\textbf{\textgamma-\texttext{Aminobutyric Acid Type B (GABA}$_{\text{B}}$\text{) Receptor Expression Is Needed for Inhibition of N-type (Ca$_{\text{v}}$2.2) Calcium Channels by Analgesic $\alpha$-Conotoxins}$^{\text{a}}$\text{b}\text{c}$

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Hartmut Cuny, Andrew de Faoite$^1$, Thuan G. Huynh, Takahiro Yasuda$^2$, Géza Berecki, and David J. Adams$^3$

\textit{From the Health Innovations Research Institute, RMIT University, Melbourne, Victoria 3083, Australia}

\textbf{Background:} A class of analgesic $\alpha$-conotoxins potently inhibits N-type calcium channels. The activity of $\alpha$-conotoxins Vc1.1 and RglA was reduced following knockdown of GABA$_{\text{B}}$ receptor expression in sensory neurons and could be reconstituted in HEK 293 cells expressing human GABA$_{\text{B}}$ receptors and Ca$_{\text{v}}$2.2.

\textbf{Conclusion:} GABA$_{\text{B}}$ receptors are needed for inhibition of Ca$_{\text{v}}$2.2 by Vc1.1 and RglA.

\textbf{Significance:} These analgesic $\alpha$-conotoxins activate human GABA$_{\text{B}}$ receptors.

\textit{\textalpha-Conotoxins Vc1.1 and RglA are small peptides isolated from the venom of marine cone snails. They have effective antinociceptive actions in rat models of neuropathic pain. Pharmacological studies in rodent dorsal root ganglion (DRG) show their analgesic effect is mediated by inhibition of N-type (Ca$_{\text{v}}$2.2) calcium channels via a pathway involving $\gamma$-aminobutyric acid type B (GABA$_{\text{B}}$) receptor. However, there is no direct demonstration that functional GABA$_{\text{B}}$ receptors are needed for inhibition of the Ca$_{\text{v}}$2.2 channel by analgesic $\alpha$-conotoxins. This study examined the effect of the GABA$_{\text{B}}$ agonist baclofen and $\alpha$-conotoxins Vc1.1 and RglA on calcium channel currents after transient knockdown of the GABA$_{\text{B}}$ receptor using RNA interference. Isolated rat DRG neurons were transfected with small interfering RNAs (siRNA) targeting GABA$_{\text{B}}$ subunits R1 and R2. Efficient knockdown of GABA$_{\text{B}}$ receptor expression at mRNA and protein levels was confirmed by quantitative real time PCR (qRT-PCR) and immunocytochemical analysis, respectively. Whole-cell patch clamp recordings conducted 2–4 days after transfection showed that inhibition of N-type calcium channels in response to baclofen, Vc1.1 and RglA was significantly reduced in GABA$_{\text{B}}$ receptor knockdown DRG neurons. In contrast, neurons transfected with a scrambled nontargeting siRNA were indistinguishable from untransfected neurons. In the HEK 293 cell heterologous expression system, Vc1.1 and RglA inhibition of Ca$_{\text{v}}$2.2 channels needed functional expression of both human GABA$_{\text{B}}$ receptor subunits. Together, these results confirm that GABA$_{\text{B}}$ receptors must be activated for the modulation of N-type (Ca$_{\text{v}}$2.2) calcium channels by analgesic $\alpha$-conotoxins Vc1.1 and RglA.}

Neuropathic pain is caused by damage or disease to the central or peripheral nervous system. It is a major health burden and poorly managed due to a lack of efficacious analgesics (1–3). Current pharmacotherapies, including opioids, anti-epileptics, and antidepressants, have low efficacy and significant side effects (4, 5). Therefore, there is a clinical need to develop novel drugs leading to better treatment of neuropathic pain.

Conotoxins (toxins isolated from the venom of marine cone snails) target a wide variety of membrane receptors and ion channels and are a rich source of potential therapeutic agents. $\alpha$-Conotoxins are small peptides that typically range in size from 12 to 19 amino acids, contain two disulfide bonds, and usually have an amidated C terminus. $\alpha$-Conotoxin Vc1.1, a synthetic derivative of a peptide derived from the venom of the marine cone snail Conus victoriae, potently suppresses signs of pain when injected intramuscularly near the site of injury in rat models (6–8), demonstrating its potential as an analgesic therapeutic. RglA, an $\alpha$-conotoxin from Conus regius, has also been shown to significantly reduce chronic constriction nerve injury-induced hyperalgesia in rats (9, 10).

How $\alpha$-conotoxins Vc1.1 and RglA relieve neuropathic pain remains controversial. Both are potent antagonists of $\alpha$9$\alpha$10 nicotinic acetylcholine receptors (nAChRs)$^3$ (9–11), and Vc1.1 antagonizes the nicotine-induced increase in axonal excitability of unmyelinated C-fibers (12). Therefore, $\alpha$9$\alpha$10 nAChRs were proposed as the target for Vc1.1 and RglA to mediate analgesia in chronic and neuropathic pain models (10). However, several structural analogs of Vc1.1 retain activity at $\alpha$9$\alpha$10 nAChRs but lose their anti-allodynic effect (13, 14). This suggests that a target other than $\alpha$9$\alpha$10 nAChR may contribute to the pain-relieving activity of $\alpha$-conotoxins. Recent studies show that the analgesic $\alpha$-conotoxins Vc1.1 and RglA potently inhibit N-type calcium channel currents in rat sensory neurons (8, 15, 16). Pharmacological studies further reveal that Vc1.1...
does not interact directly with voltage-gated calcium channels (VGCC) but instead via a voltage-independent G protein-coupled GABA<sub>B</sub> receptor-mediated mechanism (8, 15). Collectively, these findings indicate that several different membrane receptors, including the GABA<sub>B</sub> receptor, may contribute to the pain-relieving activity of a class of α-conotoxins.

To date, there is a lack of molecular studies confirming the interaction between α-conotoxins and GABA<sub>B</sub> receptor subunits and demonstrating the need for functional GABA<sub>B</sub> receptor dimers for Ca<sub>2.2</sub> channel inhibition by α-conotoxins. This study examines whether or not a functional GABA<sub>B</sub> receptor is needed to link analgesic α-conotoxins and N-type VGCC current inhibition. We took two different approaches to this study. First, we investigated VGCC currents in isolated rat DRG neurons following transient knockdown of the GABA<sub>B</sub> receptor subunit expression using small interfering RNA (siRNA). Second, we investigated Ca<sub>2.2</sub> channel modulation by Vc1.1 in HEK 293 cells stably expressing Ca<sub>2.2</sub> channels and transient transfection with both subunits of the human GABA<sub>B</sub> receptor. These approaches showed that GABA<sub>B</sub> receptor expression is necessary for the observed inhibition of N-type VGCCs by the α-conotoxins Vc1.1 and RglA. A preliminary report of some of these results has been presented in abstract form (41).

**EXPERIMENTAL PROCEDURES**

**DRG Neuron Preparation**—DRG neurons were enzymatically dissociated from the ganglia of 4–14-day-old Wistar rats according to standard protocols. Briefly, rats were killed by cervical dislocation, as approved by the RMIT University Animal Ethics committee, and the spinal cords were removed. Ganglia of all areas of the spinal cord were collected in ice-cold Hanks’ balanced salt solution (Invitrogen). They were incubated in 1 mg/ml collagenase (Sigma) in Hanks’ balanced salt solution for 30 min at 37 °C, transferred to warm Neurobasal media (Invitrogen), and then triturated using a fire-polished Pasteur pipette to obtain a cell suspension. The suspension was centrifuged at 400 × g for 5 min and immediately used for transfection.

**siRNA Knockdown of GABA<sub>B</sub> Receptor**—Mission siRNA oligonucleotides (Sigma) for the rat gabbr1 gene (catalog no. SASI_Rn01_00121458) and gabbr2 gene (catalog no. SASI_Rn01_00107052) were used for transfection. Mission siRNA oligonucleotides comprising a scramble sequence with no homology to any known genes (siRNA universal negative control 1) were used as a negative control. Mock-transfected cells (without siRNA) served as an additional negative control. The siRNAs (100 nM final concentration of each siRNA duplex) were transfected into ~5 × 10<sup>4</sup> dissociated DRG cells using the Amaxa Nucleofector II electroporation system in combination with the basic neuron SCN nucleofector kit (both Lonza, Cologne, Germany) following the manufacturer’s protocol. To identify transfected cells during electrophysiological experiments, 200 nM fluorescein-labeled oligonucleotide (Block-iT Fluorescent Oligo, Sigma) was added to the transfection reaction mixture. After transfection, the cells were suspended again in Neurobasal media containing B27 supplement (both Invitrogen), 0.5 mm L-glutamine, and 1% penicillin/streptomycin and then seeded onto poly-d-lysine-coated multiwell plates or glass coverslips. The cells were incubated under humidified conditions in 95% air and 5% CO<sub>2</sub> at 37 °C and used after 1–4 days.

**qRT-PCR**—RNA was isolated 24–48 h after transfection using the Absolutely RNA prep kit (Agilent Technologies, Santa Clara, CA), and cDNA was synthesized from the RNA using the SuperScript III first-strand synthesis supermix (Invitrogen) for qRT-PCR. Expression levels of GABA<sub>B1</sub> R1 and GABA<sub>B2</sub> R2 mRNA were analyzed by qRT-PCR (Rotor-Gene 3000, Corbett Research, Sydney, Australia) using the SensiMix SYBR No-ROX kit (Bioline, London, UK) and the following primers: 5′-TCA AGA TCA TTC TCA TGC CTG-3′ and 5′-GTG AAC TGG AGC CAT ATG AG-3′ for GABA<sub>B1</sub> R1, and 5′-GAA CGA GAC CAA CTT CTT CG-3′ and 5′-CTC TGC TGT CTT GAA ATT GAG for GABA<sub>B2</sub> R2. Additionally, in each sample, the cDNAs of the housekeeping genes succinate dehydrogenase complex, subunit A, ubiquitin C, and ribosomal protein L13a, were amplified using standard primer sets (Mouse Normalization Gene Panel, Bioline) to serve as internal references. Data were analyzed based on the comparative quantitation method (Rotor-Gene software, Corbett). For each sample, the relative expression level of GABA<sub>B</sub> receptor mRNA was calculated by comparing it with the geometric mean of the relative mRNA levels of the three housekeeping genes.

**Antibodies**—The primary antibodies used were mouse monoclonal anti-βIII tubulin (Promega, 1:2000), rabbit polyclonal anti-GABA<sub>B1</sub> R1 (Abcam, Cambridge, UK, catalog no. ab75239, 1:800), and rabbit monoclonal anti-GABA<sub>B2</sub> R2 (Abcam, catalog no. ab75838, 1:400) antibodies. The corresponding fluorescent secondary antibodies used were Alexa Fluor 488-conjugated goat polyclonal anti-rabbit IgG antibody (Invitrogen, 1:1000) and Alexa Fluor 555-conjugated goat polyclonal anti-mouse IgG antibody (Invitrogen, 1:1000).

**Double Labeling Immunocytochemistry and Confocal Microscopy**—Immunocytochemistry was performed on transfected DRG neurons 2–4 days after transfection. DRG neurons were fixed in 4% paraformaldehyde for 15 min at room temperature. After two washes with PBS, the cells were preincubated in blocking solution (10% goat serum, 1% Triton X-100 in PBS) for 30 min at room temperature, followed by overnight incubation at 4 °C with anti-β-III tubulin and either polyclonal anti-GABA<sub>B1</sub> or monoclonal anti-GABA<sub>B2</sub> antibodies in fresh blocking solution. After two washes with PBS, the fluorescein-conjugated secondary antibodies were applied for 1.5 h at room temperature. Cell nuclei were counterstained with DAPI. After four more washing steps with PBS, the cells were sealed with fluorescence mounting media (Dako, Glostrup, Denmark). Control experiments determining the specificity of the immunocytochemical procedures were validated by omitting the primary antibodies. Images were acquired using a Nikon A1 laser-scanning confocal microscope equipped with a Plan Apo VC ×60 water-immersion objective, an argon laser (488 nm), a solid state laser (561 nm), and NIS Elements software using the same settings for siRNA-transfected and control cells.

**Quantitative Analysis of GABA<sub>B</sub> Receptor Protein Levels**—DRG cells, immunolabeled as described above, were subjected to quantitative analysis of GABA<sub>B1</sub> R1 receptor protein levels using a high-content imaging system (ImageXpress TS <sup>™</sup> Micro, Molecular Devices, Sunnyvale, CA). Cells transfected with the
siRNAs targeting the GABA<sub>B</sub> R1 and R2 subunits were compared with cells transfected with the nontargeting negative control siRNA. For each siRNA treatment group, 756 sites (each containing an average of 3.3 neurons) were scanned. Neurons were identified by βIII-tubulin expression. A threshold for R1 fluorescence was set above background or nonspecific staining levels. All βIII-tubulin-positive cells with above-threshold fluorescence were counted as high R1-expressing neurons. A difference in the percentage of high R1-expressing neurons between control and knockdown of GABA<sub>B</sub>R1 and GABA<sub>B</sub>R2 (R1 + R2 KD) cell batches would indicate successful knockdown. Moreover, to detect subtle differences in expression between high R1-expressing neurons of the control and R1 + R2 KD cell batches, we further evaluated R1 fluorescence intensities indicating protein expression levels by dividing the sum of the grayscale intensity of R1 fluorescence by the total βIII-tubulin-stained area.

**DRG and HEK 293 Cell Electrophysiology**—DRG neurons were transfected with either GABA<sub>B</sub>-receptor-specific siRNAs or negative-control siRNA and grown on poly-d-lysine-coated glass coverslips for 1–4 days. Only DRG neurons exhibiting green fluorescence, due to co-transfection of the fluorescein-labeled oligonucleotide, were selected for electrophysiological recordings. Cells were transferred into a small volume (~300 μl) recording chamber, constantly perfused with a solution containing (in mM) tetraethylammonium chloride 140, BaCl<sub>2</sub> 2, d-glucose 10, and HEPES 10, pH 7.4 (with NaOH). The internal pipette solution contained (in mM) CsCl 140, MgCl<sub>2</sub> 1, MgATP 5, Na-GTP 0.1, bis(2-aminophenoxy)ethane-N,N,N′,N′-tetraacetic acid-Ca<sub>4</sub> 5, and HEPES 10, pH 7.3 (with CsOH). Whole-cell Ba<sup>2+</sup> currents were elicited from a holding potential of ~80 mV, by 0.05 Hz, 200-ms step depolarizations to ~10 mV. In each experiment, R1 + R2 KD neurons and control neurons from the same batch of DRG preparations and transfections were tested successively. They were included for analysis only if the VGCC inward current inhibition in the R1 + R2 KD neuron by baclofen was at least 10% less than the current inhibition by baclofen in the control neuron, indicating successful knockdown of the GABA<sub>B</sub> receptor.

HEK 293 cells stably expressing human Ca<sub>2.2</sub> channels (α<sub>1B</sub>, α<sub>δ1</sub>, and β<sub>3</sub> subunits) (17) were transiently co-transfected with cDNAs encoding human GABA<sub>B</sub> R1 (10 μg), human GABA<sub>B</sub> R2 (10 μg) (Origene Technologies, Inc., Rockville, MD), and enhanced green fluorescent protein (1 μg) (a reporter gene construct), using calcium phosphate precipitation (18). They were grown in T175 flasks and treated with either 1 μM Vc1.1 or 30 μM baclofen. Immediately after treatment, biotinylation of cell surface proteins was done using the Pierce cell surface protein isolation kit (Thermo Fisher Scientific, Rockford, IL) according to the manufacturer instructions. Biotinylated Ca<sub>2.2</sub> protein was detected by Western blot. Detailed methods can be found in the supplemental material.

**Statistical Analysis**—Numerical data are presented as means ± S.E. The statistical significance between the two groups was evaluated by Student’s unpaired t test. The statistical significance between three or more groups was evaluated by one-way analysis of variance followed by the Bonferroni’s post hoc test. Values of p < 0.05 were considered to be statistically significant. All statistical analyses were achieved using Prism Version 5.1 (GraphPad Software Inc., La Jolla, CA).

**RESULTS**

**Knockdown of GABA<sub>B</sub> Receptor mRNA Expression in DRG Neurons**—To effectively knockdown the expression of functional GABA<sub>B</sub> receptors (which are heterodimers of the two GABA<sub>B</sub> subunits R1 and R2), we tested siRNAs targeting either the R1 or R2 subunit in isolated DRG neurons. qRT-PCR showed that following 24–48 h post-transfection, the mRNA levels of R1 in cells transfected with an R1-specific siRNA significantly decreased to 32.6 ± 3.5% (n = 4) of control cells transfected with a scrambled, nontargeting siRNA (p < 0.0001).
Furthermore, when mRNA expression of a single GABA$_B$ receptor subunit was disrupted by siRNA, the expression level of the other subunit was not affected (Fig. 1, A and B), indicating target-specific knockdown. Co-transfection of the R1- and R2-targeting siRNAs (R1+R2 KD) demonstrated that knockdown of each subunit was equally effective compared with single subunit knockdowns without interference (33.6 ± 6.1%, n = 5; p < 0.0001 mRNA levels of R1, and 53.4 ± 13.3%, n = 5; p = 0.026 mRNA levels of R2, respectively) (Fig. 1, A and B). Given that heterodimerization of R1 and R2 subunits is essential for functional GABA$_B$ receptors (24), we used the co-transfection protocol in the following experiments, expecting more effective knockdown.

GABA$_B$ Receptor Protein Knockdown by siRNA Treatment—Immunofluorescence labeling of GABA$_B$ receptors in rat DRG neurons and visual inspection by confocal microscopy revealed an apparent decrease in fluorescence intensities for GABA$_B$ R1 and GABA$_B$ R2 in R1+R2 KD neurons compared with scrambled, nontargeting siRNA-transfected DRG neurons (Fig. 2, A and B). This indicates that there was not a nonspecific effect due to the siRNA transfection process itself.

Of 17 neurons that exhibited efficient functional knockdown of GABA$_B$ receptors, VGCC current amplitude was inhibited by 21.2 ± 2.9% in response to bath application of baclofen. However, control DRG neurons exhibited a baclofen-induced decrease of 36.6 ± 2.9% (n = 17) (p = 0.001) (Fig. 4, A and C, and Table 1). The remaining VGCC current was not inhibited more by bath application of the selective N-type VGCC antagonist, ω-conotoxin CVID (300 nM), as we observed previously (15). Bath application of 100 nM Vc1.1 to control neurons inhibited the VGCC current amplitude by 20 ± 2.6% (n = 10) (Fig. 4, A and C, and Table 1).
**GABA<sub>B</sub> Receptor Needed for α-Conotoxin Inhibition of Ca<sub>V</sub>2.2**

**FIGURE 2.** GABA<sub>B</sub> receptor immunofluorescence is reduced in GABA<sub>B</sub> knockdown DRG neurons as observed using confocal microscopy. Immunolabeling of DRG neurons and visual inspection by confocal microscopy revealed an apparent decrease in fluorescence intensities for GABA<sub>B</sub> R1 and R2 in neurons transfected with siRNAs to disrupt GABA<sub>B</sub> R1 and R2 expression. This is compared with scrambled, nontargeting siRNA-transfected DRG neurons. Cells were immunostained 3 days after transfection, using antibodies directed against R1 (green) (A) or R2 (green) (B). Immunostaining of βIII-tubulin is shown in red. Representative images are shown. Scale bars, 50 μm.

**FIGURE 3.** Quantitative evaluation of protein levels in GABA<sub>B</sub> R1 knockdown DRG neurons by high content imaging analysis revealed significantly reduced GABA<sub>B</sub> R1 expression. We compared DRG cells transfected with siRNAs targeting both GABA<sub>B</sub> receptor subunits (R1 + R2 KD) with control cells transfected with scrambled, nontargeting siRNA (control). Quantitative analysis of immunofluorescence-labeled R1 proteins in DRG neurons 2–3 days after transfection revealed significantly reduced R1 protein expression in knockdown cells (R1 + R2 KD). A, representative sites of the high content scan of a total of 756 sites per treatment group. DRG neurons showing weak green fluorescence, indicating low expression of R1 (white arrowheads), were counted as neurons with R1 expression below threshold in the statistical analysis. Scale bars, 50 μm. B, percentage of neurons exhibiting R1 immunofluorescence above threshold, which correlates to high R1 expression levels. C, R1 expression levels of neurons with R1 expression above threshold calculated from fluorescence intensities. Number of experiments indicated in parentheses (*, p < 0.05; ***, p < 0.0001 versus control).

A and D). However, in R1 + R2 KD neurons, the effect of Vc1.1 on the VGCC current was significantly impaired, with the current amplitude reduced only by 5.1 ± 3.5% (n = 10) (p = 0.003) (Fig. 4, B and D, and Table 1). Note that in all neurons of the R1 + R2 KD treatment group in which baclofen-mediated inhibition of VGCCs was impaired, Vc1.1-induced inhibition of VGCC currents decreased in all cases. The decrease in the number of functional GABA<sub>B</sub> receptors, caused by siRNA knockdown, resulted in a functional disruption of GABA<sub>B</sub> negative coupling to VGCCs. This confirms that Vc1.1, similar to baclofen, acts on N-type VGCCs via the GABA<sub>B</sub> receptor.

Disruption of VGCC current inhibition by GABA<sub>B</sub> receptor knockdown was more pronounced for Vc1.1-mediated N-type VGCC inhibition than for baclofen-mediated inhibition. In R1 + R2 KD neurons, N-type VGCC current inhibition by Vc1.1 was 74.5% (n = 10) less than the current inhibition by Vc1.1 in control neurons, whereas baclofen-mediated current inhibition in R1 + R2 KD neurons decreased by only 42.1% (n = 17), compared with the 17 corresponding control neurons (Table 1). Although the present findings substantiate previous reports that baclofen and Vc1.1 both act via the GABA<sub>B</sub> receptor (15, 16), the greater decrease in Vc1.1-mediated inhibition caused by siRNA-mediated GABA<sub>B</sub> receptor knockdown suggests that the two compounds may act via different downstream signaling cascades.

We also evaluated the activity of α-conotoxins RgIA and AuIB on the GABA<sub>B</sub> receptor in knockdown and control DRG neurons. RgIA (100 nM) reduced peak VGCC current amplitude in control neurons by 16.2 ± 2.3% (n = 5) (Table 1), which is consistent with previous studies showing RgIA activity on nontransfected DRG neurons (14, 15). In contrast, R1 + R2 KD neurons considerably decreased RgIA-mediated inhibition, with a reduction of current amplitude by 4.6 ± 3% (n = 5) (p = 0.015) (Table 1). AuIB (100 nM), an analgesic α-conotoxin active at the α3β4 nAChR (20) and GABA<sub>B</sub> receptor (8), also inhibited VGCC current amplitude in control neurons (19.9 ± 3.8%; n = 3). However, in R1 + R2 KD cells, inhibition of the VGCC current amplitude significantly decreased (7.6 ± 1.1%; n = 3) (p = 0.035). Together, these experiments show that the
GABA<sub>B</sub> Receptor Needed for α-Conotoxin Inhibition of Ca<sub>v</sub>2.2

**FIGURE 4.** Inhibition of N-type calcium channels in response to baclofen and Vc1.1 is lower in DRG neurons transfected with siRNAs that target the GABA<sub>B</sub> receptor. A and B show superimposed traces of depolarization-activated VGCC currents in scrambled, nontargeting siRNA-transfected (A) and GABA<sub>B</sub>R knockdown siRNA-transfected (B) rat DRG neurons, in the absence (control) and presence of Vc1.1 (100 nM) and baclofen (30 μM). C and D, bar graph summary of data on peak VGCC current amplitude reduction in the presence of baclofen (C) and Vc1.1 (D). Vc1.1 inhibition of VGCC in R1+R2 KD neurons was significantly lower than in control neurons. Number of experiments is indicated in parentheses (**, p < 0.01 versus control).

**TABLE 1**
Relative GPCR-mediated decrease in VGCC peak current amplitude in control and R1+R2 KD DRG neurons

| Compound | Inhibition<sup>a</sup> (control) | Inhibition<sup>a</sup> (R1+R2 KD) | % inhibition | % siRNA attenuation<sup>d</sup> |
|----------|---------------------------------|---------------------------------|-------------|---------------------------------|
| Vc1.1    | 20.0 ± 2.6                      | 5.1 ± 3.5                      | 10          | 0.003                           |
| RgIA     | 16.2 ± 2.3                      | 4.6 ± 3.0                      | 5           | 0.015                           |
| Baclofen | 36.6 ± 2.9                      | 21.2 ± 2.9                     | 17          | 0.001                           |
| DAMGO    | 15.7 ± 2.6                      | 22.3 ± 4.3                     | 10          | 0.199                           |

<sup>a</sup>Inhibition of VGCC peak current amplitude following addition of the compound indicated is calculated as 100 − I/I<sub>0</sub> × 100.

<sup>b</sup>n is number of paired observations in control and R1 + R2 KD cells.

<sup>c</sup>p value indicates statistical significance of the difference in VGCC peak current amplitude inhibition in R1 + R2 neurons versus control neurons (% inhibition of control versus inhibition of R1 + R2 KD).

<sup>d</sup>Percentage of reduction of normalized current amplitude inhibition in knockdown cells compared with control cells is calculated as 100 − 100/inhibition-(control) × inhibition(R1 + R2 KD).

activity of these α-conotoxins on N-type VGCCs is dependent on GABA<sub>B</sub> receptor expression.

To show siRNA knockdown of the GABA<sub>B</sub> receptor was specific, control experiments examined the effect of μ-opioid receptor agonists on VGCC currents in transfected DRG neurons. μ-Opioid receptor activation by agonists, such as Met-enkephalin and DAMGO, has been shown to inhibit the N-type VGCC current via activation of a pertussis toxin-sensitive G protein-coupled receptor (GPCR) (25, 26). A recent study reported that different DRG neuron sensitivity to DAMGO corresponded with different DRG neuron soma sizes (27). We also observed a wide variation in individual DRG neuron sensitivity to DAMGO, with some neurons having minimal response because no specific neuron subtype was selected. Control DRG neurons showed 15.7 ± 2.6% (n = 10) inhibition of VGCC current amplitudes by 1 μM DAMGO (n = 10) when VGCC current amplitude was compared before and after adding DAMGO (p = 0.003). The neurons we studied were chosen at random, so any statistical difference in VGCC current inhibition between R1 + R2 KD and control neurons would suggest the knockdown affected μ-opioid receptor signaling. However, we did not observe any statistically significant difference in VGCC current inhibition by 1 μM DAMGO between R1+R2 KD neurons (22.3 ± 4.3%; n = 10) and scrambled, nontargeting siRNA-transfected control neurons (15.7 ± 2.6%; n = 10) (p = 0.199) (Table 1). This confirmed that the impairment of VGCC current inhibition by baclofen, Vc1.1, RgIA, and AuIB in R1+R2 KD neurons was due to decreased GABA<sub>B</sub> receptor expression rather than the nonspecific effects of GABA<sub>B</sub> receptor-targeting siRNAs.

**Reconstitution of the Human GABA<sub>B</sub> Receptor Signaling Pathway in HEK 293 Cells**—To demonstrate that both subunits of the human GABA<sub>B</sub> receptor are needed to inhibit Ca<sub>v</sub>2.2 channels with α-conotoxin Vc1.1, we reconstituted the GABA<sub>B</sub> receptor signaling pathway in HEK 293 cells stably expressing Ca<sub>v</sub>2.2 channels (Ca<sub>v</sub>2.2 cells). This was done by co-transfecting cDNAs of cloned human GABA<sub>B</sub> receptor subunits (R1 and R2) (Fig. 5).

In HEK 293 cells with Ca<sub>v</sub>2.2 alone (control), peak Ba<sup>2+</sup> currents were not affected by α-conotoxin Vc1.1 (200 nm) (n = 10). However, the selective N-type (Ca<sub>v</sub>2.2) VGCC inhibitor,
ω-conotoxin MVIIA (200 nM), almost completely inhibited the inward Ba$^{2+}$ current ($n = 6$) (Fig. 5A). In cells expressing Ca$_{v2.2}$ and co-transfected with GABA$_B$ R1 and R2 (Ca$_{v2.2}$+R1+R2 cells), Vc1.1 and baclofen caused inward Ba$^{2+}$ current inhibition, indicated by the decrease of peak current amplitude during the administration of these compounds (40 ± 7%, $n = 5$ for Vc1.1; 65 ± 5%, $n = 7$ for baclofen) (Fig. 5, B–D). In Ca$_{v2.2}$, Ca$_{v2.2}$+R1 or Ca$_{v2.2}$+R2 cells, we did not observe inhibition of the Ba$^{2+}$ current by Vc1.1 or baclofen ($n = 6$) (Fig. 5D). Similarly, ω-conotoxin Rg1A (200 nM) inhibited inward Ba$^{2+}$ current only in Ca$_{v2.2}$+R1+R2 cells ($20 \pm 7.3\%$; $n = 4$; supplemental Fig. S1). However, the effect of Vc1.1 (200 nM) or baclofen (100 μM) was absent in Ca$_{v2.2}$ cells transiently transfected with the dopamine receptor D2 ($n = 6$; data not shown). In Ca$_{v2.2}$+R1+R2 cells, inhibition of Ca$_{v2.2}$ channels by Vc1.1 could be blocked by the selective GABA$_B$ receptor antagonist CGP55845 (1 μM), whereas baclofen (50 μM), applied after the washout of CGP55845, inhibited the inward Ba$^{2+}$ current ($n = 6$) (Fig. 5C).

In a series of experiments, Ca$^{2+}$ currents were evoked by depolarizing test pulses (50 ms, 0.1 Hz) to +20 mV from a holding potential of −80 mV, with or without a prepulse (20 ms), to +120 mV before the test pulse. Bath application of GABA (1 μM) inhibited Ca$^{2+}$ current amplitude by 64 ± 5% without a prepulse and by 3 ± 5% ($n = 8$) with a prepulse ($p < 0.0001$). Vc1.1 (300 nM) caused either a 50 ± 10% (without a

![Image of Graphs](https://example.com/graphs.png)

**FIGURE 5.** Inhibition of stably expressed Ca$_{v2.2}$ channels in HEK 293 cells transiently co-transfected with human GABA$_B$ receptor subunits R1 + R2 by α-conotoxin Vc1.1 and baclofen. A–C, peak Ba$^{2+}$ current amplitude elicited by step depolarization (0.1 Hz) to −10 mV from a holding potential of −80 mV plotted as a function of time. Bars indicate bath application of various compounds. Superimposed inward Ba$^{2+}$ currents (top insets) are shown at the times marked by lowercase letters (see below). Dotted line indicates zero current level. A, in HEK 293 cells expressing Ca$_{v2.2}$ channels alone, depolarization-activated Ba$^{2+}$ currents are not affected by α-conotoxin Vc1.1 (200 nM) but are almost completely inhibited by ω-conotoxin MVIIA (200 nM). B, in Ca$_{v2.2}$+R1+R2 cells, bath application of Vc1.1 (200 nM) inhibits inward Ba$^{2+}$ current amplitude. C, in Ca$_{v2.2}$+R1+R2 cells, the effect of Vc1.1 was antagonized by bath application of the selective GABA$_B$ receptor antagonist CGP55845 (1 μM), whereas baclofen (50 μM), applied after the washout of CGP55845, inhibited the inward Ba$^{2+}$ current. D, bar graph summary of the inhibition of peak Ba$^{2+}$ current by Vc1.1 (200 nM) and baclofen (50 μM) in the absence of GABA$_B$ receptor subunits (Ca$_{v2.2}$ alone; control) or presence of Ca$_{v2.2}$ channels and R1, R2, or R1+R2. Number of experiments indicated in parentheses (*, $p < 0.0001$ versus control).
proposed for ORL1 receptor activation (28).

transfecting both human GABAB receptor subunits into HEK unchanged compared with cells treated with either 30 to internalization of Cav2.2 channels.

ular signaling cascades, and siRNAs have already been successful because RNAi is a powerful tool for the investigation of molec-

results indicate that the observed N-type VGCC current inhi-

bition by Vc1.1 or baclofen internalizes the Cav2.2 channel and

expression system.

To determine whether prolonged GABAB receptor activation by Vc1.1 or baclofen internalizes the Ca2,2 channel and reduces N-type VGCC current amplitude, we investigated HEK 293 cells stably expressing Ca2,2 using surface biotinylation studies. A similar mechanism of Ca2,2 internalization has been proposed for ORL1 receptor activation (28).

Ca2,2 + R1 + R2 cells were exposed to 1 μM Vc1.1 for 30 min, and cell surface proteins were biotinylated immediately afterward. Vc1.1 did not appear to cause internalization of Ca2,2, with cell surface Ca2,2 protein levels detected by Western blot unchanged compared with cells treated with either 30 μM baclofen or control solution only (n = 4; supplemental Fig. S2). Furthermore, there was no difference in cell surface Ca2,2 protein levels between Ca2,2 + R1 + R2 cells and Ca2,2 cells alone (lacking GABABR) when both were treated with Vc1.1. These results indicate that the observed N-type VGCC current inhibition by α-conotoxin Vc1.1 via the GABAB receptor is not due to internalization of Ca2,2 channels.

DISCUSSION

This study demonstrates that functional GABAB receptors are needed to observe the analgesic α-conotoxins Vc1.1 and RgIA decrease N-type VGCC peak current amplitude. Although previous pharmacological studies showed that Vc1.1 and RgIA can inhibit VGCC currents in rodent DRG neurons via activation of the G protein-coupled GABAB receptor (8, 15, 16), there were no molecular studies showing the need for expression of both GABAB receptor subunits.

We chose a knockdown approach to demonstrate depend-

ence of α-conotoxin activity on GABAB receptor expression, because RNAi is a powerful tool for the investigation of molec-

ular signaling cascades, and siRNAs have already been successful for knockdown of GABAB receptor subunits (29–31) and other target receptors and ion channels in pain pathways (32, 33). In siRNA knockdown experiments, we show that the inhibition of N-type VGCC currents in rat DRG neurons by α-conotoxins decreased following a transient knockdown of GABAB receptors. The effectiveness of the siRNA knockdown was underscored by qRT-PCR and immunocytochemistry, showing a decrease in GABAB receptor mRNA and protein expression, respectively.

Results from the GABAB receptor knockdown study were reinforced by a complementary approach using HEK 293 cells. These cells represent an established expression system for vari-

ous membrane proteins (receptors and ion channels) and have been used to study signaling of heterologously expressed GABA and other G protein-coupled receptors (14, 34). By co-

transfecting both human GABAB receptor subunits into HEK 293 cells stably expressing Ca2,2 channels, we could reconsti-
tute the GABAB receptor signaling pathway. In these cells, baclofen and α-conotoxins Vc1.1 and RgIA decreased Ca2,2-mediated Ba2+ currents. However, cells expressing Ca2,2 channels alone, or only one of the two GABAsubunits, were insensitive to baclofen and Vc1.1. Thus, we have demonstrated that functional GABAB receptors are the phar-

macological target of α-conotoxins Vc1.1 and RgIA. We have also shown that functional GABAB receptor expression is needed for the inhibition of Ca2,2 channels.

Our electrophysiological findings on GABAB receptor knockdown show that in control DRG neurons, the effect of Vc1.1 and RgIA on N-type VGCC currents was substantially less than that of baclofen. Moreover, Vc1.1 and baclofen clearly differed in their susceptibility to GABAB receptor knockdown. Inhibition of N-type VGCC currents in GABAB knockdown DRG neurons by Vc1.1 was almost completely abolished. In contrast, even though the effect of baclofen was significantly impaired, inhibition of VGCC current was still observed. These observations corroborate previous findings, indicating that Vc1.1 and baclofen act on N-type VGCCs via different path-

ways, both involving the GABAB receptor (15). N-type VGCCs can be inhibited by two distinct G protein-dependent inhibi-

tory pathways, i.e. voltage-dependent and voltage-independent (35). Voltage-dependent inhibition is the most common path-

way, involving Gβγ binding directly to the α-subunit of the N-type VGCC (36, 37). The inhibition of N-type VGCCs in DRG neurons by α-conotoxin Vc1.1 is voltage-independent and activates a pathway that involves Src tyrosine kinase (15). In contrast, the GABAB receptor agonist baclofen and its inhibi-

tion of VGCC currents are well characterized (38–40) and appear to inhibit N-type VGCC currents predominantly via the voltage-independent pathway (35). These results suggest that the Vc1.1-mediated voltage-independent pathway is more sensi-
tive to GABAB receptor knockdown (partial knockdown) than the baclofen-mediated voltage-dependent pathway.

One possible way GPCR agonists could reduce VGCC cur-
ent amplitude is through receptor-mediated internalization of the channels. This mechanism has been proposed for inhibition of N-type VGCC currents after activation of the ORL1 receptor (28). However, a recent study in rat DRG neurons found no functional loss of surface N-type calcium channels after pro-

longed ORL1 activation by nociceptin/orphanin FQ (27). Our surface biotinylation studies on HEK 293 cells expressing human Ca2,2 and GABA receptor and exposed to either Vc1.1 or baclofen did not reveal any decrease in cell surface Ca2,2 protein levels. This suggests that Vc1.1 activates a GABA receptor-mediated signaling cascade that inhibits N-type VGCC currents by channel inactivation rather than channel internalization.

Our findings not only strengthen the pharmacological signifi-

ance of the observed N-type VGCC inhibition by α-conoto-
oxins, Vc1.1 and RgIA, but extend them to show that these α-conotoxins are active at human GABA receptors. In vivo studies have shown that Vc1.1, RgIA, and AuIB administered intramuscularly in rats can potently suppress neuropathic pain following partial nerve ligation (6–8). To date, there are at least five α-conotoxins that inhibit N-type VGCCs in DRG neurons, and the three (Vc1.1, RgIA, AuIB) that have been tested in ani-
mal pain models are analgesic. Further studies of the structure-activity relationships are likely to identify the specific residues that confer potency and selectivity for the GABA_B receptor and will improve understanding of how α-conotoxins act on the GABA_B receptor. In the future, other α-conotoxins and analogs activating the GABA_B receptor may be discovered, making the human GABA_B receptor a novel target for drugs used to treat neuropathic pain.

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