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Gβ2 and Gβ4 participate in the opioid and adrenergic receptor-mediated Ca^{2+} channel modulation in rat sympathetic neurons

Saifeldin Mahmoud[1], Jong K. Yun[2] and Victor Ruiz-Velasco[1]

Departments of [1] Anesthesiology and [2] Pharmacology, Penn State College of Medicine, Hershey, PA, USA

Key point

- Sympathetic stellate ganglion (SG) neurons that innervate cardiac muscle play an important role in regulating heart rate and contractility.
- In this study, we examined which Gβ protein subunit couples Ca^{2+} channels to opioid and adrenergic receptors expressed in SG neurons.
- We show that Gβ2 and Gβ4 are important signalling elements that maintain the transduction pathway of both receptor subtypes and Ca^{2+} channels, and how their expression levels are differentially compensated.
- Our results also indicate that Gβ1 subunits do not appear to play a role in either signalling pathway.
- The elucidation of specific G proteins that couple Ca^{2+} channels with opioid and adrenergic receptors will help us better understand how these receptors regulate synaptic transmission of SG neurons that innervate cardiac muscle.

Abstract Cardiac function is regulated in part by the sympathetic branch of the autonomic nervous system via the stellate ganglion (SG) neurons. Neurotransmitters, such as noradrenaline (NA), and neuropeptides, including nociceptin (Noc), influence the excitability of SG neurons by modulating Ca^{2+} channel function following activation of the adrenergic and nociceptin/orphanin FQ peptide (NOP) opioid receptors, respectively. The regulation of Ca^{2+} channels is mediated by Gβγ, but the specific Gβ subunit that modulates the channels is not known. In the present study, small interference RNA (siRNA) was employed to silence the natively expressed Gβ proteins in rat SG tissue and to examine the coupling specificity of adrenergic and NOP opioid receptors to Ca^{2+} channels employing the whole-cell variant of the patch-clamp technique. Western blotting analysis showed that Gβ1, Gβ2 and Gβ4 are natively expressed. The knockdown of Gβ2 or Gβ4 led to a significant decrease of the NA- and Noc-mediated Ca^{2+} current inhibition, while Gβ1 silencing was without effect. However, sustaining low levels of Gβ2 resulted in an increased expression of Gβ4 and a concomitant compensation of both adrenergic and opioid signalling pathways modulating Ca^{2+} channels. Conversely, Gβ4-directed siRNA was not accompanied with a compensation of the signalling pathway. Finally, the combined silencing of Gβ2 and Gβ4 prevented any additional compensatory mechanisms. Overall, our studies suggest that in SG neurons, Gβ2 and Gβ4 normally maintain the coupling of Ca^{2+} channels with the receptors, with the latter subtype responsible for maintaining the integrity of both pathways.

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Corresponding author V. Ruiz-Velasco: Department of Anesthesiology, Penn State College of Medicine, Hershey, PA 17033-0850, USA. Email: vruizvelasco@psu.edu

Abbreviations GPCR, G protein-coupled receptor; NA, noradrenaline; Noc, nociceptin; NOP, nociceptin/orphanin FQ peptide; PTX, pertussis toxin; siRNA, small interference RNA.

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Introduction

Heterotrimeric G proteins ($G\alpha\beta\gamma$) are known to play a crucial role in relaying signals (i.e. neurotransmitters, hormones) from G protein-coupled receptors (GPCR) to effectors. These effectors include kinases and ion channels, such as high-voltage activated Ca^{2+} and G protein-gated inwardly rectifying K^+ (GIRK) channels (for review see Lambert, 2008; Oldham & Hamm, 2008; Smrcka, 2008). Binding of the agonists to the GPCR leads to a conformational change of the $G\alpha$ subunit that results in exchange of GDP for GTP. The GTP-bound $G\alpha$ subunit dissociates from the $G\beta\gamma$ dimer and both functional moieties modulate downstream effector pathways. The signal is terminated following the hydrolysis of the GTP bound to the $G\alpha$ subunit, a process facilitated by regulators of G protein signalling (RGS) proteins. Furthermore, activators of G protein signalling (AGS) proteins, a newly discovered class of proteins that bind G proteins in the absence of GPCR activation, have expanded the functional role of these signalling molecules (Blumer *et al.* 2007).

Several studies have been undertaken to examine the G protein subunit coupling specificity between GPCR and effectors in various cellular systems (neurons, muscle and cell lines) by employing different experimental techniques. For example, in the pituitary GH_3 cell line, the use of antisense oligonucleotides targeting $G\alpha$ proteins revealed that muscarinic and somatostatin receptors modulate Ca^{2+} channels via $G\alpha o_1$ and $G\alpha o_2$, respectively (Kleuss *et al.* 1991). Other assays have relied on overexpression of the G protein of interest with a concomitant inhibition of native G proteins to determine the coupling ability with effectors (Caulfield *et al.* 1994, Garcia *et al.* 1998; Arnot *et al.* 2000; Jeong & Ikeda, 2000; Ruiz-Velasco & Ikeda, 2000; Zhou *et al.* 2000; Straiker *et al.* 2002; Kammermeier *et al.* 2003). More recently, the introduction of small interference RNA (siRNA) has provided a powerful approach that allows for protein function determination and signalling pathway elucidation. Simon and coworkers found that G protein-mediated signalling pathways, including chemotaxis, were blocked as a result of siRNA-mediated $G\beta$ subunit expression elimination (Hwang *et al.* 2004, 2005). Similarly, in HeLA cells, siRNA was employed in a systematic fashion to characterize the expression profile of $G\alpha$ and $G\beta$ subunits resulting from the knockdown of specific G protein subunits (Krummins & Gilman, 2006). Their results demonstrated that in some cases silencing of G protein subunits resulted in either down- or upregulation of other non-targeted G proteins.

Sympathetic neurons within the stellate ganglion (SG) innervate cardiac muscle and regulate both heart rate (chronotropy) and contractility (inotropy) (Pardini *et al.* 1990). During exercise, sympathetic discharge is increased in order to elevate cardiac output and meet the increased demand for oxygen and nutrients. Undesirable functional effects can occur in cardiac tissue as a result of an alteration in sympathetic nervous system function (Schomig & Richardt, 1990; Chen *et al.* 2001; Haunstetter *et al.* 2002). SG neuron excitability is regulated in part through the modulation of ion channels by neurotransmitters and neuropeptides. Neurotransmitter release in autonomic neurons is regulated by Ca^{2+} entry via voltage-gated Ca^{2+} channels, and in presynaptic nerve terminals N-type Ca^{2+} channels are closely involved in this process (Dunlap *et al.* 1995; Zamponi, 2001). SG neurons express both adrenergic and nociceptin/orphanin FQ peptide (NOP) opioid receptors, but do not appear to express 'classical' opioid receptors (μ, κ and δ; Fuller *et al.* 2004; Ruiz-Velasco *et al.* 2005). Nociceptin (Noc), the endogenous NOP opioid receptor ligand, has been reported to exert an inhibitory neuromodulation on transmitter release in the heart (Giuliani & Maggi, 1997; Malinowsk *et al.* 2001). In isolated guinea pig left atria, for instance, Noc inhibited the positive inotropic sympathetic response (e.g. NA release) as well as the negative inotropic parasympathetic response (e.g. ACh release) to low field stimulation (Giuliani & Maggi, 1997). Thus, Noc influences cardiac function by modulating NA release.

Previously we reported that in SG neurons, the coupling of NOP receptors and Ca^{2+} channels occurs primarily via pertussis toxin (PTX)-sensitive $G\alpha_{i1}$ protein subunits (Margas *et al.* 2008). On the other hand, the adrenergic receptors did not exhibit coupling specificity with any PTX-sensitive $G\alpha$ subunits (i.e. $G\alpha_{i1-3/oA-B}$), suggesting that adrenergic signalling in this neuron type is robust with regard to Ca^{2+} channel signalling. In the present study, the coupling specificity for both adrenergic and NOP opioid receptors was extended at the $G\beta$:Ca^{2+} channel juncture in rat SG neurons. To this end, siRNA was employed to silence the natively expressed $G\beta$ subunits. Thereafter, electrophysiological and biochemical techniques were employed to examine the functional coupling and expression levels, respectively.

Methods

Stellate ganglion (SG) tissue isolation

All the experiments performed with animals were approved by the Penn State College of Medicine Institutional Animal Care and Use Committee. Whole SG tissue or single neurons were employed in this study. Male Wistar rats (150–225 g) were anaesthetized with CO_2 and rapidly decapitated with a laboratory guillotine. Both SGs were then removed and cleared of connective tissue in ice-cold Hanks' balanced salt solution. Thereafter the ganglia were placed in ice-cold Opti-MEM (Invitrogen Corp., Carlsbad, CA, USA) supplemented with 2 mM

2,3-butanedione monoxime (BDM) until ready for siRNA transfection.

siRNA transfection

The transfection of the SG tissue with siRNA nucleotide sequences was performed by employing both electroporation and lipofection immediately and 48 h following tissue isolation. Electroporation of the ganglia was carried out first with the NEON Electroporator (Invitrogen Corp.). The ganglia were transferred from the Opti-MEM and preincubated for 15–20 min in an RNase- and DNase-free microcentrifuge tube containing the R Solution (provided with the NEON electroporator kit), Gβ siRNA (1500–2000 nM) and 2 mM BDM. In control experiments, a scrambled siRNA sequence (1500–2000 nM) was employed. Afterwards, the ganglia were drawn up into a 100 μl electroporator tip and electroporated with a single 20 ms 1000 V pulse. Following electroporation, the ganglia were placed in a 22 mm dish for lipofection containing Opti-MEM supplemented with either scrambled (control) or Gβ siRNA (1500–2000 nM), 2 mM BDM and 10 μl Lipofectamine 2000 (Invitrogen Corp.) for 4–5 h in a humidified incubator (5% CO_2–95% air) at 37°C. Following the incubation period, the tissue was rinsed 3 times with minimal essential medium (MEM), supplemented with 10% fetal bovine serum (FBS), 1% glutamine and 1% penicillin–streptomycin (all from Invitrogen Corp.) and then stored in MEM supplemented with 10% FBS, 1% penicillin–streptomycin and 1% glutamine. This protocol was performed a second time 48 h after the initial transfection.

The siRNA sequences designed to silence the Gβ protein subunits were obtained by employing a macro written by Stephen R. Ikeda (NIH/NIAA) on IGOR Pro (WaveMetrics, Inc., Lake Oswego, OR, USA) and chosen based on criteria previously described (Reynolds *et al.* 2004). The rat Gβ1 target sequence was 5-CAACTG AAGAACCAAATTA-3 corresponding to nucleotide position 37–55. The rat Gβ2 target sequences were 5-ATCAGGAGCTGCTCATGTA-3 and 5-ACATCTGGG ATGCCATGAA-3 corresponding to nucleotide positions 773–791 and 884–902, respectively. The rat Gβ4 target sequence was 5-ACAACATCTGCTCCATATA-3 corresponding to nucleotide position 353–371 (all from Ambion/Applied Biosystems, Inc., Foster City, CA, USA). Control groups were transfected with scrambled siRNA sequences (Ambion/Applied Biosystems, Inc.).

Electrophysiological recordings and data analysis

Seventy-two or ninety-six hours after siRNA transfection (i.e. the day prior to electrophysiological recording), the SG neurons were enzymatically dissociated by incubating the ganglia for 60 min in Earle's balanced salt solution containing 0.6 mg ml^{-1} collagenase (Roche Applied Science, Indianapolis, IN, USA), 0.4 mg ml^{-1} (Worthington Biochemical, Lakewood, NJ, USA) and 0.1 mg ml^{-1} DNase (Sigma-Aldrich Co., St Louis, MO, USA) in a shaking water bath at 35°C. Following the 60 min incubation, the neurons were dispersed by vigorous shaking, centrifuged twice for 6 min at ∼63 *g*, and resuspended in MEM supplemented with 10% FBS, 1% penicillin–streptomycin and 1% glutamine. The neurons were next plated onto 35 mm poly-L-lysine-coated dishes and stored overnight in a humidified incubator at 37°C.

Whole-cell Ca^{2+} currents were recorded employing the patch-clamp technique. The patch pipette electrodes, fabricated from borosilicate glass (Garner Glass Co., Claremont, CA, USA), were pulled on a P-97 micropipette puller (Sutter Instrument Co., Novato, CA, USA). The Ca^{2+} currents were acquired with the Axopatch 200B amplifier (Molecular Devices, Sunnyvale, CA, USA), analog filtered at 2 kHz (–3 dB, four pole low-pass Bessel filter) and digitized with custom-designed S5 software (Stephen R. Ikeda, NIH/NIAAA) equipped with an 18-bit AD converter board (Instrutech Corp., Elmont, NY, USA). The cell membrane capacitance and pipette series resistance were electronically compensated (80–85%).

The Ca^{2+} currents were evoked with the double-pulse voltage protocol (Ikeda, 1991) shown in Fig. 3*B* (top). Briefly, the voltage protocol consists of a test pulse (prepulse) to +10 mV, followed by a depolarizing conditioning test pulse to +80 mV, a return to −80 mV and then a second test pulse (postpulse) to +10 mV before returning to −80 mV. The peak Ca^{2+} current amplitude for both the pre- and postpulse was measured isochronally 10 ms following the initiation of each pulse. The agonist-mediated Ca^{2+} current inhibition was determined as follows: [peak Ca^{2+} current (prepulse before agonist) − peak Ca^{2+} current (prepulse after agonist)]/[peak Ca^{2+} current before agonist)] × 100. The facilitation ratio was measured by dividing the postpulse by the prepulse current amplitude. Prior to agonist application, the facilitation ratio typically ranges from 1.1 to 1.3. However, during agonist exposure, the facilitation ratio values range from 1.5 to 2.7.

The external solution consisted of (in mM): 145 tetratethylammonium hydroxide (TEA-OH), 140 methanesulfonic acid, 10 Hepes, 15 glucose, 10 $CaCl_2$, and 0.0003 tetrodotoxin. The pH was adjusted to 7.4 with TEA-OH and the osmolality of the solutions ranged from 316–321 mosmol kg^{-1}. The pipette solution contained (in mM): 80 *N*-methyl-D-glucamine (NMG), 20 TEA-OH, 20 CsCl, 40 CsOH, 11 EGTA, 10 Hepes, 1 $CaCl_2$, 4 MgATP, 0.3 Na_2GTP and 14 Tris-creatine phosphate. The pH was adjusted to 7.2 with methanesulfonic acid and the osmolality of the solution was 306 mosmol kg^{-1}. Stock solutions of nociceptin (Noc, Tocris Cookson,

Ellisville, MO, USA) and noradrenaline (NA, from Sigma-Aldrich Co.) were prepared in water and diluted in the external solution to their final concentration prior to use. The agonists were applied to the neuron under study employing a custom-designed gravity-fed perfusion system that was positioned ∼100 mm from the cell.

Data and statistical analyses were performed with Igor Pro 6.0 (Lake Oswego, OR, USA) and Prism 4.0 (GraphPad Software, Inc., San Diego, CA, USA) software packages with $P < 0.05$ considered statistically significant. Graphs and current traces were generated with Igor Pro and Canvas 8.0 (Deneba Software, Miami, FL, USA) software packages. The results are presented as means ± SEM or SD.

Western blot analysis

Total protein from SG tissues was prepared with the Nucleospin RNA/Protein Kit (Macherey-Nagel, Germany) following the standard protocol. Total protein concentrations were determined employing the Pierce Plate BCA Protein Assay (Thermo Fisher Scientific, Inc., Rockford, IL, USA). Protein samples (20 or 25 μg) were electrophoretically separated on NuPAGE 10% Bis-Tris pre-cast gels (Invitrogen Corp.) employing 200 V for 55 min and then transferred to PVDF membranes. The membranes were blocked with 5% milk in Tris buffered saline-Tween20 (TBS-T) solution for 1–2 h and incubated with anti Gβ1 (1/2000), β2 (1/2000), β3 (1/500) and β4 (1/500 or 1/750) rabbit polyclonal antibodies (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) and mouse monoclonal antibody to actin (1/2500; Abcam, Cambridge, MA, USA) in 5% dry-milk in TBS-T solution for 60 min at room temperature or overnight at 4°C. Subsequently, the membrane was incubated with horse-radish peroxidase conjugated anti-rabbit or mouse IgG antibody (both from GE Healthcare, Piscataway, NJ, USA; 1/5000) for 1 h. The Gβ and actin protein bands were visualized employing enhanced chemiluminescent (ECL) reagents SuperSignal West Dura or SuperSignal West Femto (both from Thermo Scientific, Rockford, IL, USA). It should be noted that the latter reagent was necessary to detect Gβ4 subunits and is likely the reason for the higher background signal compared to the signal observed for Gβ1, Gβ2 and actin. The images were acquired on either X-ray film (Figs 2 and 4) or with a ChemiDoc-It Imaging system (UVP, LLC, Upland, CA, USA) equipped with a 16-bit CCD camera (Figs 1, 6, 8 and 10), and processed with VisionWorksLS software (UVP).

In a separate set of experiments, human embryonic kidney (HEK 293) cells were transiently transfected with the cDNA plasmid coding for the enhanced yellow fluorescent protein (EYFP, Clontech Laboratories, Palo Alto, CA, USA) fused to the human Gβ3 (Ruiz-Velasco & Ikeda, 2003). Approximately 20 h following transfection, the cells were homogenized and expression of Gβ3 in non-transfected and Gβ3-YFP-expressing cells was determined as described above.

Quantitative real-time-PCR (QRT-PCR)

Total RNA was extracted from SG tissue employing the Nucleospin RNA/Protein Kit (Macherey-Nagel) following the standard protocol. Equal quantities of total RNA were DNAse treated (Ambion/Applied Biosystems, Inc.) and cDNA synthesis was performed using the High Capacity cDNA RT Kit (Applied Biosystems, Inc.). For QRT-PCR reactions, the TaqMan Gene Expression Assays (Applied Biosystems, Inc.) specific for rat Gβ1, Gβ2, Gβ4 and GAPDH were performed according to the manufacturer's instructions and carried out with equal quantities of cDNA. The assays were run on a 7900HT PCR system (Applied Biosystems, Inc.) and analysed using the comparative C_t method. The results were normalized to internal GAPDH mRNA controls.

Results

The coupling of NOP and adrenergic receptors with Ca^{2+} channels is significantly decreased by Gβ2 knockdown and subsequently restored by Gβ4 subunits

There are five known Gβ subunit isoforms (Gβ1–β5) though Gβ5 binds preferentially with RGS proteins that contain a Gγ-like (ggl) domain (Jayaraman *et al.* 2009). Thus, we initially determined the expression of Gβ1–β4 in SG tissue employing Western blotting analysis. The Western blot in Fig. 1 illustrates that SG tissue expresses Gβ1, Gβ2 and Gβ4 (∼37–39 kDa) while Gβ3 was not detected. Unlike Gβ1 and Gβ2, an ultra-sensitive chemiluminescent substrate was necessary for the detection of Gβ4 (see Methods above). In addition, as a positive control for the anti-Gβ3 antibody, Gβ3-YFP cDNA was transfected in HEK 293 cells. While untransfected HEK 293 cells do not appear to express Gβ3, detection of Gβ3-YFP was confirmed with the anti-Gβ3 antibody. The results show that Gβ1, Gβ2 and Gβ4 subunits were detected in SG tissue. As a result, we focused our efforts on silencing the expression of these three sub-units to examine the coupling specificity of Ca^{2+} channels to NOP and adrenergic receptors.

In the next set of experiments, Gβ2 subunits were initially targeted for knockdown given their apparent higher expression. Figure 2*A* shows the relative mRNA levels of the three Gβ subunits isolated from SG tissue 72 h post-transfection with either scrambled or Gβ2 siRNA. There was a significant ($P < 0.05$) decrease in Gβ2 mRNA, while Gβ1 and Gβ4 mRNA levels were not significantly altered. The immunoblot in Fig. 2*B* shows

that Gβ2 protein expression in SG tissue was decreased by ∼25% when compared to scrambled siRNA-transfected tissue. In addition, it can be seen that the Gβ2 knockdown resulted in an increase of Gβ1 and Gβ4 expression levels (Fig. 2*B*) but more pronounced with the latter. These results demonstrate that transfection with Gβ2 siRNA led to the specific decrease of the subunit at the nucleotide and protein levels.

In the next series of experiments, we examined the effect of Gβ2 knockdown on the Ca^{2+} channel modulation by NOP and adrenergic receptors in SG neurons transfected with Gβ2 siRNA 72 h post-transfection. Figure 3*A* shows the time course of peak Ca^{2+} currents that were evoked every 10 s with the voltage protocol (depicted in Fig. 3*B* top and described in Methods) from an SG neuron transfected with scrambled siRNA. The corresponding numbered traces (1–8) are shown in Fig. 3*B*. Prior to the application of Noc, the prepulse amplitude was slightly less than the postpulse amplitude, which is a result of low-level tonic G protein activation (Ikeda, 1991). Further, the activation phase of the prepulse current (trace 1, Fig. 3*B*) was fast and reached a plateau within 5 − 8 ms following the onset of the pulse. Exposure to Noc (1 μM) led to an approximate 70% block of the prepulse current and to the kinetic slowing of the prepulse rising phase (trace 3, Fig. 3*B*). The kinetic slowing is thought to result from a voltage-dependent relief of block during the test pulse. The time course in Fig. 3*A* shows that following recovery of the Noc-mediated Ca^{2+} current inhibition, the neuron was exposed next to NA (10 μM). Similar to Noc, the Ca^{2+} currents were inhibited (∼57%) in a voltage-dependent manner in the presence of NA (Fig. 3*A* and traces 5–8, Fig. 3*B*). Figure 3*C* shows the time course of prepulse and postpulse Ca^{2+} currents recorded from an SG neuron transfected with rat Gβ2 siRNA and the corresponding numbered traces (1–8) are shown in Fig. 3*D*. Unlike the neuron transfected with scrambled siRNA, application of Noc (1 μM) or NA (10 μM) to the neuron showed a diminished inhibition of the Ca^{2+} currents (cf. traces 1, 3, 5 and 7 in Fig. 3*B* and *D*). The traces in Fig. 3*D* show that the Ca^{2+} currents were inhibited in a voltage-dependent manner similar to the control SG neuron. Figure 3*E* is a summary graph showing the mean (±SEM) Noc- and NA-mediated Ca^{2+} current inhibition in SG neurons transfected with scrambled and Gβ2 siRNA. The results show that silencing Gβ2 subunits significantly decreased the agonist-mediated, voltage-dependent Ca^{2+} current inhibition mediated by Noc ($P = 0.015$) and NA ($P = 0.0003$). Overall, the data suggest that Gβ2 is an essential signalling element in the pathways that couple Ca^{2+} channels with NOP and adrenergic receptors.

The facilitation of the Ca^{2+} currents can be observed by comparing the prepulse and postpulse current amplitudes of the superimposed traces. Before exposure to Noc, the conditioning pulse to +80 mV had a minor effect on the postpulse current amplitude (traces 1 and 2, Fig. 3*B*) and the basal facilitation ratio was 1.03. However, during Noc application, the amplitude of the postpulse was greater than that of the prepulse (traces 3 and 4, Fig. 3*B*) and there was a 'relief' of the Noc-mediated Ca^{2+} current inhibition as indicated by the elevated facilitation ratio of

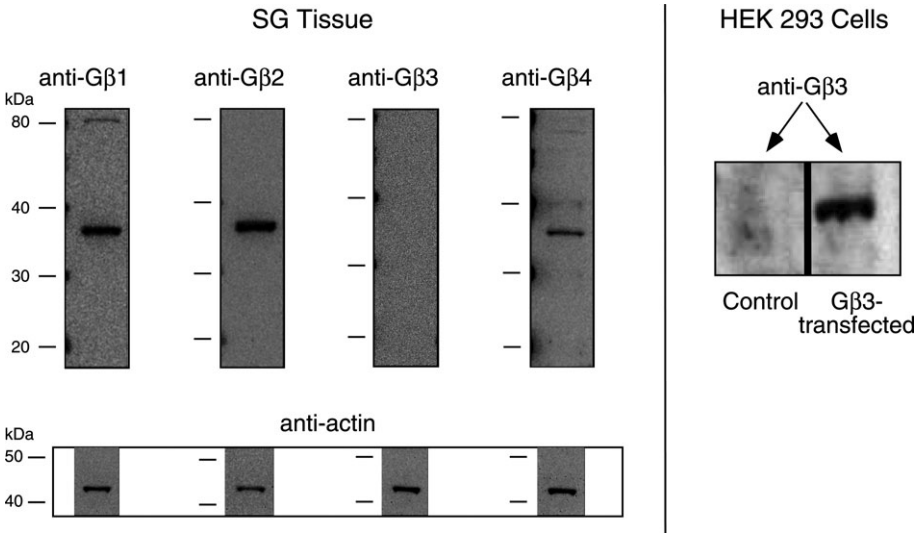

Figure 1. Detection of Gβ subunit expression by Western blot analysis in SG tissue and HEK 293 cells
Western blot experiments illustrating Gβ subunits natively expressed in SG tissue (left) and Gβ3-YFP cDNA-expressing HEK 293 cells (20 h post-transfection). The Western blots used anti-Gβ1, -Gβ2, -Gβ3, -Gβ4 and -actin. The lanes containing SG tissue and HEK 293 cells were loaded with approximately 20 and 25 μg protein, respectively. Gβ3 was not detected in SG tissue or untransfected HEK 293 cells but was present in Gβ3-YFP-transfected cells (right). Lines indicate approximate molecular masses (kDa).

2.66. In this group of experiments, the mean (±SEM) basal facilitation ratios for control and siRNA-transfected SG neurons were (1.08 ± 0.02) and (1.06 ± 0.01), respectively (t test, $P = 0.43$).

Given the relatively short half-life of siRNA nucleotides, we monitored Gβ subunit expression for an additional 24 h period (i.e. 96 h post-transfection) to ensure Gβ2-targeted siRNA was still effective. The QRT-PCR results in Fig. 4*A* show that Gβ2 mRNA levels remained significantly ($P < 0.05$) lower in Gβ2 siRNA-transfected SG tissue when compared to those transfected with scrambled siRNA. Furthermore, it can be observed that

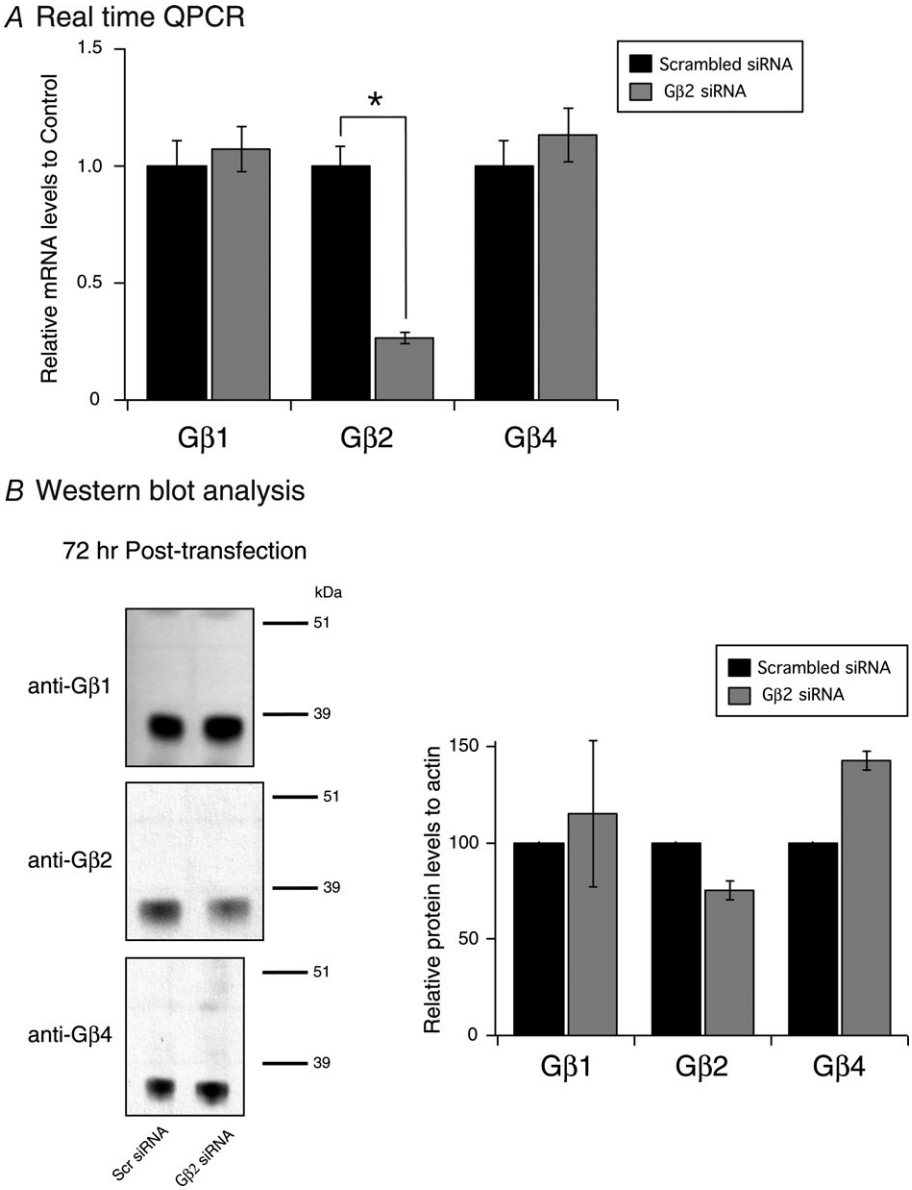

Figure 2. Detection of Gβ subunit expression by QRT-PCR and Western blot analysis in SG 72 h post-Gβ2 siRNA transfection

A, quantitative assessment of Gβ mRNA expression by QRT-PCR in SG tissue 72 h post-siRNA transfection. The ΔC_T values were determined for both groups ($n = 6$; 2 rats per group) and the Gβ mRNA levels were measured relative to GAPDH mRNA levels. The mRNA levels are expressed as fold-change compared to SG tissue transfected with control scrambled siRNA. Three independent experiments were performed. *B*, Western blot (left panel) experiments illustrating results with anti-Gβ1, -Gβ2 and -Gβ4 in SG tissue transfected with either control scrambled or Gβ2 siRNA. Each lane was loaded with approximately 20 μg protein (2–3 rats per group). Lines indicate approximate molecular masses (kDa). Right panel shows the densitometric analysis of Western blots for actin and Gβ subunits of 2 independent experiments. The normalized values represent mean with standard deviation. $^*P < 0.05$ compared to neurons transfected with control scrambled siRNA, Student's t test.

Gβ4 mRNA levels increased significantly ($P < 0.05$) in SG tissue transfected with Gβ2 siRNA. The Western blot in Fig. 4*B* also illustrates that Gβ2 protein levels were lower than SG tissue transfected with scrambled siRNA while the Gβ1 and Gβ4 protein levels remained higher in Gβ2 siRNA-transfected tissue (cf. Figs 2 and 4). This was more apparent with Gβ4 subunits. These data suggest that the expression of Gβ2 and Gβ4 are closely linked so that when

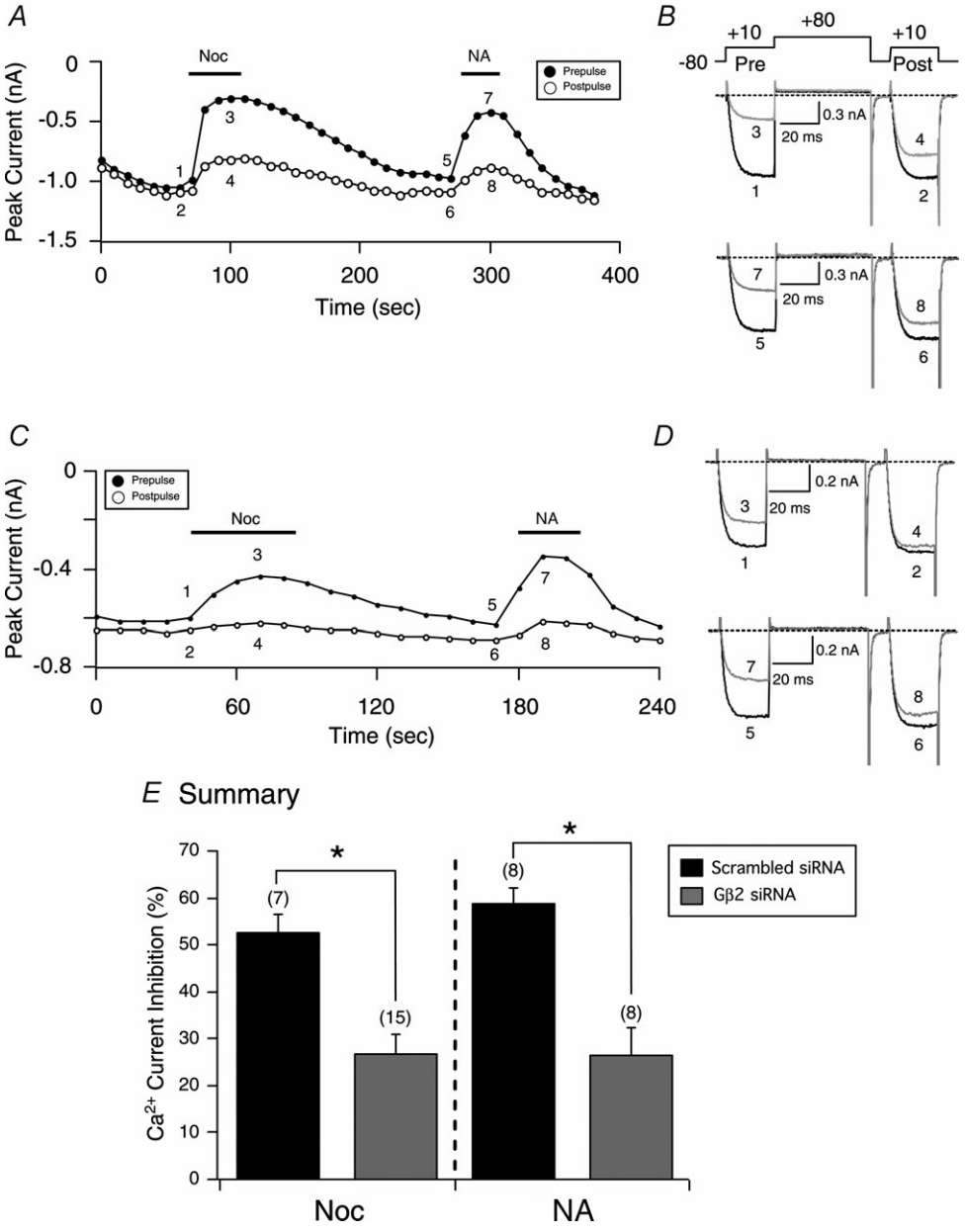

Figure 3. Effect of Gβ2 silencing 72 h post-siRNA transfection on the Noc- and NA-mediated Ca^{2+} current inhibition in SG neurons

A and *C*, time courses of peak Ca^{2+} current amplitude inhibition for pre- (●) and postpulse (○) acquired from the sequential application of Noc (1 μM) and NA (10 μM) in neurons transfected with control scrambled (*A*) and Gβ2 (*C*) siRNA, respectively. *B* and *D*, superimposed Ca^{2+} current traces evoked with the 'double-pulse' voltage protocol (shown at the top of *B*) in the absence (1 and 2; 5 and 6) or presence (3 and 4; 7 and 8) of Noc and NA, respectively. Currents were evoked every 10 s. The filled bars indicate the application of agonists. *E*, summary graph of mean (±SEM) Ca^{2+} current inhibition produced by application of Noc or NA in neurons trasfected with control scrambled or Gβ2 siRNA. Inhibition was determined from the Ca^{2+} current amplitude measured isochronally at 10 ms into the prepulse (+10 mV) in the absence or presence of Noc or NA. Numbers in parenthesis indicate the number of experiments. *$P < 0.05$ compared to neurons transfected with control scrambled siRNA, Student's *t* test.

levels of the former are reduced, there is an increased expression of the latter.

The functional coupling between Ca^{2+} channels and both NOP and adrenergic receptors was examined next in SG neurons 96 h post-transfection. Figure 5*A* shows the time course of peak Ca^{2+} currents from a control SG neuron transfected with scrambled siRNA and the corresponding numbered traces are shown in Fig. 5*B*. Exposure of the neuron to NA (10 μM) resulted in a 75% current inhibition (traces 1 and 3, Fig. 5*B*). After a recovery

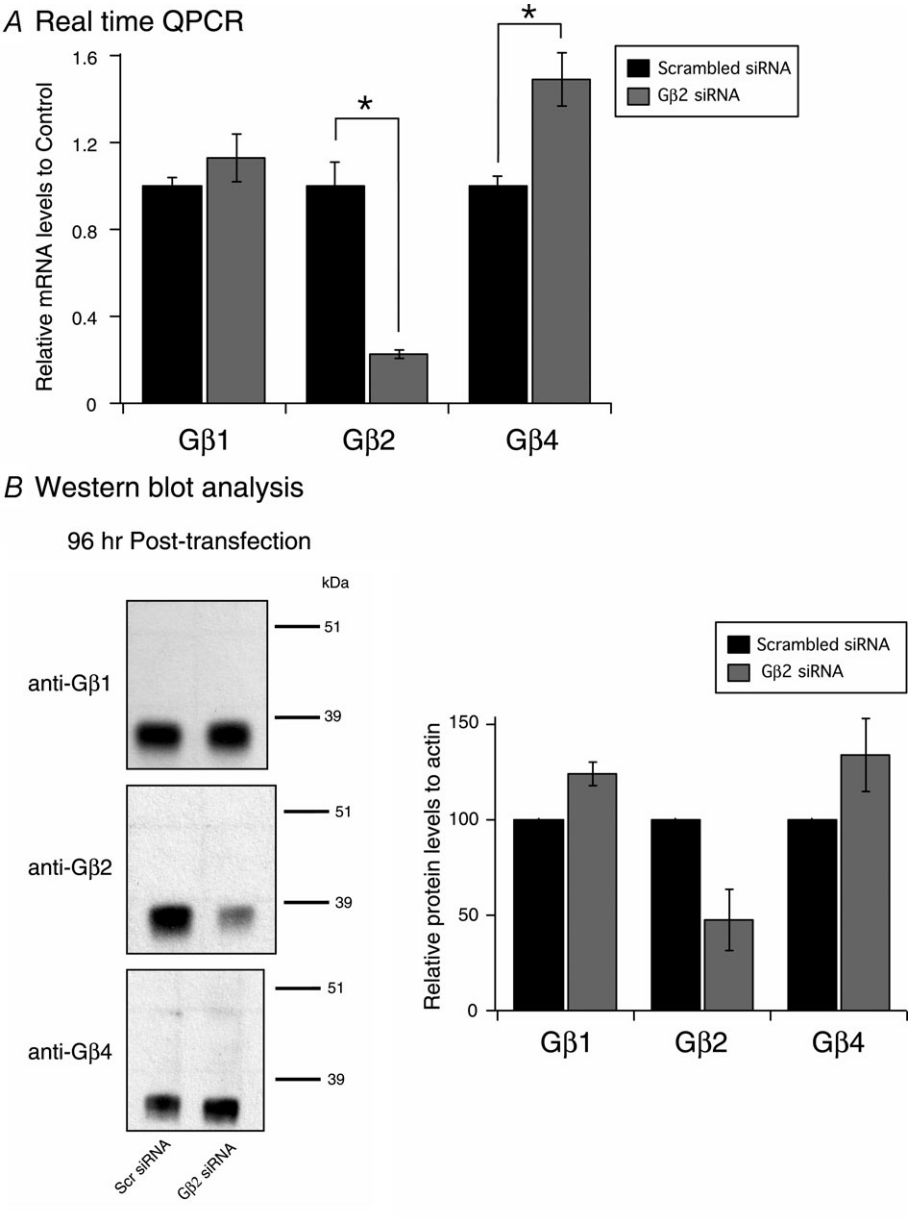

Figure 4. Detection of Gβ subunit expression by QRT-PCR and Western blot analysis in SG 96 h post-Gβ2 siRNA transfection

A, quantitative assessment of Gβ mRNA expression by QRT-PCR in SG tissue 96 h post-Gβ2 siRNA transfection. The ΔC_T values were determined for both groups ($n = 6$; 2 rats per group) and the Gβ mRNA levels were measured relative to GAPDH mRNA levels. The mRNA levels are expressed as fold-change compared to SG tissue transfected with scrambled siRNA. Three independent experiments were performed. *B*, Western blot (left panel) experiments illustrating results with anti-Gβ1, -Gβ2 and -Gβ4 in SG tissue transfected with either control scrambled or Gβ2 siRNA. Each lane was loaded with 20 μg protein and the lines indicate approximate molecular masses (kDa). Right panel shows the densitometric analysis of Western blots for actin and Gβ subunits of 2 independent experiments. The normalized values represent mean with standard deviation. $^*P < 0.05$ compared to neurons transfected with control scrambled siRNA, Student's *t* test.

period, Noc (1 μM) was then applied to the neuron and the Ca^{2+} currents were inhibited by 54%. The time course shown in Fig. 5*C* was acquired from an SG neuron transfected with Gβ2 siRNA. The results show that exposure of the neuron to either Noc or NA led to similar inhibition of the Ca^{2+} currents observed in control neurons. In addition, unlike the results observed in SG neurons with a 72 h post-transfection period, coupling between opioid

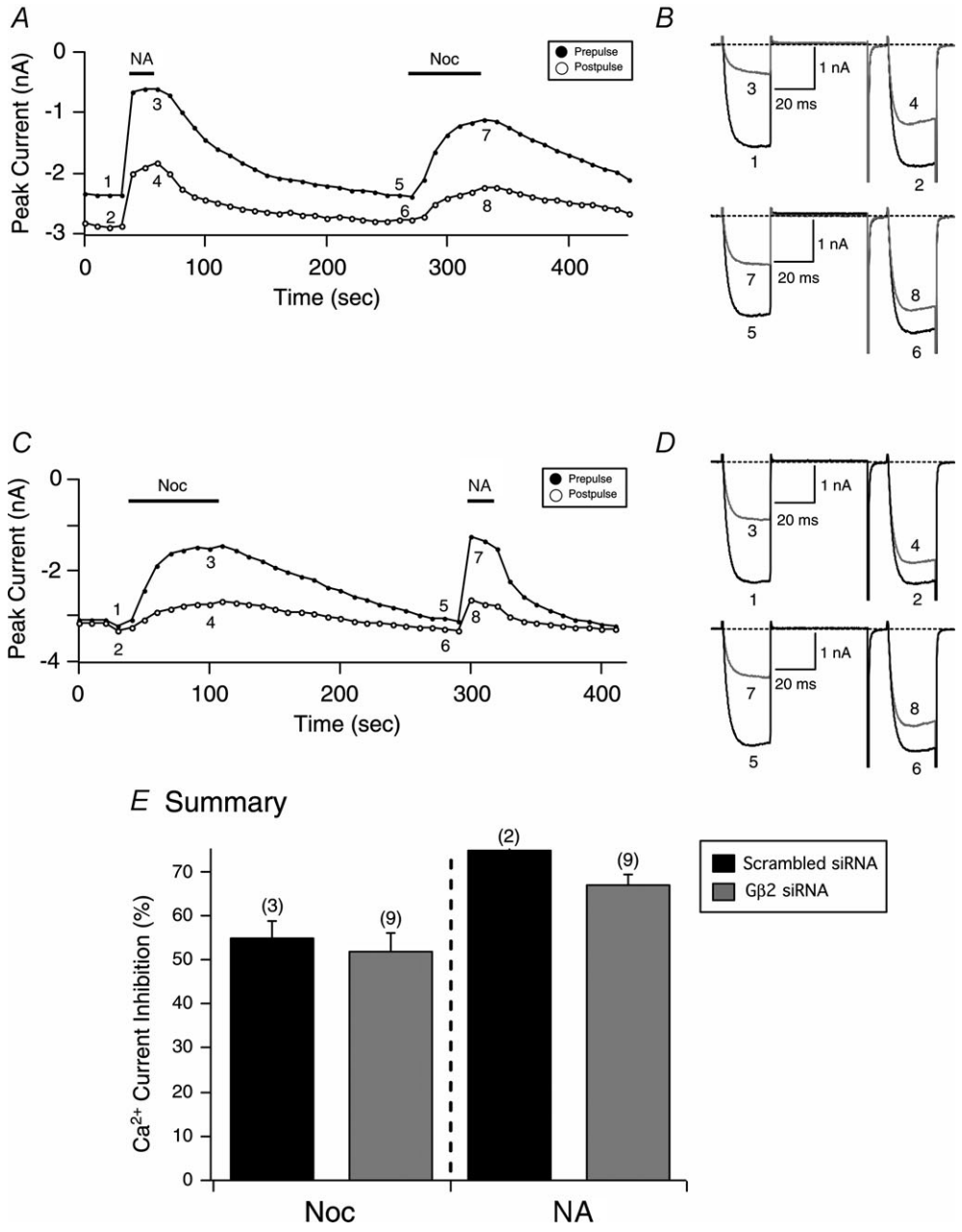

Figure 5. Effect of Gβ2 silencing 96 h post-siRNA transfection on the Noc- and NA-mediated Ca^{2+} current inhibition in SG neurons

A and *C*, time courses of peak Ca^{2+} current amplitude inhibition for pre- (●) and post-pulse (○) acquired from the sequential application of NA (10 μM) and Noc (1 μM) in neurons transfected with control scrambled (*A*) and Gβ2 (*C*) siRNA, respectively. *B* and *D*, superimposed Ca^{2+} current traces evoked with the 'double-pulse' voltage protocol (shown at the top of Fig. 3*B*) in the absence (1 and 2; 5 and 6) or presence (3 and 4; 7 and 8) of either NA or Noc. Currents were evoked every 10 s. The filled bars indicate the application of agonists. *E*, summary graph of mean (±SEM, except for NA control scrambled siRNA group where $n = 2$) Ca^{2+} current inhibition produced by application of Noc or NA in neurons transfected with scrambled or Gβ2 siRNA. Inhibition was determined from the Ca^{2+} current amplitude measured isochronally at 10 ms into the prepulse (+10 mV) in the absence or presence of Noc or NA. Numbers in parenthesis indicate the number of experiments.

and adrenergic receptors had been restored to control levels (cf. Figs 3*E* and 5*E*). Comparison of the basal facilitation ratios for both control- and siRNA-transfected neurons depicted in Fig. 5*A* and *C* reveals that the neurons with low Gβ2 expression levels had lower tonic G protein activation. The mean (±SEM) basal facilitation ratio for control neurons was 1.15 ± 0.02 ($n = 3$) while the ratio measured in Gβ2 siRNA-transfected neurons was significantly lower 1.04 ± 0.01 ($n = 9$, $P = 0.006$). The plot depicted in Fig. 5*E* summarizes the Noc and NA-mediated Ca^{2+} current inhibition for both control- and Gβ2 siRNA-transfected neurons. The results show that coupling between both receptors and Ca^{2+} channels was restored due to the compensatory overexpression of Gβ4 subunits.

Silencing of Gβ4 significantly decreases the coupling of opioid and adrenergic receptors with Ca^{2+} channels with no apparent compensation provided by other Gβ subunits

Given the observed changes in Gβ4 expression levels following Gβ2 knockdown, the next set of experiments was designed to examine the effect of silencing this subunit on the modulation of Ca^{2+} channels and the receptors. Figure 6*A* shows the Western blot for scrambled and Gβ4 siRNA-transfected SG tissue 96 h post-transfection. The results show that Gβ4 protein levels were lower (~40%) in the tissue transfected with Gβ4 siRNA while we did not observe a significant change (increase or decrease) in either Gβ1 or Gβ2 expression.

Coupling of Ca^{2+} channels and both receptors was examined next in Gβ4 siRNA-transfected neurons. The time courses in Fig. 7*A* show the peak Ca^{2+} currents from a control (left) and a Gβ4 siRNA transfected SG neuron (right) 72 h post-transfection. Application of NA (10 μM) and Noc (1 μM) led to a 76% and 70% inhibition, respectively, of the Ca^{2+} currents. Exposure of the Gβ4 siRNA transfected neuron to Noc and NA inhibited the currents, but the degree of block was less than that observed for control neurons. The summary plot in Fig. 7*C* (left) indicates that Gβ4 silencing significantly ($P < 0.05$) decreased the agonist-mediated inhibition of Ca^{2+} currents. In addition, the mean (±SEM) basal facilitation ratio of Gβ4 siRNA-transfected neurons (1.06 ± 0.02) was significantly ($P = 0.014$) lower when compared to control neurons (1.17 ± 0.03). Figure 7*B* shows time courses of peak Ca^{2+} currents for control (left) and Gβ4 siRNA-transfected (right) neurons 96 h post-transfection. Unlike the control neuron, application of Noc or NA did not lead to a significant modulation of the Ca^{2+} currents. The summary plot in Fig. 7*C* (right) illustrates that the additional 24 h Gβ4 silencing period resulted in a significant loss of coupling between both receptors and Ca^{2+} channels, similar to that observed for Gβ2 silencing described above (cf. Figs 3*E* and 7*C*, right). Finally, Gβ4 knockdown also led to a significantly ($P = 0.009$, Student's t test) lower basal facilitation ratio (0.97 ± 0.01) when compared to control neurons (1.07 ± 0.05). The data suggest that, unlike the Gβ2 silencing set of experiments, neither Gβ1 nor Gβ2 exert a compensatory effect to restore coupling of the receptors and Ca^{2+} channels following Gβ4 silencing.

The combined knockdown of Gβ2 and Gβ4 protein subunits abolishes any Gβ-mediated compensatory effects for receptor-channel coupling

In the next set of experiments, both Gβ2 and Gβ4 proteins in SG tissue were silenced as described above. Figure 8*A* and *B* shows Western blots of the Gβ subunit protein levels for scrambled- and Gβ2/Gβ4-silenced SG tissue. Not surprisingly, the Gβ1 protein levels increased 72 h post-transfection while Gβ2 and Gβ4 levels were lower than control tissue. By 96 h post-transfection, Gβ2 and Gβ4 levels decreased further and Gβ1 expression remained relatively normal.

Ca^{2+} currents were measured next in SG neurons transfected with either scrambled or both Gβ2 and Gβ4 siRNA. The time course of peak Ca^{2+} currents in Fig. 9*A* (left) from a control SG neuron shows the Noc- and NA-mediated current inhibition from a control SG neuron 72 h post-transfection. Exposure of the neuron to either agonist resulted in a voltage-dependent inhibition of the current similar to that observed above for control neurons. On the other hand, Fig. 9*A* (right) shows that the agonist-mediated current inhibition was abrogated in an SG neuron with reduced Gβ2 and Gβ4 expression levels. The application of NA (10 μM) and Noc (1 μM) led to approximately a 13% current inhibition for both agonists. Note that the basal facilitation ratio for this neuron was 1.02, indicating that tonic G protein activation was reduced, while that in the control neuron was 1.10 (Fig. 9*A*, left).

Figure 9*B* shows time courses of peak Ca^{2+} currents for SG neurons transfected with scrambled (left) and both Gβ2 and Gβ4 siRNA (right). Similar to the results described for 72 h post-transfection group, the double Gβ knockdown resulted in a significant loss of Ca^{2+} current modulation following activation of either receptor (Fig. 9*C*, right). In both sets of experiments, the mean (±SE) basal facilitation ratios for control and Gβ2 and Gβ4 siRNA-transfected neurons were 1.17 ± 0.03 ($n = 10$) and 0.98 ± 0.02 ($n = 11$, $P = 0.0003$, Student's t test), respectively, 72 h post-transfection, while the ratios measured 96 h post-transfection in control and both Gβ2 and Gβ4 siRNA-transfected neurons were 1.13 ± 0.03 ($n = 17$) and 0.97 ± 0.02 ($n = 28$, $P < 0.0001$, Student's

t test). Figure 9*C* is a summary plot of the Noc- and NA-mediated Ca^{2+} current inhibition for control- and Gβ2 and Gβ4-directed siRNA 72 (left) and 96 h (right) post-transfection. The results indicate that coupling of opioid and adrenergic receptors decreased significantly ($P < 0.05$).

Knockdown of Gβ1 subunits does not alter the opioid- and adrenergic receptor-mediated Ca^{2+} current inhibition

In the final set of experiments we examined whether silencing Gβ1 would affect the opioid and adrenergic coupling to Ca^{2+} channels. To this end, Gβ1 protein expression levels were determined. Figure 10*A* and *C* are Western blots obtained from SG tissue that had been transfected with Gβ1 siRNA 72 and 96 h post-transfection, respectively. The intensity analysis of the blots indicated that Gβ1 protein decreased ∼5% and 45%, respectively, 72 and 96 h post-transfection. Moreover, Gβ2 and Gβ4 expression levels increased 19% and 9% (72 h), respectively, but decreased 10% and 2% (96 h), respectively.

The effect of Gβ1 knockdown on the Noc- and NA-mediated Ca^{2+} current inhibition 72 and 96 h post-transfection is summarized in Fig. 10*B* and *D*. The results show that silencing of Gβ1 did not lead to significant changes in response to both agonists between the control and Gβ1 siRNA-transfected groups. Compared with the results described above for Gβ2, Gβ4 and combined Gβ2/4, silencing of Gβ1 does not appear to play a significant role in the adrenergic/NOP receptor/G protein/Ca^{2+} channel signalling pathways.

Discussion

In the present study we describe the transfection of SG tissue with siRNA designed to silence natively expressed Gβ subunits and determine the coupling specificity of Ca^{2+} channels with NOP opioid and adrenergic receptors. Western blot analysis of SG tissue revealed that of the four Gβ proteins assayed, Gβ1, Gβ2 and Gβ4 isoforms were expressed while Gβ3 subunit expression was not detected. Gβ2 subunits exhibited higher expression levels than either Gβ1 or Gβ4. Our results show that 72 or 96 h post-siRNA transfection, both mRNA and protein levels of the intended siRNA target were decreased when compared to tissue transfected with scrambled siRNA. It is clear that protein expression of the targeted Gβ subunit was not entirely abolished. It should be kept in mind that these biochemical assays were performed with the entire tissue, which includes neuronal and glial cell types. Thus, the simplest explanation for the incomplete knockdown is that the absolute amount of Gβ protein expression in SG neurons is significantly less than that contributed by glial cells. The contribution of Gβ subunits from each cell type is technically difficult to determine. Given this limitation, the facilitation of Ca^{2+} currents (described

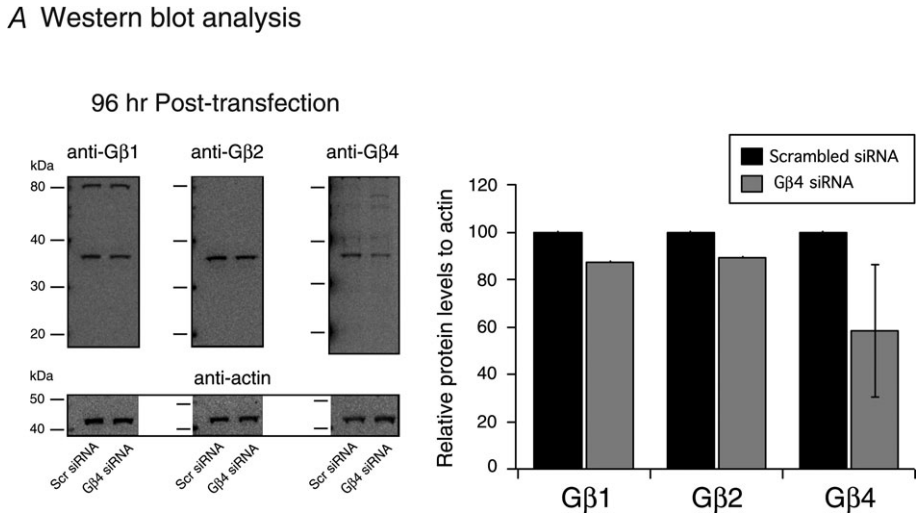

Figure 6. Detection of Gβ subunit expression by Western blot analysis in SG 96 h post-Gβ4 siRNA transfection
A, quantitative assessment of Gβ protein expression in SG tissue 96 h post siRNA transfection. Western blot experiments illustrating results with anti-actin (loading control), -Gβ1, -Gβ2 and -Gβ4 in SG tissue transfected with either scrambled or Gβ4 siRNA. Each lane was loaded with 20 μg protein and the lines indicate approximate molecular masses (kDa). The right panel shows the intensity analysis of Western blots for actin and Gβ subunits. Each Gβ subunit was normalized to actin and the relative protein levels to actin are plotted. The normalized values represent mean (±SD) of 2 independent experiments. The Western blots were quantified by measuring area density (i.e. intensity) employing VisionWorks LS software.

above) can, however, be employed as an indicator of Gβ subunit expression. Our results showed that the basal facilitation ratios of SG neurons transfected with Gβ2, Gβ4 and Gβ2/β4 siRNA were significantly lower than control neurons.

The significant decrease of coupling between both NOP and adrenergic receptors with Ca^{2+} channels following Gβ2, Gβ4 and Gβ2/β4 knockdown suggests that both subunits are normally involved in mediating the inhibition of the Ca^{2+} currents. However, when either subunit was silenced, the results revealed two different effects. First, we observed that the available Gβ1 or Gβ4 protein levels did not compensate for the Gβ2-mediated loss of coupling within the 72 h post-transfection period. However, as the Gβ2 levels became persistently low (i.e. 96 h post siRNA-transfection), then the increased Gβ4 subunit expression restored both opioid and adrenergic signalling pathways. This finding suggests that the functional redundancy observed with Gβ2 knockdown may impart a 'protective' mechanism under conditions where expression levels of a subunit are significantly decreased or the protein structure is compromised. For example, in some human populations, the Gβ3 gene (*GNB3*) contains a polymorphism (C825T) that results in the deletion of 41 amino acids (Siffert *et al.* 1998). The expression of the mutant subunit, Gβ3-s, has been

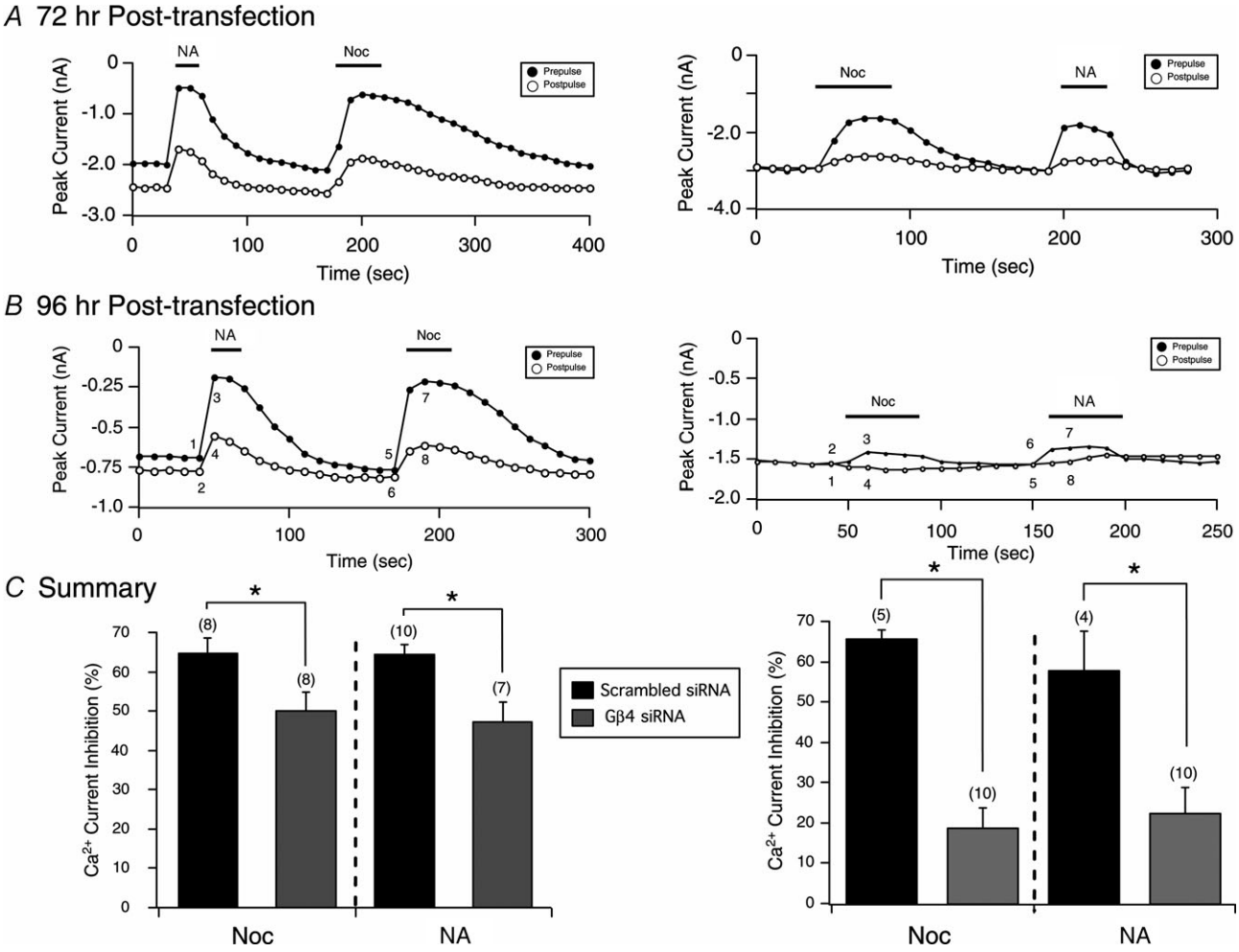

Figure 7. Effect of Gβ4 silencing 72 and 96 h post-siRNA transfection on the Noc- and NA-mediated Ca^{2+} current inhibition in SG neurons

A and *B*, time courses of peak Ca^{2+} current amplitude inhibition for pre- (●) and postpulse (○) acquired from the sequential application of either Noc (1 μM) or NA (10 μM) in neurons transfected with control scrambled (*A* and *B*, left) and Gβ4 siRNA (*A* and *B*, right) 72 (*A*) and 96 h (*B*) post-transfection. Currents were evoked every 10 s with the 'double-pulse' voltage protocol (shown at the top of Fig. 3*B*). The filled bars indicate the application of agonists. *C*, summary graph of mean (±SEM) Ca^{2+} current inhibition produced by application of Noc or NA in control and neurons transfected with Gβ4 siRNA 72 (left) and 96 h (right) post-transfection. Inhibition was determined from the Ca^{2+} current amplitude measured isochronally at 10 ms into the prepulse (+10mV) in the absence or presence of Noc or NA. Numbers in parenthesis indicate the number of experiments. $^*P < 0.05$ compared to neurons transfected with control scrambled siRNA, Student's *t* test.

linked to several cardiovascular disorders (for review see Siffert, 2005). Nevertheless it has been reported that the heterologously expressed mutant Gβ3-s in sympathetic neurons does not modulate Ca^{2+} or G protein-gated K^+ channels when compared to wild-type Gβ3 (Ruiz-Velasco & Ikeda, 2003). Thus, although the Gβ3-s subunit is normally expressed in individual subpopulations, a different Gβ isoform may aid in counteracting the diminished signalling that results from the mutant isoform. This compensation, however, may not be achieved altogether given the correlation of the presence of this polymorphism and cardiovascular disease (Siffert, 2005).

The second outcome was that the loss of coupling between both receptor types and Ca^{2+} channels mediated by Gβ4 knockdown did not lead to a 'rescue' by either Gβ2 or Gβ1, at least during the time scale of our experiments. This finding was unexpected given that Gβ2 knockdown exhibited reciprocity between Gβ2 and Gβ4. This lack of functional redundancy has been previously shown to occur in mouse macrophages (Hwang *et al.* 2004; Hwang *et al.* 2005) and mouse neutrophils (Zhang *et al.* 2010), both of which primarily express Gβ1 and Gβ2 isoforms. In these *in vitro* studies, the former found that Gβ2 knockdown eliminated the complement factor C5a-mediated chemotactic response as well as other Gβ2-mediated signalling pathways. The latter study reported that silencing Gβ1, and not Gβ2, affected the ability of neutrophils to ingest bacteria, while Gβ2 silencing affected chemotaxis but not motility (Zhang *et al.* 2010). Furthermore, a recent study found that knockdown of any Gβ1–β4 subunits in HeLa cells did not lead to marked changes in the expression profile of non-targeted Gβ proteins (Krummins & Gilman, 2006). However, a significant up- or down-regulation of some of the Gβ subunits occurred after silencing a small

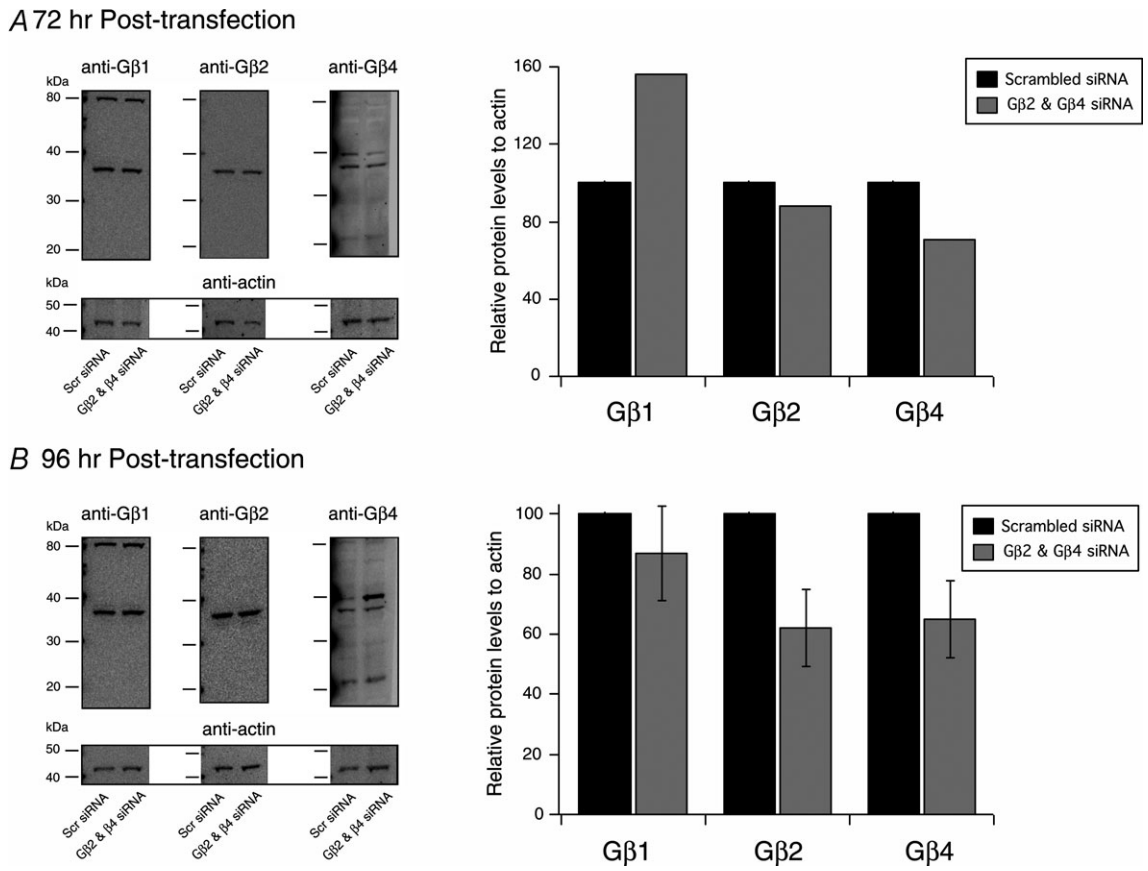

Figure 8. Detection of Gβ subunit expression by Western blot analysis in SG tissue transfected with Gβ2 and Gβ4 siRNA

Quantitative assessment of Gβ protein expression in SG tissue 72 (*A*) and 96 h (*B*) post-siRNA transfection. Western blot experiments illustrating results with anti-actin (loading control), -Gβ1, -Gβ2 and -Gβ4 in SG tissue transfected with either scrambled or Gβ2 and Gβ4 siRNA. Each lane was loaded with 20 μg protein and the lines indicate approximate molecular masses (kDa). The right panels show the intensity analysis of Western blots for actin and Gβ subunits. Each Gβ subunit was normalized to actin and the relative protein levels to actin are plotted. The normalized values represent mean of 1 (*A*) and 3 (*B*) independent experiments. The Western blots were quantified by measuring area density (i.e. intensity) employing VisionWorks LS software.

number of Gα subunit isoforms. Another report found that during mouse neonatal development, a compensatory mechanism was absent when Gβ1 genes were silenced (Okae & Iwakura, 2010). The study showed that Gβ1 plays a major role in embryonic neurogenesis because the absence of the protein resulted in perinatal lethality. The authors reasoned that death resulted from defects in neurotransmitter release mechanisms (Okae & Iwakura, 2010). With regard to our observations, one can speculate that the specific composition of the heterotrimeric G protein, to which adrenergic and NOP receptors couple, exhibits selectivity for Gβ4.

The results shown also suggest that Gβ1 subunits do not normally couple Ca^{2+} channels with either NOP or adrenergic receptors in SG neurons. This is based on the inability of Gβ1 to rescue the loss of coupling when Gβ2 and Gβ4 were simultaneously silenced. Also, Gβ1 knockdown failed to exert any effect on the modulation of Ca^{2+} channels mediated by NA or Noc exposure. It is possible that SG neurons do not express this subunit and that the source of Gβ1 detected via Western blots is principally of glial origin. Thus, we did not observe that Gβ1 plays a role in both signalling pathways.

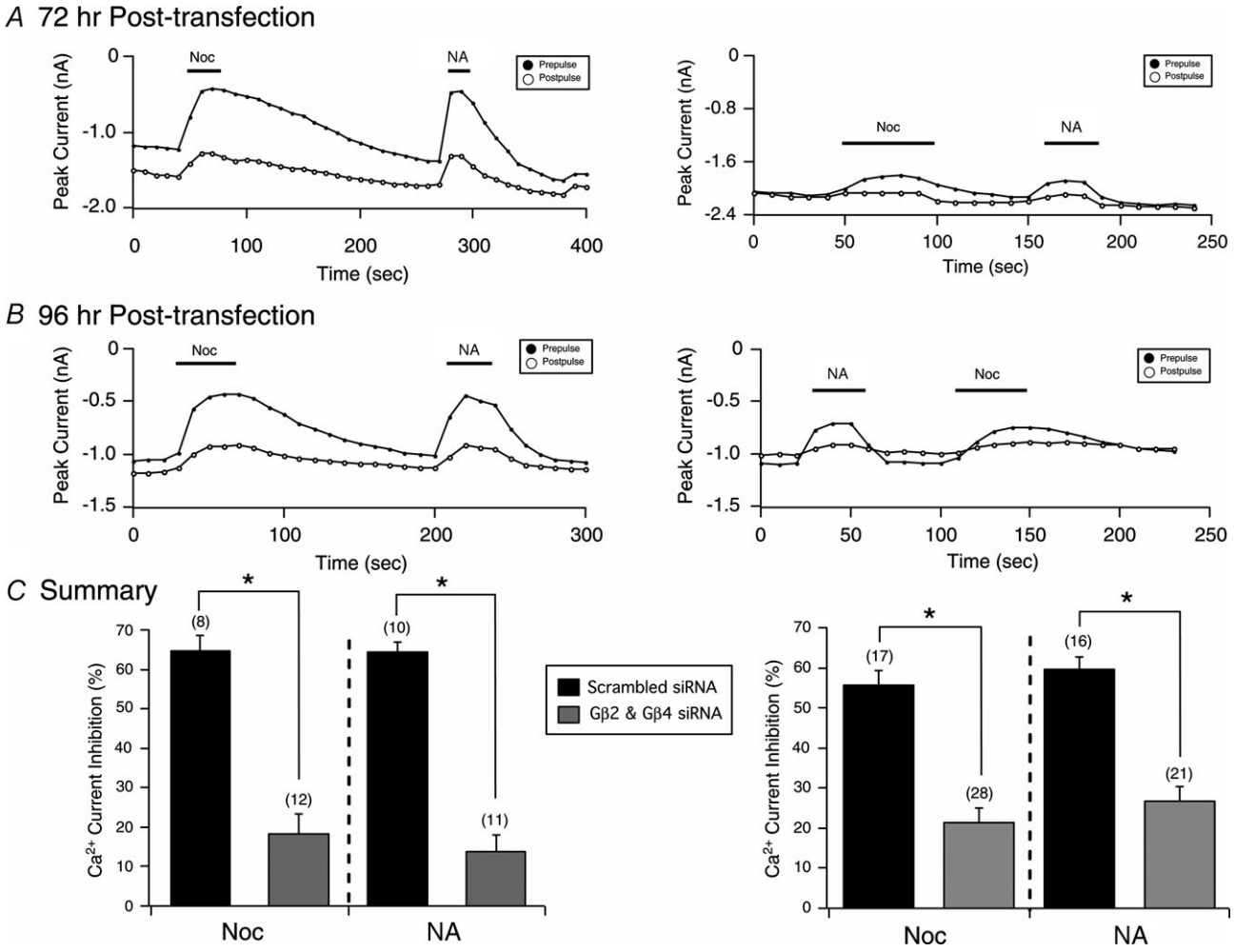

Figure 9. Effect of Gβ2 and Gβ4 silencing 72 and 96 h post-siRNA transfection on the Noc- and NA-mediated Ca^{2+} current inhibition in SG neurons
A and *B*, time courses of peak Ca^{2+} current amplitude inhibition for pre- (●) and postpulse (○) acquired from the sequential application of either Noc (1 μM) or NA (10 μM) in neurons transfected with control scrambled (*A* and *B*, left) and Gβ2 and Gβ4 siRNA (*A* and *B*, right) 72 (*A*) and 96 h (*B*) post-transfection. Currents were evoked every 10 s with the 'double-pulse' voltage protocol (shown at the top of Fig. 3*B*). The filled bars indicate the application of agonists. *C*, summary graph of mean (±SEM) Ca^{2+} current inhibition produced by application of Noc or NA in control and neurons transfected with Gβ2 and Gβ4 siRNA. Inhibition was determined from the Ca^{2+} current amplitude measured isochronally at 10 ms into the prepulse (+10 mV) in the absence or presence of Noc or NA. Numbers in parenthesis indicate the number of experiments. $^*P < 0.05$ compared to neurons transfected with control scrambled siRNA, Student's *t* test.

The adrenergic receptor-mediated inhibition of Ca^{2+} channels in rat superior cervical ganglion neurons has been shown to arise by $G\beta\gamma$ dimers coupled primarily to $G\alpha_o$ subtypes and to a lesser extent with $G\alpha_i$ (Delmas *et al.* 1999). We have previously reported that knocking out the PTX-sensitive $G\alpha_{i/o}$ proteins individually does not perturb the coupling of the adrenergic receptors with the Ca^{2+} channels (Margas *et al.* 2008). On the other hand, we found that silencing $G\alpha_{i1}$ subunits eliminated the Noc-mediated Ca^{2+} current inhibition, suggesting that SG neurons require a robust adrenergic pathway at the receptor:$G\alpha$ juncture. Collectively, the present study and the previous report (Margas *et al.* 2008) show that NOP opioid receptors, unlike adrenergic receptors, exhibit selectivity with regard to coupling of $G\alpha$ subunits with Ca^{2+} channels while both receptor signalling pathways normally rely on either $G\beta2$ or $G\beta4$ isoform. This suggests that receptor-mediated Ca^{2+} channel modulation in SG neurons is likely to be derived from a $G\alpha_{i1}\beta2(4)\gamma$ heterotrimer.

Several studies have shown that all $G\beta$ subunits when heterologously expressed are capable of modulating Ca^{2+} channels (Garcia *et al.* 1998; Arnot *et al.* 2000; Ruiz-Velasco & Ikeda, 2000; Zhou *et al.* 2000). Thus, coupling specificity of $G\beta$ subunits and Ca^{2+} channels appears to exhibit stringency with regard to the expression profile within the tissue rather than a specific subunit *per se*. However, our results suggest that $G\beta$ subunit selectivity cannot be completely ruled out. Alternatively, it is also possible that specificity may arise from the $G\gamma$ subunit that dimerizes with $G\beta$. At present, the $G\gamma$ expression profile in SG neurons is not known.

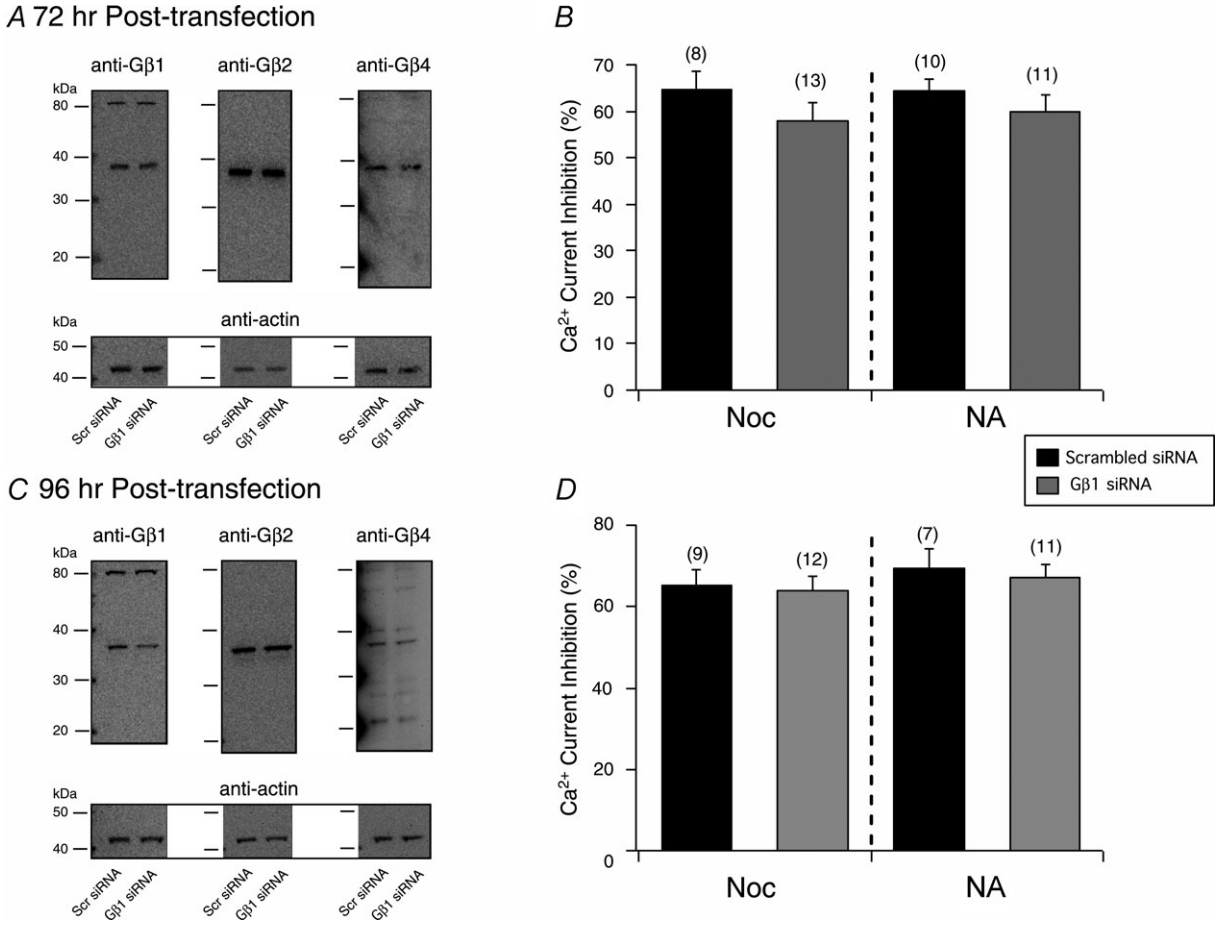

Figure 10. Effect of Gβ1 silencing 72 and 96 h post-siRNA transfection on the Noc- and NA-mediated Ca^{2+} current inhibition in SG neurons
Western blot experiments illustrating results with anti-Gβ1, -Gβ2, -Gβ4 and -actin in SG tissue transfected with either control scrambled or Gβ1 siRNA 72 (*A*) and 96 h (*C*) post-transfection. Each lane was loaded with 20 μg protein (1–2 rats per group). Lines indicate approximate molecular masses (kDa). The blots were stripped and reprobed with anti-actin (loading control). *B* and *D*, summary graphs of mean (±SEM) Ca^{2+} current inhibition produced by application of Noc or NA in neurons transfected with scrambled or Gβ1 siRNA 72 (*B*) and 96 h (*D*) post-transfection. Inhibition was determined from the Ca^{2+} current amplitude in the absence or presence of Noc or NA. Numbers in parentheses indicate the number of experiments.

In summary, the results from the present study show the successful transfection of SG tissue with siRNA targeting Gβ proteins. Of the three Gβ isoforms expressed, Gβ2 and Gβ4 were demonstrated to play an important role in coupling opioid and adrenergic receptors to Ca^{2+} channels. Knockdown of either Gβ2 or Gβ4 led to the loss of coupling for both receptors. Whereas the continued diminution of Gβ2 levels resulted in a compensatory increase of Gβ4 expression with a concomitant restoration of the coupling for both receptors, the inverse was not observed. Further, when Gβ2 and Gβ4 were simultaneously silenced, coupling of the receptors and channels was also significantly reduced. Finally, Gβ1 subunits do not appear to play a crucial role in either pathway. Thus, Gβ2 and Gβ4 are necessary for maintaining the NOP opioid/adrenergic receptors:Ca^{2+} channel signal transduction pathways. However, Gβ4 subunits have the unique ability to sense Gβ2 expression levels and maintain adequate function. The preservation of these signalling events is likely to be crucial in SG neurons that innervate cardiac muscle and help to regulate blood pressure and heart rate.

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Author contributions

V.R.-V. conceived and designed the experiments, analysed the data, performed the electrophysiological and western blotting assays, and co-wrote the manuscript. S.M. performed the transfection and electrophysiological experiments, and analysed the data. J.K.Y. helped in the design of both the western blotting and QRT-PCR experiments and co-wrote the manuscript. All authors approved final version for publication.

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