBS), and incubated overnight at 4°C with primary antibodies. Images were obtained with an inverted confocal microscope Zeiss Axioobserver / LSM 5 (40x or 63x EC plan NeoFluar Oil objective) or with with a PL-Apochromat 20x/0.8 objective on an upright Zeiss microscope equipped with an AxioCam MRm CCD camera (Zeiss, Le Pecq, France), using Zen software. For neurite analysis, images were obtained with a PL-Neofluar Zeiss 10X 0.3 objective.

Immunoblot analysis

For protein extraction, contralateral and ipsilateral lumbar L4-L5 dorsal root ganglia together with the peripheral nerves and dorsal roots from 6 adult mice were collected and pooled in 50 μ l RIPA 2 days post left sciatic nerve transection. For primary cultures, neurons from 12-14 DRG (~ 28.000 neurons) were lysed with 150 μ l RIPA buffer after 1 DIV. RIPA contains: 50 mM Tris, 150 mM NaCl, 0.1% SDS, 0.5% sodium deoxycholate, 1% Triton X-100. BCA Protein Assay (Pierce) was used for protein quantification. Protein extracts were separated by electrophoresis on a 4-15% TGX gel (Bio-Rad) and blotted onto a PVDF membrane (Bio-Rad). The membranes were blocked in 5% nonfat milk in TBST (10 mM Tris-HCl pH 8.0, 150 mM NaCl, and 0.1% Tween 20). Membranes were incubated overnight at 4°C with primary antibody, followed by 1 hour incubation with the secondary antibody. All washes were performed with TBST. Signals were visualized using the chemiluminescence HRP substrate (Millipore). Exposure times were chosen based on the

saturation of the highest amounts of protein. The primary antibodies used were mouse anti-actin (Sigma; 1:1000) and rabbit anti-Gem (1:500). The secondary antibodies used were: peroxidase goat anti rabbit or anti mouse (Dako Denmark; 1:1000).

Statistical analysis.

All values are reported as mean \pm s.e.m. Statistical significance was evaluated using one way ANOVA (followed by Dunnett's or Bonferroni's test), Mann Whitney test or Fisher's test as indicated. $P < 0.05$ was considered as significant (Graphpad Prism 5, Graphpad software Inc., La Jolla CA).

Results

RGK GTPase Gem is up-regulated early upon nerve injury of the dorsal root ganglia

To determine the RGK GTPases present in DRG, we quantified their mRNA level by using quantitative RT-PCR (qRT-PCR). In lumbar L4-L5 DRGs obtained from uninjured mice DRG, the C_t values obtained for Rad, Gem, Rem1 and Rem2 were: 29.98, 29.18, 26.15, and 24.25, respectively. This rank of expression order was conserved with different cDNA samples.

To investigate the effects of nerve injury on the transcriptional expression of RGK GTPases, qRT-PCR analysis of lumbar L4-L5 DRGs from control and axotomized adult mice was performed at different time points following sciatic nerve transection. For mRNA quantification, we calculated the relative mRNA levels of RGK GTPase by using the $2^{-\Delta C_t}$ method. To correct for cDNA fluctuations between different samples, the level of expression for each gene was normalized to *Polr2j*, a previously validated stable control gene [28]. Our

1 results show that peripheral nerve injury produced a transient three-fold increase in the *Gem*
2 transcript as early as 24 hours following injury, returning to basal level three days post-injury
3 (Fig 1A). *Rad* and *Rem1* transcripts were not significantly modified while *Rem2* transcript was
4 significantly decreased three days following injury (Fig. 1A). To confirm that up-regulation of
5 *Gem* was an early event induced by nerve injury, we performed qRT-PCR on DRG 6 hours
6 following nerve injury and still found a 3.5-fold increase (data not shown).

7 As the level of *Gem* transcript appeared low relative to *Rem1* and *Rem2* and to confirm
8 that *Gem* regulation is a general cellular mechanism induced by nerve injury, we performed a
9 new statistical analysis from a publicly available microarray experiments studying the
10 transcriptional profile of genes regulated early following sciatic nerve crush on rats [37]. Our
11 analysis, which focused on the RGK genes family, shows that the *Gem* probe is significantly
12 up-regulated 24 hours after nerve crush, while the other members did not show any significant
13 variation (Fig. 1B).

14 Therefore, our results strongly support *Gem* as being the only RGK GTPase up-
15 regulated following nerve injury, which led us to investigate the role, expression and
16 localization of this protein in the peripheral nervous system in both uninjured and injured
17 environments.

18 ***In vivo* and *in vitro* expression of Gem protein**

19 Under control conditions, *Gem* could not be detected by immunoblot analysis in DRG or in the
20 peripheral sciatic nerve (Fig. 2A). In dorsal roots, *Gem* expression was detected as a doublet,
21 as frequently reported in other systems [7, 25, 26]. Two days after nerve injury, protein
22 expression in DRG and peripheral sciatic nerve was clearly amplified, while *Gem* expression
23 remained constant in dorsal roots. Interestingly, the higher weight band detected by the *Gem*
24 antibody was clearly and specifically upregulated in DRG after nerve injury (Fig. 2A), while in
25

peripheral nerve the up-regulation was recorded for both bands of the doublet. Consistent with other studies [51], the antibody also recognized a band at the expected molecular weight (33-35 kDa) in tissue extracts from mouse brain but not from spleen (Fig. 2B). Gem is therefore constitutively expressed in dorsal roots and up-regulated in DRG and peripheral nerve after nerve injury.

To analyze the role of Gem at a cellular and molecular level, we used DRG primary cultures, a convenient *in vitro* model that reproduces the short-term effects of an *in vivo* peripheral nerve axotomy. This choice was validated by the analysis of the transcript of the small proline-rich protein 1A (*Sprr1A*), a known early up-regulated gene following injury [39]. Expression of *Sprr1A* showed a 20-fold increase in 1 day *in vitro* (DIV) primary culture relative to control DRG (Fig. 2C). We then analyzed the expression level of Gem transcript in DRG primary cultures. Consistent with our *in vivo* data, *Gem* expression displayed a 4-fold increase in 1 DIV DRG culture compared to control DRG and was not significantly different from DRG after 1 day post-axotomy *in vivo* (Fig. 2C). Gel electrophoresis analysis of the amplified *Gem* fragments confirmed the up-regulation in primary culture and axotomized DRG (Fig. 2D). We then verified that the expression of *Gem* in DRG primary cultures was also observed at the protein level. Immunoblot analysis of 1 DIV culture allowed detecting one faint band consistent with the expression of the Gem protein. However, the presence of only one band did not allow to state whether it was the phosphorylated form (Fig. 2E).

Gem is expressed in axotomized sensory neurons

Since Gem has been reported to be increased in glial Schwann cells following sciatic nerve crush [51], we used immunocytochemistry to assess its cellular and sub-cellular expression in DRG primary cultures at 2 DIV to optimize the effects of RNA interference on protein expression. Nuclear, cytoplasmic and plasma membrane staining were observed with the anti-

1 Gem antibody amongst a subset of neurons (Fig. 3A), while staining was never observed in non-
2 neuronal cells (data not shown). Quantitative analysis showed that ~70 % of sensory neurons
3 were Gem-positive with 40% having cytoplasmic and/or membrane-associated staining (Fig.
4 3A right). Nuclear staining was observed in ~30 % of neurons (Fig. 3A, left). DRG neurons
5 form a morphologically and functionally heterogeneous population coding for different
6 modalities such as pain, touch, temperature and proprioception [24]. A soma size criterion is
7 commonly used in *in situ* and in *in vitro* analyses to rapidly discriminate the nociceptive
8 neuronal population from the innocuous population. Acutely dissociated DRG cells have been
9 classified into small (<27 μm) for the nociceptive population , medium (30-40 μm) for a mixed
10 of innocuous and nociceptive mechanoreceptors and large size neurons (> 45 μm) for innocuous
11 mechanoreceptors and proprioceptors [46]. For simplicity, we classified our neurons into two
12 populations: smaller neurons (< 30 μm) and larger neurons (> 30 μm). Therefore, using
13 frequency histogram of staining distribution relative to cell size, we show that cytoplasmic and
14 membrane-associated Gem staining was predominant in the population of larger sensory
15 neurons (37 %), while nuclear staining was most frequently observed amongst smaller neurons
16 (Fig. 3B). Staining specificity was evaluated by RNA interference using the single-cell
17 electroporation method. Two days after transfection with Gem siRNA or control siRNA (with
18 the fluorescent dextran marker simultaneously), cultures were stained with the anti-Gem
19 antibody. In the presence of Gem siRNA, the number of dextran-positive neurons having a
20 cytoplasmic and/or membrane-associated staining (17%, 7/41 neurons) was significantly
21 decreased compared to control siRNA (53%, 18/34), while the number of neurons having a
22 nuclear staining was not modified 26%, 9/34 (control siRNA) and 29%, 12/41 (Gem siRNA)
23 (Fig. 3C). These results suggest that endogenous cytoplasmic/plasma membrane Gem, and not
24 nuclear, is distinctively targeted by Gem siRNA. To assess specificity of Gem siRNA, we
25 transfected N2A neuroblastoma cells, reported to endogenously express Gem, with Gem or

1 control siRNAs. Consistent with a specific knockdown, one day after transfection, the
2 expression of Gem transcript was decrease by 60% compared to the control value (Fig. 3D). No
3 effect was observed on the expression of other related transcripts of the RGK GTPase family,
4 such as Rad and Rem2 (Fig. 3D). Lastly, we used HEK-293 cells that do not express Gem
5 protein. The anti-Gem antibody recognized the exogenously expressed pGem-GFP, but not
6 pRem2 (Fig. 3E). Importantly, in the presence of both pGem-GFP and Gem siRNA, no protein
7 was detected (Fig. 3E). **To confirm the cellular and sub-cellular Gem expression *in situ*, we**
8 **carried out Gem labeling in control and axotomized L4-L5 DRG. In control DRG, no distinctive**
9 **staining was observed among sensory neurons (Fig. 3F). Five days following nerve injury,**
10 **cytoplasmic Gem staining was mainly observed in a subset of large sensory neurons. No nuclear**
11 **staining was observed in control and axotomized DRG neurons. We performed Gem co-**
12 **labelling experiments with a known marker of large proprioceptive neurons, the high affinity**
13 **receptor for neurotrophin-3, TrkC [48]. We confirmed the expression of TrkC in a subset of**
14 **large control and axotomized DRG sensory neurons (Fig. 3F-G) and show Gem colocalization**
15 **with numerous TrkC positive neurons as well as in some large negative TrkC neurons (see**
16 **merge image Fig. 3F).**

17
18 Our data thus demonstrates that Gem protein is expressed in a subset of axotomized sensory
19 neurons, **including TrkC positive sensory neurons**, with a cytoplasmic/plasma membrane
20 subcellular localization.

21
22 **Gem inhibits P/Q type Ca^{2+} current amplitude in sensory neurons of large somatic**
23 **diameter**

To analyze the role of endogenous Gem in sensory neurons, we used our validated RNA interference strategy. Gem silencing did not affect cell survival, at least up to three days after siRNA transfection (Fig. 4A). At 2DIV, resting membrane potential was -58 ± 3 mV and -61 ± 2 mV and current threshold for action potential triggering was 2.2 ± 0.2 nA, $n=4$ and 1.9 ± 0.1 nA, $n=9$, under control and Gem siRNAs, respectively. Action potential duration measured at 50 % of repolarization was not significantly modified (1.56 ± 0.28 ms and 1.92 ± 0.29 ms under control and Gem siRNAs, respectively) and amplitude of the after hyperpolarization was -73 ± 1 mV and -74 ± 1 mV, under control and Gem siRNAs, respectively (Fig. 4B). Overall, no significant effects of Gem silencing could be observed on electrical activity. However, since Gem has been reported to highly inhibit HVA Ca^{2+} channels *in vitro*, we then focused our electrophysiological analysis on high and low voltage-activated (LVA) Ca^{2+} currents. Two days after siRNA electroporation, electrophysiological recordings indicated that Gem siRNA induced a significant 55% increase in HVA Ca^{2+} current amplitude compared to neurons transfected with control siRNA (Fig. 4C), leaving LVA channels almost unaffected (not shown but see below). These effects were observed among the population of larger sensory neurons and correlated with the cytoplasmic-membrane associated Gem staining observed in this neuronal subpopulation. Analysis of smaller sensory neurons showed no significant effects of Gem silencing on HVA Ca^{2+} current amplitude, which correlated with a preferential nuclear localization of Gem in this subpopulation (Fig. 4C).

We next analyzed whether Gem specifically inhibited a particular subtype of HVA Ca^{2+} current expressed in larger sensory neurons. Global HVA Ca^{2+} current recorded in sensory neurons at 2 DIV reflects the activity of Cav1.x (L-type), Cav2.1 (P/Q type), Cav2.2 (N-type) and Cav2.3 (R-type) Ca^{2+} channels. When recorded in the presence of nifedipine and AgaIVA to inhibit L- and P/Q types HVA, respectively, Gem siRNA had no significant effects on current amplitude compared to control siRNA (Fig. 4D). When recorded in the presence of nifedipine

1 and GVIA to inhibit L- and N-type currents, current amplitude of remaining current was
2 significantly increased when treated with Gem siRNA compared to control (Fig. 4D).
3 Therefore, this pharmacological and deduction analysis suggests that Gem specifically inhibit
4 the P/Q type Ca^{2+} current in sensory neurons.

5 Knowing that, under our experimental conditions, HVA Ca^{2+} current expressed in
6 smaller sensory neurons was not sensitive to siRNA Gem, we thus compared the
7 pharmacological profile of HVA channels between small and large neurons at 2DIV. For this
8 experiment, each inhibitor was sequentially applied to a neuron and the percentage of inhibition
9 was calculated. Our results clearly show that smaller neurons preferentially express N-type Ca^{2+}
10 current (53 ± 8 % of global HVA, $n=4$) versus P/Q type (13 ± 3 %, $n = 6$); while P/Q type Ca^{2+}
11 current was a clear marker of larger sensory neurons (40 ± 5 % of global HVA, $n=11$) versus
12 N-type (13 ± 4 %, $n = 7$). No significant differences were observed for L- and R-type currents
13 expression between different sizes of sensory neurons (Fig. 4E). Altogether, these results
14 suggest that endogenous upregulation of Gem specifically inhibits the ω -AgaIVA-sensitive P/Q
15 type current expressed in larger sensory neurons after nerve damage.

18 **Overexpressed full-length Gem localizes to the membrane and inhibits HVA Ca^{2+} current** 19 **amplitude in sensory neurons**

21 To further analyze the subcellular expression together with functional analysis of Gem
22 expression in sensory neurons, we overexpressed Gem in sensory neurons using a GFP tagged
23 Gem cDNA. Experiments were performed at 1 DIV, as a strong GFP fluorescence was already
24 observed one day after cDNAs electroporation. Compared with the uniform cytosolic pEGFP
25 staining, exogenous expression of EGFP-tagged Gem in live adult sensory neurons was

characterized by a more granular display in the cytoplasm and increased signal intensity close to the plasma membrane. No nuclear staining was observed (Fig. 5A). Gem is reported to inhibit HVA Ca^{2+} currents when expressed in heterologous system [7, 8, 26]. Therefore, we investigated the effects of overexpressed Gem on Ca^{2+} currents in sensory neurons. Electrophysiological recordings of Ca^{2+} currents show that pEGFP-transfected sensory neurons express low voltage activated (LVA) and HVA Ca^{2+} currents (Fig. 5B). In the presence of pEGFP-Gem, there was a significant 94 % and 82 % decrease in the HVA Ca^{2+} current peak amplitude and current measured at 500 ms, respectively (-3.2 ± 0.7 nA and -1.1 ± 0.3 nA, $n = 11$, for maximal peak and 500 ms current amplitudes, respectively, under pEGFP; -0.2 ± 0.1 nA and -0.2 ± 0.1 nA, $n = 11$, for maximal peak and 500 ms current amplitudes, respectively, under Gem) (Fig. 5C). The effects were observed at all potentials and in all transfected neurons. In agreement with specific Gem's interaction with HVA Ca^{2+} currents [56], the amplitude of LVA Ca^{2+} current recorded in a subset of sensory neurons [1] was not affected by Gem overexpression (Fig. 5B and D). Furthermore, random recordings of the Down-hair neurons, a mechanosensory population characterized by the expression of a large T-type Cav3.2 LVA-current [15], confirmed the lack of inhibitory effects of Gem on Cav3.x gene family (Fig. 5E). In addition, we could trigger Na^{+} -dependent action potentials in Gem-transfected neurons (data not shown). Altogether, our results suggest that overexpressed Gem in sensory neurons localizes to the cytoplasm and plasma membrane and inhibits all endogenous HVA Ca^{2+} currents, leaving T-type currents unaffected.

High affinity block of Cav2.1/Cav β 4 Ca^{2+} channels by expression of Gem

Gem overexpression experiments clearly demonstrate a nearly complete inhibition of HVA Ca^{2+} currents recorded in DRG neurons, while our knockdown of endogenous Gem highlights a specific effect on P/Q type Ca^{2+} channels. We thus assessed how Gem targets P/Q type channels in the large neuron population. The preferential cytoplasmic/membrane-associated staining of Gem in larger sensory neurons may explain Gem's specific effects on HVA Ca^{2+} currents in this subpopulation. However, how Gem targets P/Q type channels in the larger neuron population remains unclear. In heterologous expression systems, the effects of Gem on HVA Ca^{2+} channels, including the Cav2.1 (P/Q type), have been shown to require, albeit not necessarily the interaction with, the auxiliary Cav β subunits [14, 17, 26]. However, how any of the four possible Cav β subunits modulate the strength of the Ca^{2+} current inhibition by Gem is not known. In rat DRG sensory neurons among the four Cav β genes, Cav β 3 and Cav β 4 are the most prominent subtypes expressed [27]. We indeed confirmed this major expression relative to Cav β 1 and -2 in mice DRG (Fig. 6A). To determine whether Gem showed any preferential sensitivity for a given Cav α -Cav β x subunit combination, we used the *Xenopus* oocytes expression system. As P/Q and N-types Ca^{2+} currents are the predominant types of current present in sensory neuron cultures (see Fig. 4E), the comparative study was performed with Cav2.1 (P/Q type) and Cav2.2 (N-type) co-expressed with Cav β 3 or Cav β 4 subunits. Our results confirm that Gem is able to inhibit both Ca^{2+} channel types in the presence of Cav β but the inhibition was slightly more pronounced for Cav2.1 combined with Cav β 4 (compared to Cav2.2) and was equally effective when combined with Cav β 3 (Fig. 6B).

We next challenged the sensitivity of these two types of channels to different concentration of Gem. This study was performed with Cav2.1 and Cav2.2 co-expressed with the Cav β 4 subunit. The results (Fig. 6C) clearly demonstrated a higher sensitivity of Cav2.1 versus Cav2.2 Ca^{2+} channels to Gem inhibition. This higher sensitivity is highlighted at the low-intermediate concentrations of Gem (see the 1/4 Gem dilution in Fig. 6C, which represents an

1 approximate 1/1 w/w mRNA ratio between Gem and the Cav β subunit), that may be prevalent
2 in neurons after nerve injury. At higher concentration, these differences tended to vanish, and
3 complete inhibition of both channel types could be recorded in conditions of massive
4 overexpression, similar to those obtained following Gem overexpression in DRG (see Figure
5 5).

6 We then examined by immunofluorescence the distribution of Cav β 3 or Cav β 4 subunits
7 in sensory neurons at 2DIV. Intense cytoplasmic Cav β 3 staining was observed in most sensory
8 neurons as previously reported in DRG tissue [27] (data not shown), while staining of Cav β 4
9 was mainly close to the plasma membrane in a subset of sensory neurons (see Fig. 6D). As
10 Cav β 4 appeared as a specific marker of a sensory neuron subpopulation, we used co-labeling
11 and confocal microscopy to determine the expression of Cav β 4 in Gem-positive sensory
12 neurons. All Cav β 4-positive neurons were also Gem-positive (Fig. 6D). Among 55 neurons
13 analyzed, 17 were Gem-positive, with 15 colocalizing with Cav β 4. Mean somatic diameter was
14 $33.5 \pm 1.6 \mu\text{m}$, $n = 15$ (ranged from $25 \mu\text{m}$ to $50 \mu\text{m}$). Few smaller sensory neurons were found
15 to be Gem-positive, but did not express Cav β 4 ($n = 4$). Therefore, the preferential expression
16 of Gem and Cav2.1/Cav β 4 Ca $^{2+}$ channel subunit in larger sensory neurons after nerve section
17 may explain Gem's preferential inhibition of the P/Q type Ca $^{2+}$ current in this subpopulation,
18 leaving the Cav2.2 (N-type) channels almost unaffected.

19 20 **P/Q type Ca $^{2+}$ current regulates neurite branching**

21 Following nerve injury, transcriptional downregulation of L-type Cav1.2 Ca $^{2+}$ current causes
22 an increase in axon outgrowth of axotomized sensory neurons [16]. Therefore, we determined
23 whether, in our conditions, inhibition of P/Q-type current had relevant effects on neurite
24 morphology. After cell dissociation, the specific P/Q-type Ca $^{2+}$ current inhibitor, AgaIVA, was
25 added to the culture medium and neurite morphology of larger sensory neurons was analyzed

at 1 DIV. Complete inhibition of P/Q-type Ca^{2+} current did not modify the longest primary neurite length but did significantly decrease by half the number of secondary branches (Fig. 7). Our results thus suggest that Gem-induced P/Q-type Ca^{2+} current inhibition after nerve injury controls neurite branching of sensory neurons.

Discussion

We report here that peripheral nerve injury leads to an up-regulation of Gem expression in a particular set of sensory neurons from DRG, including the proprioceptive population. This up-regulation specifically inhibit P/Q type Ca^{2+} current in these neurons, leaving other HVA and LVA channels unaffected, as opposed to overexpression of exogenous Gem which inhibits all HVA channels. This specificity of endogenous Gem inhibition relies at least partly on the higher sensitivity of the $\text{Cav}2.1+\text{Cav}\beta 4$ Ca^{2+} channel subunits pair to Gem. This preferential inhibition of the P/Q-type current over other Ca^{2+} channel types by Gem contributes to neurite branching and thus could participate to general homeostatic plasticity mechanisms of large axon groups following peripheral nerve injury (aimed to promote a successful regeneration).

Our results show that among the four members of the RGK family analyzed; only Gem is consistently increased after peripheral nerve injury. Under physiological conditions, Gem is constitutively expressed in the dorsal roots, but not, or at undetectable levels, in DRG and peripheral nerves. As soon as six hours following peripheral nerve injury, increased Gem transcription probably accounts for up-regulation of the protein in DRG and peripheral nerve, while the protein level remains constant in the dorsal roots. Increased expression of Gem was recently reported in sciatic nerve following a crush and was shown to be predominant in Schwann cells and macrophages, suggesting that it may promote Schwann cell proliferation in peripheral nerve [51]. In the present study, Gem appears to be also up-regulated in DRG

1 supporting a role in neuronal function. A strong up-regulation of the higher molecular weight
2 (phosphorylated) band of Gem was noted in DRG. Gem migration as a doublet is typically
3 observed and is attributed to protein phosphorylation [7, 26, 52], which was also established
4 for the RGK Rem2 in embryonic cortices [21]. In our study, the signaling pathway leading to
5 Gem phosphorylation is unknown, but could involve the cytokine pathway that we have
6 previously shown to contribute to protein phosphorylation and neurite outgrowth observed after
7 sensory neurons axotomy [41, 42]. Gem phosphorylation modulates its subcellular localization
8 by preventing nuclear accumulation [7, 32, 33]. It is suggested that preventing nuclear
9 sequestration of Gem is required for its inhibitory action on Ca^{2+} channel activity [7]. Therefore,
10 phosphorylation and sub-plasma-membrane-cytoplasmic localization of Gem may both
11 contribute to the inhibition of Ca^{2+} current in sensory neurons.

12 A major finding from this study is that endogenous Gem is mainly expressed in larger
13 sensory neurons. Gem thus appears to be excluded from pain sensing small diameter neurons
14 that project to the lamina I of the dorsal spinal cord. The larger neurons are mainly composed
15 of mechano- and proprioceptors and we show *in situ* that Gem colocalized with TrkC, a known
16 marker of proprioceptors. Gem was also present in a subset of TrkC-negative neurons that could
17 include the innocuous mechanoreceptors as well as a subset of large myelinated TrkA positive
18 nociceptors [5, 34]. Gem specifically inhibits the P/Q type HVA Ca^{2+} current among this
19 population. Cellular and functional analysis of endogenous Gem in sensory neurons points to
20 several key features of this regulation. First, the preferential expression of Gem in larger
21 neurons is undoubtedly the major factor accounting for localized inhibition of Ca^{2+} influx in
22 this particular subset of sensory neurons. Second, following Gem mRNA silencing, the specific
23 up-regulation of only the P/Q type HVA Ca^{2+} channel is consistent with a dose-dependent
24 inhibition of the Cav2.1-Cav β 4 complex by endogenous Gem [47], while the less sensitive and
25 mildly expressed Cav2.2 channel was not affected. Although our deductive approach supports

an exclusive role of Gem on P/Q type, we do not totally exclude a contribution of Gem on the well-expressed Cav2.3 R-type current as we did not use a pharmacological inhibitor of this HVA Ca^{2+} current. However, the R-type Ca^{2+} channels have been reported to be less sensitive than the other Cav2.x channels to the effect of Gem expression [11]. Third, co-expression of Gem and Cav β 4 along the plasma membrane supports the existence of a highly sensitive form of the P/Q type channel that includes this auxiliary subunit, and leads to the specific current inhibition reported herein. This is in agreement with numerous studies, showing that Ca^{2+} channel inhibition by Gem not only requires the auxiliary Cav β subunit [26, 47, 57], but is also dependent on specific pore channel subunit sequences [17, 57]. In addition, we show that, although both Cav β 3 and Cav β 4 are expressed in DRG, only Cav β 4 appears to be a molecular marker of the larger sensory neurons subpopulation.

Therefore, the preferential Cav β 4 subunit expression among Gem-positive sensory neurons, together with an apparent higher sensitivity of the Cav2.1-Cav β 4 complex to Gem, could explain, at least in part, the preferential inhibition of P/Q type Ca^{2+} current. Additional factors, such as the expression of specific alternative splice variants or the existence of specific channel partners, may also be put forward and clearly need further studies. In all cases however, the fact that overexpression of exogenous Gem completely blocked all HVA channels, argues strongly in favor of a dose-dependent effect with a preferential inhibition of Cav2.1 channels.

The model of peripheral nerve section has been widely used for the identification of genes involved in survival and regeneration [13, 43] and also in neuropathic pain [54] because some changes in molecules are reported to be implicated in generation and maintenance of pain. Therefore, the decreased amplitude of P/Q type in this model could have implications in regeneration or neuropathic pain. Concerning the role of P/Q type in afferent processing, it is established that P/Q-type Ca^{2+} channels are not of major importance for the control of transmitter release from small peptidergic nociceptive primary afferents. In the dorsal horn, the

1 highest density of terminals labeled with P/Q-directed antibodies is in laminae II-VI, with
2 minimal immunoreactivity present in lamina I, an observation in agreement with our
3 electrophysiological results [22, 38, 53]. Its preferential functional expression among large
4 somatic sized neurons together with the demonstration of Gem colocalization with TrkC
5 positive DRG sensory neurons suggests that it could contribute to proprioception in addition to
6 the various mechano-sensations processed in lamina II-VI.

7 Regarding neuropathic pain behavior, studies with antagonists for the P/Q- channels have
8 shown fewer efficacies than inhibition of N-type or T-type voltage-gated channels [49, 55].
9 Actually, P/Q type seems to play a role in central sensitization in painful models, but not in pain
10 at afferent presynaptic site [30]. Therefore, decreased P/Q type current amplitude in presynaptic
11 afferents following nerve injury is probably not relevant for pain expression.

12 Interestingly, we show that pharmacological inhibition of P/Q type leads to a reduction of the
13 number of secondary neurite branching. HVA channels have been implicated in regulating axon
14 growth or regeneration and dendrite morphology. Calcium entry through Cav2.2 is involved in
15 sensory innervation during development [45]. Interaction of Gem with Cav1.2 L-type Ca^{2+}
16 current has been reported to be important for the ability of the channel to control activity-
17 dependent dendritic arborization of cortical neurons [23]. Down-regulation of Cav1.2 caused
18 an increase in axon outgrowth after nerve injury [16]. Here, the control of neurite arborization
19 via Gem interaction with Cav2.1 P/Q type current could be part of a general homeostatic
20 mechanism aimed to restructure afferent projections of large axon groups following a peripheral
21 nerve injury. Whether Cav2.1 regulates branching by electrical activity, Ca^{2+} -dependent
22 regulation and/or Ca^{2+} -channel dependent transcriptional regulation remains to be determined.

23 Overall the present study provides new mechanistic insights into the regulation of
24 neurite plasticity induced by nerve injury. Our results establishes for the first time that the RGK
25 GTPase Gem is constitutively expressed in DRG neurons, transcriptionally regulated and

phosphorylated following peripheral nerve injury. The molecular combination of the RGK GTPase Gem-Cav2.1- Cavβ4 leads to specific inhibition of the P/Q-type Ca²⁺ current in a subset of sensory neurons that contributes to neurite plasticity. **These results open perspectives on activation of Ca²⁺ dependent intracellular mechanisms linking P/Q type Ca²⁺ current to plasticity.**

References

1. Andre S, Puech-Mallie S, Desmadryl G, Valmier J, Scamps F (2003) Axotomy differentially regulates voltage-gated calcium currents in mice sensory neurones. *Neuroreport* 14(1): 147-150.
2. Andre S, Boukhaddaoui H, Campo B, Al-Jumaily M, Mayeux V, Greuet D, Valmier J, Scamps F (2003) Axotomy-induced expression of calcium-activated chloride current in subpopulations of mouse dorsal root ganglion neurons. *J Neurophysiol* 90(6): 3764-73
3. Arikath J, Campbell KP (2003) Auxiliary subunits: essential components of the voltage-gated calcium channel complex. *Curr Opin Neurobiol* 13(3): 298-307
4. Baccei ML, Kocsis JD (2000) Voltage-gated calcium currents in axotomized adult rat cutaneous afferent neurons. *J Neurophysiol* 83(4): 2227-2238.
5. Bachy I, Franck MC, Li L, Abdo H, Pattyn A, Ernfors P (2011) The transcription factor Cux2 marks development of an A-delta sublineage of TrkA sensory neurons. *Dev Biol* 360(1): 77-86
6. Bauer CS, Tran-Van-Minh A, Kadurin I, Dolphin AC (2010) A new look at calcium channel alpha2delta subunits. *Curr Opin Neurobiol* 20(5): 563-71
7. Beguin P, Mahalakshmi RN, Nagashima K, Cher DH, Takahashi A, Yamada Y, Seino Y, Hunziker W (2005) 14-3-3 and calmodulin control subcellular distribution of Kir/Gem and its regulation of cell shape and calcium channel activity. *J Cell Sci* 118(Pt 9): 1923-34
8. Beguin P, Nagashima K, Gono T, Shibasaki T, Takahashi K, Kashima Y, Ozaki N, Geering K, Iwanaga T, Seino S (2001) Regulation of Ca²⁺ channel expression at the cell surface by the small G-protein kir/Gem. *Nature* 411(6838): 701-6
9. Bolstad BM, Collin F, Simpson KM, Irizarry RA, Speed TP (2004) Experimental design and low-level analysis of microarray data. *Int Rev Neurobiol* 60: 25-58
10. Boudes M, Pieraut S, Valmier J, Carroll P, Scamps F (2008) Single-cell electroporation of adult sensory neurons for gene screening with RNA interference mechanism. *J Neurosci Methods* 170(2): 204-11
11. Charnet P, Scamps F, Rousset M, Menard C, Bellis M, Cens T, *Regulation of Cav2 channels by small GTPases*, in *Modulation of presynaptic calcium channels*, S.a.S. Mochida, G., Editor. 2013, Springer Publishing.
12. Chen H, Puhl HL, 3rd, Niu SL, Mitchell DC, Ikeda SR (2005) Expression of Rem2, an RGK family small GTPase, reduces N-type calcium current without affecting channel surface density. *J Neurosci* 25(42): 9762-72
13. Chen ZL, Yu WM, Strickland S (2007) Peripheral regeneration. *Annu Rev Neurosci* 30: 209-33

- 1 14. Correll RN, Pang C, Niedowicz DM, Finlin BS, Andres DA (2008) The RGK family of GTP-binding
2 proteins: regulators of voltage-dependent calcium channels and cytoskeleton remodeling.
3 Cell Signal 20(2): 292-300
- 4 15. Dubreuil AS, Boukhaddaoui H, Desmadryl G, Martinez-Salgado C, Moshourab R, Lewin GR,
5 Carroll P, Valmier J, Scamps F (2004) Role of T-type calcium current in identified d-hair
6 mechanoreceptor neurons studied in vitro. J Neurosci 24(39): 8480-4
- 7 16. Enes J, Langwieser N, Ruschel J, Carballosa-Gonzalez MM, Klug A, Traut MH, Ylera B,
8 Tahirovic S, Hofmann F, Stein V, et al. (2010) Electrical activity suppresses axon growth
9 through Ca(v)1.2 channels in adult primary sensory neurons. Curr Biol 20(13): 1154-64
- 10 17. Fan M, Buraei Z, Luo HR, Levenson-Palmer R, Yang J (2010) Direct inhibition of P/Q-type
11 voltage-gated Ca²⁺ channels by Gem does not require a direct Gem/Cavbeta interaction.
12 Proc Natl Acad Sci U S A 107(33): 14887-92
- 13 18. Finlin BS, Crump SM, Satin J, Andres DA (2003) Regulation of voltage-gated calcium channel
14 activity by the Rem and Rad GTPases. Proc Natl Acad Sci U S A 100(24): 14469-74
- 15 19. Finlin BS, Shao H, Kadono-Okuda K, Guo N, Andres DA (2000) Rem2, a new member of the
16 Rem/Rad/Gem/Kir family of Ras-related GTPases. Biochem J 347 Pt 1: 223-31
- 17 20. Finlin BS, Mosley AL, Crump SM, Correll RN, Ozcan S, Satin J, Andres DA (2005) Regulation of
18 L-type Ca²⁺ channel activity and insulin secretion by the Rem2 GTPase. J Biol Chem 280(51):
19 41864-71
- 20 21. Ghiretti AE, Kenny K, Marr MT, 2nd, Paradis S (2013) CaMKII-dependent phosphorylation of
21 the GTPase Rem2 is required to restrict dendritic complexity. J Neurosci 33(15): 6504-15
- 22 22. Heinke B, Balzer E, Sandkuhler J (2004) Pre- and postsynaptic contributions of voltage-
23 dependent Ca²⁺ channels to nociceptive transmission in rat spinal lamina I neurons. Eur J
24 Neurosci 19(1): 103-11
- 25 23. Krey JF, Pasca SP, Shcheglovitov A, Yazawa M, Schwemberger R, Rasmusson R, Dolmetsch RE
26 (2013) Timothy syndrome is associated with activity-dependent dendritic retraction in rodent
27 and human neurons. Nat Neurosci 16(2): 201-9
- 28 24. Lawson S, *Morphological and biochemical cell types of sensory neurons*, in *Sensory Neurons.*
29 *Diversity, Development, and Plasticity*, S. Scott, Editor. 1992, Oxford University Press: New
30 York. p. 27-59.
- 31 25. Leone A, Mitsiades N, Ward Y, Spinelli B, Poulaki V, Tsokos M, Kelly K (2001) The Gem GTP-
32 binding protein promotes morphological differentiation in neuroblastoma. Oncogene 20(25):
33 3217-25
- 34 26. Leyris JP, Gondeau C, Charnet A, Delattre C, Rousset M, Cens T, Charnet P (2009) RGK
35 GTPase-dependent CaV2.1 Ca²⁺ channel inhibition is independent of CaVbeta-subunit-
36 induced current potentiation. Faseb J 23(8): 2627-38
- 37 27. Li L, Cao XH, Chen SR, Han HD, Lopez-Berestein G, Sood AK, Pan HL (2012) Up-regulation of
38 Cavbeta3 subunit in primary sensory neurons increases voltage-activated Ca²⁺ channel
39 activity and nociceptive input in neuropathic pain. J Biol Chem 287(8): 6002-13
- 40 28. Lucas O, Hilaire C, Delpire E, Scamps F (2012) KCC3-dependent chloride extrusion in adult
41 sensory neurons. Mol Cell Neurosci 50(3-4): 211-220
- 42 29. Luo ZD, Chaplan SR, Higuera ES, Sorkin LS, Stauderman KA, Williams ME, Yaksh TL (2001)
43 Upregulation of dorsal root ganglion (alpha)2(delta) calcium channel subunit and its
44 correlation with allodynia in spinal nerve-injured rats. J Neurosci 21(6): 1868-1875.
- 45 30. Luvisetto S, Marinelli S, Panasiti MS, D'Amato FR, Fletcher CF, Pavone F, Pietrobon D (2006)
46 Pain sensitivity in mice lacking the Ca(v)2.1alpha1 subunit of P/Q-type Ca²⁺ channels.
47 Neuroscience 142(3): 823-32
- 48 31. Maguire J, Santoro T, Jensen P, Siebenlist U, Yewdell J, Kelly K (1994) Gem: an induced,
49 immediate early protein belonging to the Ras family. Science 265(5169): 241-4
- 50 32. Mahalakshmi RN, Nagashima K, Ng MY, Inagaki N, Hunziker W, Beguin P (2007) Nuclear
51 transport of Kir/Gem requires specific signals and importin alpha5 and is regulated by
52 calmodulin and predicted serine phosphorylations. Traffic 8(9): 1150-63

- 1 33. Mahalakshmi RN, Ng MY, Guo K, Qi Z, Hunziker W, Beguin P (2007) Nuclear localization of
2 endogenous RGK proteins and modulation of cell shape remodeling by regulated nuclear
3 transport. *Traffic* 8(9): 1164-78
- 4 34. Marmigere F, Ernfors P (2007) Specification and connectivity of neuronal subtypes in the
5 sensory lineage. *Nat Rev Neurosci* 8(2): 114-27
- 6 35. Martin DE, Demougin P, Hall MN, Bellis M (2004) Rank Difference Analysis of Microarrays
7 (RDAM), a novel approach to statistical analysis of microarray expression profiling data. *BMC*
8 *Bioinformatics* 5: 148
- 9 36. McCallum JB, Wu HE, Tang Q, Kwok WM, Hogan QH (2011) Subtype-specific reduction of
10 voltage-gated calcium current in medium-sized dorsal root ganglion neurons after painful
11 peripheral nerve injury. *Neuroscience* 179: 244-55
- 12 37. Michaelievski I, Segal-Ruder Y, Rozenbaum M, Medzihradsky KF, Shalem O, Coppola G, Horn-
13 Saban S, Ben-Yaakov K, Dagan SY, Rishal I, et al. (2010) Signaling to transcription networks in
14 the neuronal retrograde injury response. *Sci Signal* 3(130): ra53
- 15 38. Murakami M, Nakagawasai O, Suzuki T, Mobarakeh, II, Sakurada Y, Murata A, Yamadera F,
16 Miyoshi I, Yanai K, Tan-No K, et al. (2004) Antinociceptive effect of different types of calcium
17 channel inhibitors and the distribution of various calcium channel alpha 1 subunits in the
18 dorsal horn of spinal cord in mice. *Brain Res* 1024(1-2): 122-9
- 19 39. Nilsson A, Moller K, Dahlin L, Lundborg G, Kanje M (2005) Early changes in gene expression in
20 the dorsal root ganglia after transection of the sciatic nerve; effects of amphiregulin and PAI-
21 1 on regeneration. *Brain Res Mol Brain Res* 136(1-2): 65-74
- 22 40. Paradis S, Harrar DB, Lin Y, Koon AC, Hauser JL, Griffith EC, Zhu L, Brass LF, Chen C, Greenberg
23 ME (2007) An RNAi-based approach identifies molecules required for glutamatergic and
24 GABAergic synapse development. *Neuron* 53(2): 217-32
- 25 41. Pieraut S, Lucas O, Sangari S, Sar C, Boudes M, Bouffi C, Noel D, Scamps F (2011) An autocrine
26 neuronal interleukin-6 loop mediates chloride accumulation and NKCC1 phosphorylation in
27 axotomized sensory neurons. *Journal of Neuroscience* 31(38): 13516-13526
- 28 42. Pieraut S, Laurent-Matha V, Sar C, Hubert T, Mechaly I, Hilaire C, Mersel M, Delpire E,
29 Valmier J, Scamps F (2007) NKCC1 phosphorylation stimulates neurite growth of injured adult
30 sensory neurons. *J Neurosci* 27(25): 6751-9
- 31 43. Raivich G, Makwana M (2007) The making of successful axonal regeneration: genes,
32 molecules and signal transduction pathways. *Brain Res Rev* 53(2): 287-311
- 33 44. Reynet C, Kahn CR (1993) Rad: a member of the Ras family overexpressed in muscle of type II
34 diabetic humans. *Science* 262(5138): 1441-4
- 35 45. Sann SB, Xu L, Nishimune H, Sanes JR, Spitzer NC (2008) Neurite outgrowth and in vivo
36 sensory innervation mediated by a Ca(V)2.2-laminin beta 2 stop signal. *J Neurosci* 28(10):
37 2366-74
- 38 46. Scroggs RS, Fox AP (1992) Calcium current variation between acutely isolated adult rat dorsal
39 root ganglion neurons of different size. *J Physiol* 445: 639-658.
- 40 47. Seu L, Pitt GS (2006) Dose-dependent and isoform-specific modulation of Ca²⁺ channels by
41 RGK GTPases. *J Gen Physiol* 128(5): 605-13
- 42 48. Snider WD (1994) Functions of the neurotrophins during nervous system development: what
43 the knockouts are teaching us. *Cell* 77(5): 627-38
- 44 49. Vanegas H, Schaible H (2000) Effects of antagonists to high-threshold calcium channels upon
45 spinal mechanisms of pain, hyperalgesia and allodynia. *Pain* 85(1-2): 9-18
- 46 50. Wang HG, Wang C, Pitt GS (2011) Rem2-targeted shRNAs reduce frequency of miniature
47 excitatory postsynaptic currents without altering voltage-gated Ca²⁺(+) currents. *PLoS One*
48 6(9): e25741
- 49 51. Wang Y, Cheng X, Zhou Z, Wu H, Long L, Gu X, Xu G (2012) Increased expression of Gem after
50 rat sciatic nerve injury. *J Mol Histol* 44(1): 27-36

- 1 52. Ward Y, Spinelli B, Quon MJ, Chen H, Ikeda SR, Kelly K (2004) Phosphorylation of critical
2 serine residues in Gem separates cytoskeletal reorganization from down-regulation of
3 calcium channel activity. *Mol Cell Biol* 24(2): 651-61
- 4 53. Westenbroek RE, Hoskins L, Catterall WA (1998) Localization of Ca²⁺ channel subtypes on rat
5 spinal motor neurons, interneurons, and nerve terminals. *Journal of Neuroscience* 18(16):
6 6319-6330
- 7 54. Xiao HS, Huang QH, Zhang FX, Bao L, Lu YJ, Guo C, Yang L, Huang WJ, Fu G, Xu SH, et al.
8 (2002) Identification of gene expression profile of dorsal root ganglion in the rat peripheral
9 axotomy model of neuropathic pain. *Proc Natl Acad Sci U S A* 99(12): 8360-8365.
- 10 55. Yaksh TL (2006) Calcium channels as therapeutic targets in neuropathic pain. *J Pain* 7(1 Suppl
11 1): S13-30
- 12 56. Yang T, Colecraft HM (2013) Regulation of voltage-dependent calcium channels by RGK
13 proteins. *Biochim Biophys Acta* 1828(7): 1644-54
- 14 57. Yang T, Puckerin A, Colecraft HM (2012) Distinct RGK GTPases differentially use alpha1- and
15 auxiliary beta-binding-dependent mechanisms to inhibit CaV1.2/CaV2.2 channels. *PLoS One*
16 7(5): e37079
17
18

Competing interests

The authors declare no competing financial interests

Author contributions

F.S. and P.C. conceived and designed the experiments. F.S., S.S., M.B., M.R., P.C. collected and analyzed the data. T.C. designed the plasmids. F.S. and P.C. drafted the article. Experiments were performed in the laboratories of F.S. at INSERM U-1051 and of P.C. at CRBM, Montpellier. All authors approved the final version of this manuscript.

Acknowledgments

This work was supported by the Institut National de la Santé et la Recherche Médicale (INSERM), Centre National de la Recherche Scientifique (CNRS), Association contre les Myopathies (AFM) and EMBO Long-Term Fellowship (MB). We thank the regional imaging platform RIO and the experimental histology platform RHEM for technical assistance.

Figure Legends

Fig. 1

Gem is a nerve injury up-regulated gene in dorsal root ganglia. **A**, qRT-PCR results for the effects of mice sciatic nerve transection on RGK genes demonstrate a specific and transient up-regulation of *Gem* in DRG as early as 1 day post operation (DPO). At 3 DPO, *Gem* recovered its initial value. *Rem2* expression was significantly decreased at 3 DPO. Variations in *Rad* and *Rem1* were not significant at the time points used ($n = 6$, *** $p < 0.001$, * $p < 0.05$, ANOVA). Analysis of gene expression levels between different conditions is normalized relative to *Polr2j*, a stable control gene. **B**, Rank Difference Analysis of Microarray following rat sciatic nerve

crush shows that one gene (*Gem*) has a significant positive variation 24 hours after injury (FDR<10%). Standardized variations (Zvar) of 9.75 for *Gem* correspond to 1.30 fold change (signal ratio).

Fig. 2.

Gem protein is up-regulated in DRG after peripheral nerve injury. **A**, Immunoblot analysis of lysates from 12 lumbar L4-L5 dorsal root ganglia (DRG) and their peripheral sciatic nerves (PN) and dorsal roots (DR) (from 6 mice contralateral (control) and ipsilateral (axotomized), 55 µg protein per lane). **B**, The antibody confirmed *Gem* expression in brain (positive control, 10 µg protein) and no signal in spleen (negative control, 10 µg protein). **C**, Q-PCR results show that, at 1 DIV, DRG primary cultures highly expressed the early up-regulated injury marker *sprr1A* compared to control DRG (n = 3, * p < 0.05, Mann Whitney test). *Gem* is up-regulated in primary cultures at 1 DIV to a similar level than axotomized DRG at 1 DPO (Data normalized relative to *Polr2j*. n = 3, *** p < 0.001, ** p < 0.01, * p < 0.05, ANOVA). **D**, Agarose gel electrophoresis showing the presence of the cDNA product of *Gem* transcript in 1 DIV control cultures, control DRG and axotomized DRG at 1 DPO. **E**, Immunoblot of *Gem* protein in 1 DIV DRG culture (30 µg protein). The anti-*Gem* antibody (1:500) detected a single band at the expected molecular weight 33-35 kDa.

Fig. 3

Gem is expressed in axotomized sensory neurons. **A**, Confocal images of 2 DIV cultured sensory neurons stained with anti-*Gem* antibody at 1:500 (scale bar 20 µm). Staining shows nuclear (left image, white star) and cytoplasmic/plasma membrane (left and right images, white arrows) *Gem* localization. **B**, Roughly 70 % of sensory neurons are *Gem*-positive with 35% having nuclear and 36 % cytoplasmic and plasma membrane staining. Frequency distribution

of Gem staining according to somatic size of sensory neurons shows preferential membrane expression in the large sensory neurons (25-35 μm), while nuclear localization is more frequently observed in the small diameter neurons (15-20 μm). **C**, single-cell electroporation of control siRNA or Gem siRNA shows a preferential inhibition of the cytoplasmic/plasma membrane staining with Gem siRNA (** $p < 0.01$, Fisher's test,). **D**, N2A cells show specific *Gem* transcript inhibition following Gem siRNA transfection without effects on other related GTPases, *Rad* and *Rem2*. **E**, Immunoblot of Gem protein in HEK-293 cells transfected with plasmid Gem-GFP or Rem2. Under control conditions or in Rem2-transfected HEKs, no signal was detected with anti-Gem antibody (1:500). In the presence of Gem-GFP (58 kD), a clear signal was observed at the expected molecular weight. The signal was totally abolished when Gem siRNA was transfected together with Gem-GFP. **F**, Transverse sections of L4-L5 DRG immunolabeled with anti-Gem antibody (green) and anti-TrkC antibody (red); no Gem signal is observed in control DRG. Five days after nerve injury, there is Gem expression mainly among large diameter sensory neurons. No signal is observed in nucleus, nor in glial satellite cells surrounding sensory neurons. TrkC, a marker of mechano-and proprioceptors is expressed in large sensory neurons in control and axotomized DRG. Gem is expressed in a subset of TrkC positive and negative sensory neurons. (scale bar, 50 μm). **G**, Confocal image of Gem staining in transverse section of axotomized DRG shows both cytoplasmic and sub-plasma membrane expression in a large TrkC-positive sensory neuron (scale bar 25 μm).

Fig. 4

Gem regulates P/Q type Ca^{2+} current amplitude expressed in large somatic diameter sensory neurons. **A**, Gem inhibition with Gem siRNA did not affect neuronal death at two and three days after electroporation. Live fluorescent dextran-positive neurons (control or Gem siRNAs) were counted under fluorescence microscopy at the time of electroporation (2 hours after

dissociation) and two or three days later. **B**, Two days after transfection, neurons were processed for electrical activity recordings. Typical action potentials recorded under control or Gem siRNAs show no obvious differences. **C**, Gem inhibition with Gem siRNA did not modify HVA Ca^{2+} current amplitude in small somatic diameter neurons ($< 30 \mu\text{m}$) but induces a significant increase in HVA Ca^{2+} current amplitude in large somatic diameter neurons ($> 30 \mu\text{m}$) compared to control siRNA measured at 3 DIV in sensory culture. **D**, following Gem siRNA transfection in large somatic diameter neurons, HVA Ca^{2+} currents were recorded in the presence of specific inhibitors of subtypes: $3 \mu\text{M}$ ω -conotoxin GVIA (GVIA) for N-type current; $1 \mu\text{M}$ AgaIVA for P/Q type current, $0.5 \mu\text{M}$ nifedipine for L-type current. Compared to control siRNA, Gem siRNA induces a significant increase in HVA amplitude in the presence of GVIA and nifedipine, and had no effects in the presence of $1 \mu\text{M}$ AgaIVA and $0.5 \mu\text{M}$ nifedipine. **E**, Pharmacological analysis of subtypes expression among small ($< 30 \mu\text{m}$) and large ($> 30 \mu\text{m}$) somatic diameter neurons demonstrates that P/Q type HVA Ca^{2+} current is preferentially expressed in large neurons while N-type is a marker of small diameter neurons. L- and R-types appear equally distributed among sensory neurons subpopulations. (* $p < 0.05$, ** $p < 0.01$, Mann Whitney tests).

Fig. 5

Exogenously-expressed Gem inhibits global HVA Ca^{2+} -current expressed in sensory neurons. **A**, confocal images of live sensory neurons at 1 DIV transfected with pEGFP or pEGFP-Gem. GFP fluorescence shows that Gem localized close to plasma membrane (white arrow) and in the cytosol, not in the nucleus (white star), (scale bars $10 \mu\text{m}$). **B**, Typical current-voltage relationships and corresponding current traces recorded from pEGFP (a-b) and pEGFP-Gem (c-d) transfected neuron following activation of Ca^{2+} current with a 1 sec step pulse to -40 mV to evidence low voltage activated, LVA, Ca^{2+} current and to -10 mV to record high voltage

activated, HVA, Ca^{2+} current. pEGFP-Gem transfection decreased the amplitude of HVA Ca^{2+} current compared to transfection with pEGFP and did not modify LVA Ca^{2+} current amplitude. **C**, Overexpressed Gem induced a nearly complete inhibition of HVA Ca^{2+} currents, (***) $p < 0.001$, Mann Whitney test). **D**, Gem did not inhibit LVA Ca^{2+} current amplitude among the low expressing T-current population of sensory neurons ($p > 0.05$, Mann Whitney test). **E**, Random recordings of D-hair neurons (a), a subset of mechano-sensitive neurons characterized by the expression of large amplitude Cav3.2 LVA Ca^{2+} current (high expressing T-current population), confirm the specific inhibition of HVA currents by Gem (b). A ramp protocol from -80 mV to +40 mV was used to simultaneously visualize both LVA and HVA Ca^{2+} currents.

Fig 6

The Cav β 4 subunit of HVA Ca^{2+} channels is a molecular determinant of Gem-induced P/Q type Cav2.1 current inhibition. **A**, qRT-PCR results for Cav β 1-4 subunits transcripts, *Cacn β 1-4*, expression in DRG. For each run, data are normalized relative to the transcript having the highest expression (n = 3 mice). **B**, Percentage of Gem inhibition of Ba^{2+} currents from Cav2.1 or Cav2.2 expressing *Xenopus* oocytes in combination with either Cav β 3x or Cav β 4 (coexpressed with α 2 δ 1, more than 20 oocytes from 2 to 6 experiments in each case, * $p < 0.05$, Mann Whitney test). For these experiments, oocytes are injected with 15ng/oocyte Gem RNA (see methods). **C**, Dose-response curves of Gem coexpressed with Cav2.1+Cav β 4 or Cav2.2+Cav β 4 in *Xenopus* oocytes. Ca^{2+} channels subunits were coinjected Gem 1:1, 1/4, 1/10 and 1/100 w/w dilution. In all cases, the Ca^{2+} channels subunits RNA mix was at 1ng/nl and 30 ng were injected by oocyte. (n= 3, * $p < 0.05$, Mann Whitney test). Below are representative Ba^{2+} current traces showing a stronger inhibitory effect of Gem at 1/4 dilutions on Cav2.1 current compared to Cav2.2 current. **D**, Representative confocal images show Cav β 4 immunoreactivity close to plasma membrane (red) together with Gem staining (green) in a large

1 sensory neuron at 2DIV. DAPI staining confirms that Cav β 4 is also present in the nucleus.
2 (Scale bar, 20 μ m).

3
4 **Fig. 7**
5

6 P/Q type Ca²⁺ current inhibition reduces neurite branching. **A-B**, Immunocytochemistry with
7 anti- β III-tubulin antibody shows typical neurite branching of sensory neurons at 1 DIV with
8 somatic diameter superior to 30 μ m (large neurons) under control or in the presence of Aga-
9 IVA. **C**, Overnight incubation with 500 nM Aga-IVA in culture medium did not change neurite
10 length, but significantly reduced the number of secondary branching on the longest neurite
11 measured at 1 DIV (**D**) (***) ($p < 0.001$, t' test). (Scale bars, 50 μ m).