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**Novel Mechanism of Voltage-Gated N-type (Ca<sub>v</sub>2.2) Calcium Channel Inhibition  
Revealed Through  $\alpha$ -Conotoxin Vc1.1 Activation of the GABA<sub>B</sub> Receptor**

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**Running Title:** Mechanism of Ca<sub>v</sub>2.2 inhibition revealed by  $\alpha$ -conotoxin Vc1.1

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**List of non-standard abbreviations:**

DRG, dorsal root ganglion

GABA<sub>B</sub>R,  $\gamma$ -aminobutyric acid type B receptor

GIRK channel, G protein-gated inwardly rectifying potassium channel

GPCR, G protein-coupled receptor

HVA, high voltage-activated

IC<sub>50</sub>, half-maximal inhibitory concentration

PCT, proximal carboxyl terminus

PTX, pertussis toxin

SSI, steady-state inactivation

V<sub>0.5, act</sub>, half-maximal activation voltage

V<sub>0.5, inact</sub>, half-maximal inactivation voltage

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## Abstract

Neuronal voltage-gated N-type ( $\text{Ca}_v2.2$ ) calcium channels are expressed throughout the nervous system and regulate neurotransmitter release and hence synaptic transmission. They are predominantly modulated via G protein-coupled receptor activated pathways, and the well-characterized  $\text{G}\beta\gamma$  subunits inhibit  $\text{Ca}_v2.2$  currents. Analgesic  $\alpha$ -conotoxin Vc1.1, a peptide from predatory marine cone snail venom, inhibits  $\text{Ca}_v2.2$  channels by activating pertussis toxin (PTX)-sensitive  $\text{G}_{i/o}$  proteins via the  $\text{GABA}_B$  receptor ( $\text{GABA}_B\text{R}$ ) and potently suppresses pain in rat models. Using a heterologous  $\text{GABA}_B\text{R}$  expression system, electrophysiology and mutagenesis, we showed  $\alpha$ -conotoxin Vc1.1 modulates  $\text{Ca}_v2.2$  via a different pathway from that of the  $\text{GABA}_B\text{R}$  agonists GABA and baclofen. In contrast to GABA and baclofen, Vc1.1 changes  $\text{Ca}_v2.2$  channel kinetics by increasing the rate of activation and shifting its half-maximum inactivation to a more hyperpolarized potential. We then systematically truncated the  $\text{GABA}_{B1a}$  C-terminus and discovered that by removing the proximal carboxyl terminus (PCT) of the  $\text{GABA}_{B1a}$  subunit, Vc1.1 inhibition of  $\text{Ca}_v2.2$  currents was significantly reduced. We propose a novel mechanism by which Vc1.1 activates  $\text{GABA}_B\text{R}$  and requires the  $\text{GABA}_{B1a}$  PCT domain to inhibit  $\text{Ca}_v2.2$  channels. These findings provide important insights into how  $\text{GABA}_B\text{Rs}$  influence  $\text{Ca}_v2.2$  channel inhibition and nociceptive transmission.

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## Introduction

The G protein-coupled GABA<sub>B</sub> receptor (GABA<sub>B</sub>R) is widely expressed in the brain and plays an important role in regulating neurotransmission. The neurotransmitter  $\gamma$ -aminobutyric acid (GABA) binds to GABA<sub>B</sub>Rs leading to activation of G<sub>i/o</sub> G proteins, which dissociate into activated G $\alpha_{i/o}$ -GTP and the G $\beta\gamma$  dimer. G $\alpha_{i/o}$  inhibits adenylyl cyclase while the G $\beta\gamma$  dimer inhibits high voltage-activated (HVA) calcium channels (Menon-Johansson et al., 1993; Ikeda, 1996; Sun and Chiu, 1999) and activates G protein-gated inwardly rectifying potassium (GIRK) channels (Luscher and Slesinger, 2010). The GABA<sub>B</sub>R is a potential target for drugs that treat neurological illnesses such as pain, epilepsy, spasticity and psychiatric disorders (Marshall et al., 1999; Bowery et al., 2002). For example, inhibition of presynaptic HVA calcium channels such as Ca<sub>v</sub>2.2 has been linked to suppression of neurotransmitter release and block of nociception transmission (Winqvist et al., 2005; Gribkoff, 2006).

A 16 amino acid peptide derived from venom of the marine cone snail *Conus victoriae*,  $\alpha$ -conotoxin Vc1.1 (Clark et al., 2006), potently suppresses neuropathic pain when injected intramuscularly near the site of injury in rat models (Sandall et al., 2003; Satkunanathan et al., 2005; Klimis et al., 2011). Initially, the analgesic effect was attributed to inhibition of neuronal nicotinic acetylcholine receptors (Satkunanathan et al., 2005; Livett et al., 2006; Vincler et al., 2006). However, subsequent studies using rodent dorsal root ganglion (DRG) neurons demonstrated that the analgesic effect of Vc1.1 is more likely due to inhibition of Ca<sub>v</sub>2.2 channels via GABA<sub>B</sub>R activation (Callaghan et al., 2008; Callaghan and Adams, 2010). In support of this, siRNA knockdown of GABA<sub>B</sub>R expression in DRG neurons significantly reduced Vc1.1-dependent inhibition of Ca<sub>v</sub>2.2 (Cuny et al., 2012). In addition, Vc1.1 inhibition of Ca<sub>v</sub>2.2 can be reconstituted in HEK293 cells co-expressing only GABA<sub>B</sub>Rs and Ca<sub>v</sub>2.2 channels (Cuny et al., 2012). Thus, Vc1.1 inhibition minimally requires both GABA<sub>B</sub>R subunits, G proteins and Ca<sub>v</sub>2.2 channels. We have recently shown

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that Vc1.1 also inhibits Ca<sub>v</sub>2.3 but not Ca<sub>v</sub>2.1 channels via GABA<sub>B</sub>R activation in HEK293 cells (Berecki et al., 2014). However, little is known about how Vc1.1 activates GABA<sub>B</sub>Rs.

Here, we examined the mechanism of Vc1.1 inhibition in a heterologous expression system that reconstitutes the human Ca<sub>v</sub>2.2/GABA<sub>B</sub>R signaling pathway (Cuny et al., 2012). We provide further details of the mechanism by which Vc1.1 inhibits Ca<sub>v</sub>2.2 channels via activation of GABA<sub>B</sub>R. We discovered that Vc1.1 activation of GABA<sub>B</sub>Rs occurs via a signaling pathway distinct from that of classical agonists, such as GABA and baclofen. Our findings support an emerging model of signaling bias, which results from allosteric ligand binding to G protein-coupled receptors (GPCRs) at sites different to endogenous ligand binding (Wootten et al., 2013). Numerous studies have shown that agonists do not uniformly activate cellular signaling pathways (biased signaling) and GPCRs produce multiple effector pathways due to their association with secondary messengers, such as G proteins and β-arrestin (Bohn and McDonald, 2010; Rajagopal et al., 2011).

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## Materials and Methods

### *Clones, cell culture and transfections*

Human GABA<sub>B1</sub> and GABA<sub>B2</sub> subunit cDNA clones were obtained from OriGene Technologies, Inc., Rockville, MD. Rat Ca<sub>v</sub>2.2 channel,  $\alpha_{1B}$  splice variant e37a and auxiliary subunit  $\beta_3$  were provided by Dr Diane Lipscombe (Brown University), and the auxiliary subunit  $\alpha_2\delta_1$  by Dr Gerald W. Zamponi (University of Calgary). Rat wild-type GABA<sub>B</sub>R and the mutant GABA<sub>B1a</sub>- $\Delta$ PCT, GABA<sub>B1a</sub>- $\Delta$ 887, GABA<sub>B1a</sub>- $\Delta$ 863 (Laviv et al., 2011) and GABA<sub>B2</sub>-R576D (Binet et al., 2007; Boyer et al., 2009) constructs have been described previously. Site-directed mutagenesis on GABA<sub>B1a</sub> was carried out using the GENEART® Site-Directed Mutagenesis System Kit (Invitrogen Life Technologies) with the following primers: 5'-TCTCATATGGCTCCAGTGCACCAGCCTTGTCAAACCG-3' and 5'-CGGTTTGACAAGGCTGGTGCACCTGGAGCCATATGAGA-3' for rat GABA<sub>B1a</sub>-S270A; 5'-CCTTATGCCTGGCTGCGCCTCTGTCTCCACGCTGG-3' and 5'-CCAGCGTGGAGACAGAGGCGCAGCCAGGCATAAGG-3' for human GABA<sub>B1a</sub>-S246A. Correct mutation of the cDNAs was verified by automated DNA sequencing (Australian Genome Research Facility, Melbourne, Australia).

Human Embryonic Kidney 293 cells expressing the SV40 large T-antigen (HEK293T) were cultured in Dulbecco's Modified Eagle's Medium (Invitrogen Australia Pty Ltd., Mt. Waverley, Victoria, Australia) supplemented with 10% (v/v) fetal bovine serum (Invitrogen, Australia), 50 IU/ml penicillin and 50  $\mu$ g/ml streptomycin (Invitrogen, Australia) at 37°C in 5% CO<sub>2</sub>. HEK293 cells stably expressing human Ca<sub>v</sub>2.2 channels ( $\alpha_{1B}$ ,  $\alpha_2\delta_1$  and  $\beta_3$  subunits) were obtained from Merck and cultured according to procedures described previously (Dai et al., 2008).

HEK293T cells were transiently co-transfected by calcium phosphate transfection, as described previously (Cuny et al., 2012), with plasmid cDNAs (3  $\mu$ g) encoding the rat Ca<sub>v</sub>2.2

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channel  $\alpha_{1B}$ ,  $\alpha_{2\delta 1}$  and  $\beta_{1b}$  auxiliary subunits, 5  $\mu\text{g}$  rat wild-type or mutant  $\text{GABA}_{B1aR}$ ,  $\text{GABA}_{B2R}$  and 0.1  $\mu\text{g}$  eGFP. Stable  $\text{Ca}_v2.2$  expressing HEK293 cells were seeded into 12-well plates and transiently co-transfected with human  $\text{GABA}_{B1}$  and  $\text{GABA}_{B2}$  subunits (2  $\mu\text{g}$  each) and 0.2  $\mu\text{g}$  eGFP using Lipofectamine (Invitrogen) according to the manufacturer's protocol.

Two to three days after transfection cells were seeded onto glass coverslips and incubated at 37°C in 5%  $\text{CO}_2$  for at least 6 hours before recording. Effects of Vc1.1 or baclofen on the percentage of peak current inhibition were independent of either transfection method or cell line (transiently or stably expressed  $\text{Ca}_v2.2$ ) used.

### ***Electrophysiological patch clamp recording***

Whole-cell patch clamp recording from transfected HEK293 cells has been described previously (Cuny et al., 2012). Briefly, HEK293 cells were superfused with a solution containing (in mM): NaCl 90,  $\text{BaCl}_2$  10, CsCl 5, TEA-Cl 30,  $\text{MgCl}_2$  1, D-glucose 10 and HEPES 10, pH 7.4 with TEA-OH. Fire-polished borosilicate patch pipettes (2–3 M $\Omega$  tip resistance) were filled with a solution containing (in mM): K-gluconate 120, NaCl 5,  $\text{MgCl}_2$  2, EGTA 5, MgATP 2,  $\text{Na}_2\text{GTP}$  0.6 and HEPES 10, pH 7.2 with CsOH. Typically, cells were voltage-clamped at –90 mV (adjusted for liquid junction potential) and membrane currents elicited by 200 ms step depolarizations to either +5 mV or +10 mV at 0.1 or 0.05 Hz. Whole-cell patch clamp recordings were made using a MultiClamp 700B amplifier (Molecular Devices, Sunnyvale, CA), controlled by a Clampex 10/ DigiData1332 acquisition system at room temperature (22–23°C). Membrane currents were filtered at 1 or 2 kHz and sampled at 5 or 8 kHz. Leak and capacitive currents were subtracted using a –P/4 pulse protocol. Data were stored digitally on a computer for further analysis. Peak current amplitude in response to the depolarizing pulse was measured once a steady state was achieved (3–5 min).

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### ***Data analysis and statistics***

Data were analyzed using Clampfit 10 software (Molecular Devices), 2007 Excel (Microsoft) and Prism 6 (GraphPad Software Inc., La Jolla, CA). Peak current amplitude in response to a depolarization step was calculated and plotted against time. A steady-state peak current was recorded for at least 2–3 min before the drug was applied.

Activation curves were plotted from the I–V relationship by calculating conductance using the equation:

$$G = I/(V_t - E_{rev})$$

where  $G$  is the conductance,  $V_t$  the test potential, and  $E_{rev}$  the extrapolated reversal potential.

The voltage dependence of the peak  $Ca_v2.2$  current density was fitted with the Boltzmann function:

$$I = G_{max}(V_t - E_{rev})/(1 + \exp[(V_{0.5} - V_t)/k])$$

where  $G_{max}$  is the maximum conductance,  $V_{0.5}$  the midpoint or half-maximal activation, and  $k$  the slope factor.

For steady-state inactivation, data was fitted with a single Boltzmann function:

$$I = 1/[1 + \exp((V_{0.5, inact} - V_t)/k)]$$

where  $V_{0.5, inact}$  is the midpoint or half-maximal inactivation, and  $k$  the slope factor.

Using Clampfit 10 (Molecular Devices), activation kinetics were determined from a single exponential fit of the current activating phase. Concentration–response relationships were obtained by plotting normalized current amplitude as a function of drug concentration, and fitted using a logistic equation. All voltage potentials were adjusted for liquid junction potential. Unless stated otherwise, all data are represented as mean  $\pm$  SEM. The statistical significance between two groups was evaluated by student's  $t$ -test. One-way analysis of

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variance (ANOVA) followed by Bonferroni's post hoc test was used to compare three or more groups. Differences were considered statistically significant if  $P < 0.05$ .

### ***Chemicals and drugs***

$\alpha$ -Conotoxin Vc1.1 was synthesized as described previously (Schnolzer et al., 1992; Luo et al., 1998; Clark et al., 2006; Clark et al., 2008). It was provided as a stock concentration of ~1 mM in H<sub>2</sub>O by Dr Ray Norton (Monash University, Melbourne). Baclofen, GABA and pertussis toxin (PTX) were purchased from Sigma-Aldrich and CGP54626 [*S*-(*R*\*,*R*\*)][3-[[1-(3,4-dichlorophenyl)ethyl]amino]-2-hydroxypropyl]([3,4]-cyclohexylmethyl) phosphinic acid hydrochloride was purchased from Tocris Bioscience (Bristol, UK). All drugs were diluted to the appropriate final concentration and applied via perfusion in the bath solution. Vc1.1 was applied at a concentration of 200 nM for maximum response. As a positive control for functional expression of GABA<sub>B</sub>R with Ca<sub>v</sub>2.2 channels, baclofen was applied after Vc1.1 application. Only cells responding to baclofen with at least 50% peak current inhibition were included in our analysis.

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## Results

### *Comparison of Vc1.1-dependent inhibition with canonical GABA-dependent inhibition of Ca<sub>v</sub>2.2 channels*

Confirming previous reports (Cuny et al., 2012), bath application of Vc1.1 (200 nM) reduced peak Ba<sup>2+</sup> current (I<sub>Ba</sub>) amplitude by  $45 \pm 7\%$  ( $n = 9$ ) in HEK293 cells stably expressing human Ca<sub>v</sub>2.2 ( $\alpha_{1B}$ ,  $\beta_{1b}$  and  $\alpha_2\delta$ ) and co-expressing human GABA<sub>B1a/B2</sub> receptors (Fig. 1A,B). The concentration-response relationship for Vc1.1 inhibition of Ca<sub>v</sub>2.2 has an IC<sub>50</sub> of 19.1 nM, which is approximately ten-fold higher than that reported for rat DRG neurons (Callaghan et al., 2008) (Fig. 1D). This shift most likely reflects differences between human and rat GABA<sub>B</sub>R. However, typically 200 nM Vc1.1 was used as it gave maximal response with either rat or human GABA<sub>B</sub>R. Inhibition of I<sub>Ba</sub> by baclofen was maximal in < 1 min and rapidly reversed upon washout. By contrast Vc1.1 inhibition was slower, reaching steady-state after ~15 min. Furthermore, Vc1.1 inhibition of I<sub>Ba</sub> was not reversed following washout of Vc1.1 peptide but the amplitude was subsequently further reduced by application of baclofen (Fig. 1B,C). Taken together, these results suggest Vc1.1 inhibition of Ca<sub>v</sub>2.2 channels occurs via a signaling pathway different from GABA/baclofen-mediated inhibition.

Baclofen and GABA both bind to the extracellular Venus Fly Trap of the GABA<sub>B1</sub> subunit (Galvez et al., 1999; Galvez et al., 2000). Therefore we introduced an alanine substitution at serine 270 of the GABA<sub>B1a</sub> subunit to test whether Vc1.1 interacts with this binding site. S270A was shown previously to substantially reduce agonist and antagonist binding affinities to the rat GABA<sub>B</sub>R (Galvez et al., 1999). As expected, S270A significantly reduced baclofen inhibition of Ca<sub>v</sub>2.2-mediated currents ( $14.7 \pm 3.0\%$ ,  $n = 8$ ) compared with wild-type GABA<sub>B</sub>R ( $43.3 \pm 8.6\%$ ,  $n = 6$ ;  $P < 0.05$ ). However, the S270A mutation in GABA<sub>B1a</sub> did not significantly alter Vc1.1-dependent inhibition of Ca<sub>v</sub>2.2 currents ( $47.1 \pm 14.4\%$ ,  $n = 7$ ), and was similar to wild-type GABA<sub>B</sub>R ( $52.3 \pm 8.6\%$ ,  $n = 6$ ;  $P = 0.75$ ) (Fig. 2).

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We therefore tested another mutation, S246A, in the Venus Flytrap domain of GABA<sub>B1a</sub>, which causes complete insensitivity to baclofen and GABA (Galvez et al., 2000). Expression of GABA<sub>B1a</sub>-S246A with GABA<sub>B2</sub> abolished baclofen-dependent inhibition of Ca<sub>v</sub>2.2 currents ( $2.4 \pm 4.8\%$ ,  $n = 6$ ), as compared with wild type GABA<sub>B</sub>R ( $76.9 \pm 12.5\%$ ,  $n = 5$ ). In contrast, Vc1.1 continued to inhibit Ca<sub>v</sub>2.2 currents in cells co-expressing GABA<sub>B1a</sub>-S246A/GABA<sub>B2</sub> ( $26.6 \pm 7.9\%$ ,  $n = 6$ ). Inhibition was to a similar extent as that observed at wild type GABA<sub>B</sub>R ( $30.4 \pm 1.8\%$ ,  $n = 4$ ), supporting our findings obtained with the S270A mutant. Vc1.1 inhibition of Ca<sub>v</sub>2.2, however, was strictly dependent on co-expression with the GABA<sub>B</sub>R subunits (Fig. 1C) and consistent with previous results (Cuny et al., 2012). These findings raise the possibility that Vc1.1 interacts with a different region of the GABA<sub>B</sub>R. In rat DRG neurons, the selective GABA<sub>B</sub>R antagonists CGP54626 and CGP55845 blocked Vc1.1 inhibition of Ca<sub>v</sub>2.2 currents (Callaghan et al., 2008), suggesting that these receptor antagonists interfere with Vc1.1 binding. In HEK293 cells expressing GABA<sub>B1</sub>-S270A, CGP54626 was inactive and unable to antagonize Vc1.1 inhibition of Ca<sub>v</sub>2.2 ( $n = 3$ ; data not shown). This is consistent with previous studies showing the S270A mutation prevents binding of CGP54626 to GABA<sub>B</sub>R (Galvez et al., 1999; Geng et al., 2013) and suggests that CGP54626 and CGP55845 occlude Vc1.1 binding to a site physically distinct from S270 and S246 of GABA<sub>B1</sub>.

GABA<sub>B</sub>R-dependent inhibition of voltage-gated calcium channels has been examined extensively (Tedford and Zamponi, 2006). This form of inhibition typically involves Gβγ subunits binding to Ca<sub>v</sub> channels, shifting the voltage-dependence of activation and slowing the activation kinetics. In addition, prepulse potentiation can antagonize Gβγ-dependent inhibition of Ca<sub>v</sub> channels (Ikeda, 1996; Weiss et al., 2007). We next examined if these features were also observed with Vc1.1-dependent inhibition. The voltage-dependence and kinetics of activation/inactivation of Ca<sub>v</sub>2.2 channels were analyzed in the presence of Vc1.1

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or baclofen. Similar to previous studies (Jones et al., 1997; Colecraft et al., 2000), baclofen shifted the half-maximum activation ( $V_{0.5, \text{act}}$ ) of the conductance-voltage relationship by +10.0 mV ( $+3.7 \pm 1.5$  mV,  $n = 7$ ) as compared to that of control ( $-6.3 \pm 0.7$  mV,  $n = 8$ ) (Fig 3A and Table 1). By contrast, Vc1.1 did not alter the I–V relationship or voltage-dependence of activation with  $V_{0.5, \text{act}}$  of  $-5.1 \pm 0.9$  mV ( $n = 6$ ) (Fig. 3A and Table 1). We also analyzed the activation curves generated from  $\text{Ca}_v2.2$  tail currents in the presence of baclofen or Vc1.1 (Fig. 3B). The normalized peak tail current amplitudes were fitted with the sum of two Boltzmann functions (Lin et al., 2004; Castiglioni et al., 2006). In the presence of baclofen,  $\text{Ca}_v2.2$  channels were activated at significantly more depolarized voltages than in control conditions. Baclofen, but not Vc1.1, caused a shift in both the  $V_{0.5}$  and  $k$  of each component of the Boltzmann fit (Table 1 and Fig. 3B).

We next examined whether Vc1.1 inhibition altered the voltage-dependence of steady-state inactivation (SSI) of  $\text{Ca}_v2.2$ . We used a 10 s prepulse at varying potentials to evaluate SSI. This prepulse duration was sufficient to allow complete recovery from inactivation. The inactivation curve obtained in the presence of Vc1.1 gave a half-maximal inactivation ( $V_{0.5, \text{inact}}$ ) of  $-78.8 \pm 0.5$  mV ( $n = 10$ ), ~13 mV more hyperpolarized than that of control ( $-65.5 \pm 0.6$  mV,  $n = 8$ ;  $P < 0.05$ ) (Table 1 and Fig. 3C). Because of this shift in voltage, we examined the extent of Vc1.1-dependent inhibition produced by the shift in SSI. Therefore, we investigated SSI in individual HEK293 cells with pre-pulses of  $-110$ ,  $-80$  and  $-50$  mV in the absence and presence of Vc1.1. Vc1.1 significantly reduced P2/P1 at both  $-50$  mV and  $-110$  mV ( $P < 0.01$ , paired  $t$ -test) (Fig. 3D). The shift in voltage-dependence of inactivation over a wide range of pre-pulse potentials may account, in part, for Vc1.1 inhibition of  $\text{Ca}_v2.2$  channels. Thus, Vc1.1 significantly affected SSI by shifting the inactivation curve to more hyperpolarized potentials.

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We next examined the kinetics of activation after Vc1.1 inhibition of Ca<sub>v</sub>2.2 channels. We measured the time constant of activation ( $\tau$ ) of Ca<sub>v</sub>2.2 current in the absence (control) and then presence of baclofen or Vc1.1 (Fig. 4A,B). Baclofen (50  $\mu$ M) significantly increased  $\tau$  from  $1.63 \pm 0.18$  ms to  $2.13 \pm 0.36$  ms ( $n = 7$ ;  $P = 0.022$ , paired  $t$ -test) (Fig. 4C). These values were comparable to those in previous reports (Bean, 1989; Toselli and Taglietti, 1993). Studies using prepulse facilitation to examine the involvement of G $\beta\gamma$  in Ca<sub>v</sub>2 modulation also reported similar changes in kinetics of activation (Doupnik and Pun, 1994; Ikeda, 1996). In contrast, Vc1.1 (100 nM), significantly reduced  $\tau$  from  $1.69 \pm 0.25$  ms to  $1.26 \pm 0.23$  ms ( $n = 8$ ;  $P < 0.05$ ). Thus, these results show that Vc1.1 inhibition produces the opposite effect on the kinetics of Ca<sub>v</sub>2.2 activation; baclofen slows whereas Vc1.1 speeds activation.

Previous studies suggested that binding of G $\beta\gamma$  to the pore-forming Ca<sub>v</sub> $\alpha$  subunit and the subsequent slowing of activation are reversed by a strong depolarizing prepulse (Bean, 1989; Ikeda, 1996; Furukawa et al., 1998; Zamponi and Currie, 2012). We therefore compared the effect of prepulse facilitation on GABA- and Vc1.1-dependent inhibition of Ca<sub>v</sub>2.2 channels. In the absence of agonist, a prepulse ( $pp$ ) to +120 mV led to an increase in the current amplitude evoked by the test pulse ( $20.9 \pm 1.9\%$ ,  $n = 6$ ), compared with the current amplitude recorded in the absence of a prepulse. This observation suggests some basal inhibition of Ca<sub>v</sub>2.2 occurs in HEK293 cells similar to previous reports of G $\beta\gamma$  modulation of Ca<sub>v</sub>2.2 in the absence of GPCR activation (Meza and Adams, 1998; McDavid and Currie, 2006). As expected, the prepulse to +120 mV significantly relieved GABA-dependent inhibition of peak current from  $64 \pm 4\%$  [ $-pp$ ] to  $4 \pm 5\%$  [ $+pp$ ] ( $n = 8$ ;  $P < 0.05$ ) (Fig. 5B). Remarkably, a prepulse to +120 mV did not significantly relieve Vc1.1 inhibition, which was  $50 \pm 11\%$  [ $-pp$ ] compared with  $36 \pm 6\%$  [ $+pp$ ] ( $n = 5$ ;  $P = 0.283$ ) (Fig. 5D). Taken together, these experiments demonstrate Vc1.1 inhibition differs from that of

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GABA/baclofen modulation of Ca<sub>v</sub>2.2 channels in voltage-dependence of activation and inactivation, time course of activation, and sensitivity to recovery from prepulse potentiation.

We therefore suggest that Vc1.1 inhibits Ca<sub>v</sub>2.2 channels via a novel mechanism that does not involve Gβγ G proteins.

### ***Unique role for G<sub>i/o</sub> G proteins in Vc1.1 inhibition of Ca<sub>v</sub>2.2 channels***

To elucidate the signaling pathway(s) underlying Vc1.1-dependent inhibition of Ca<sub>v</sub>2.2 channels, we first explored the role of PTX-sensitive G<sub>i/o</sub> G proteins. G<sub>i/o</sub> G proteins are essential components of GABA inhibition of Ca<sub>v</sub>2.2 channels via GABA<sub>B</sub>Rs (Tedford and Zamponi, 2006; Katada, 2012). PTX ADP-ribosylates the Gα<sub>i/o</sub> subunit, uncoupling it from the receptor (Katada, 2012). To examine the potential role of G<sub>i/o</sub> G proteins in Vc1.1-dependent inhibition of Ca<sub>v</sub>2.2, we treated HEK293 cells co-expressing human GABA<sub>B</sub>Rs and Ca<sub>v</sub>2.2 channels with PTX (3 μg/ml) for 16 hours. As expected, PTX almost completely prevented baclofen-dependent inhibition of Ca<sub>v</sub>2.2 currents (5.0 ± 2.3%, *n* = 4), as compared to untreated controls (70.7 ± 2.3%, *n* = 11; *P* < 0.05, unpaired *t*-test). Similarly, PTX attenuated Vc1.1 (200 nM) inhibition of peak Ca<sub>v</sub>2.2 currents (0.9 ± 3.5%, *n* = 6) compared with control (42.0 ± 5.7%, *n* = 7; *P* < 0.05, unpaired *t*-test) (Fig. 6). Therefore, it appears Vc1.1 inhibition of Ca<sub>v</sub>2.2 channels requires G<sub>i/o</sub> G protein coupling with GABA<sub>B</sub>Rs (see also Callaghan et al., 2008).

We next examined whether activating Gα<sub>i/o</sub> through the GABA<sub>B</sub>R is also needed for Vc1.1-dependent inhibition of Ca<sub>v</sub>2.2 channels. Previously, Binet et al. (2007) demonstrated that an arginine to aspartate mutation in the third transmembrane segment (TM3) of GABA<sub>B2</sub> dramatically reduces G protein activation. Consistent with such an effect, baclofen inhibition of peak current amplitude in HEK293 cells co-expressing GABA<sub>B2</sub>-R576D/GABA<sub>B1a</sub> and Ca<sub>v</sub>2.2 channels was decreased to 11.2 ± 4.3% (*n* = 7), compared to control peak current

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amplitude of  $64.7 \pm 4.0\%$  ( $n = 6$ ;  $P < 0.05$ ). Surprisingly, Vc1.1-dependent inhibition persisted in the presence of GABA<sub>B2</sub>-R576D. Here, Vc1.1 decreased peak current amplitude by  $35.6 \pm 8.8\%$  ( $n = 8$ ) and this did not differ significantly from that of wild-type GABA<sub>B</sub>R ( $52.9 \pm 8.9\%$ ,  $n = 6$ ;  $P = 0.204$ ) (Fig. 7). Thus, Vc1.1 inhibition of Ca<sub>v</sub>2.2 channel current involves activation of GABA<sub>B</sub>Rs through a novel mechanism that requires G $\alpha_{i/o}$  G proteins (i.e., PTX-sensitive) but not the canonical pathway for stimulating G $\alpha_{i/o}$ .

### ***Important role for the C-terminal domain of the GABA<sub>B1a</sub> subunit in Vc1.1 inhibition of Ca<sub>v</sub>2.2 channels***

The observation that Vc1.1 inhibition occurred even with the R576D mutation in GABA<sub>B2</sub>, shifted our focus to other regions of the GABA<sub>B</sub>R. The C-terminal domain of the GABA<sub>B1a</sub> subunit contains an endoplasmic reticulum (ER) retention signal, a coiled-coil domain for binding with GABA<sub>B2</sub> and shielding the ER retention signal, and a putative domain in the proximal C-terminus (PCT) that interacts with Ca<sub>v</sub> channels (Laviv et al., 2011). To examine the role of the coiled-coil domain in Vc1.1 inhibition, we truncated the C-terminus of the GABA<sub>B1a</sub> receptor subunit from S887 (GABA<sub>B1a</sub>- $\Delta$ 887). Compared to wild-type GABA<sub>B</sub>R, co-expression of GABA<sub>B1a</sub>- $\Delta$ 887 with GABA<sub>B2</sub> and Ca<sub>v</sub>2.2 channels did not significantly alter Vc1.1-dependent inhibition of peak current ( $37.9 \pm 5.6\%$ ,  $n = 11$  compared to  $24.9 \pm 6.9\%$ ,  $n = 7$ ;  $P = 0.171$ , unpaired *t*-test). Similarly, baclofen inhibition of peak current was not affected by the GABA<sub>B1a</sub>  $\Delta$ 887 deletion ( $62.0 \pm 7.3\%$ ,  $n = 7$  for  $\Delta$ 877 compared with control  $57.5 \pm 5.2\%$ ,  $n = 11$ ;  $P = 0.613$ ) (Fig. 8B,E). Truncating the GABA<sub>B1a</sub> C-terminal domain at G863 ( $\Delta$ 863), which removes the PCT as well as the coiled-coil domain, significantly reduced Vc1.1 inhibition of peak current ( $9.5 \pm 4.0\%$ ,  $n = 12$  for  $\Delta$ 863 compared with control  $37.9 \pm 5.6\%$ ,  $n = 11$ ;  $P < 0.05$ ) (Fig. 8C,E). However, baclofen-dependent inhibition was unaffected by the truncation at G863 (Fig. 8C,E).

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To further explore the role of the PCT, we examined the effect of specifically deleting 21 amino acids within this domain (R857 – S877;  $\Delta$ PCT) on Vc1.1 inhibition. In HEK293 cells co-expressing  $\text{Ca}_v2.2$  and  $\text{GABA}_{\text{B}1\text{a}}\text{-}\Delta\text{PCT}/\text{GABA}_{\text{B}2}\text{-wt}$ , Vc1.1 inhibition of peak current was significantly less ( $5.3 \pm 3.9\%$ ,  $n = 5$ ) than that of cells co-expressing wild-type  $\text{GABA}_{\text{B}}\text{R}$  ( $37.9 \pm 5.7\%$ ;  $n = 11$ ;  $P < 0.05$ ). In contrast, baclofen inhibition of peak current in cells expressing the  $\text{GABA}_{\text{B}1\text{a}}\text{-}\Delta\text{PCT}$  mutant was unchanged ( $60 \pm 2.7\%$ ,  $n = 5$  for  $\Delta\text{PCT}$  compared with  $57.5 \pm 5.2\%$  for control,  $n = 11$ ;  $P > 0.05$ ) (Fig. 8D,E). These results suggest that the proximal C-terminal domain of  $\text{GABA}_{\text{B}1\text{a}}$  is involved in Vc1.1-dependent inhibition of  $\text{Ca}_v2.2$  channels.

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## Discussion

### *$\alpha$ -Conotoxin Vc1.1 and GABA/baclofen differentially inhibit Ca<sub>v</sub>2.2*

The GABA<sub>B</sub>R agonists, GABA and baclofen, are known to bind to the GABA<sub>B1</sub> Venus Fly Trap, activating the heterodimer of GABA<sub>B1a/B2</sub> and inhibiting Ca<sub>v</sub>2.2 channels via a pathway involving G $\beta\gamma$  subunits (Galvez et al., 1999). Binding of G $\beta\gamma$  to the Ca<sub>v</sub> $\alpha_{1B}$  pore shifts the channels from 'willing' to 'reluctant' gating states, thus changing the channel's inactivation to a hyperpolarized potential and slowing the rate of activation to reduce peak current (Ikeda, 1996). Prepulse potentiation can recover the inhibition produced by G $\beta\gamma$  subunits (Zamponi and Currie, 2013). Using an established HEK293 system that co-expresses GABA<sub>B</sub>R and Ca<sub>v</sub>2.2 channels (Cuny et al., 2012), we discovered the mode of Vc1.1 inhibition is distinctly different from that of GABA/baclofen inhibition of Ca<sub>v</sub>2.2 channels.

First, GABA and baclofen inhibition of Ca<sub>v</sub>2.2 via GABA<sub>B</sub>R activation is predominantly voltage-dependent (Ikeda, 1996); that is, activation is slowed and shifted to more depolarized potentials. In comparison, Vc1.1 inhibition of Ca<sub>v</sub>2.2 channels does not shift the voltage-dependence of activation, regardless of whether or not G $\beta\gamma$  is activated, and the kinetics of activation are accelerated following Vc1.1 inhibition. Second, Vc1.1 inhibition of Ca<sub>v</sub>2.2 via GABA<sub>B</sub>R activation cannot be reversed by peptide washout or strong depolarization. These findings indicate that Vc1.1 inhibition of Ca<sub>v</sub>2.2 channels does not occur via the traditional pathway of activated G $\beta\gamma$  voltage-dependent inhibition.

The slow onset and recovery of Vc1.1 inhibition of Ca<sub>v</sub>2.2-mediated currents are suggestive of a phosphorylation-dependent mechanism, similar to that of tyrosine kinase-dependent G $\beta\gamma$ -independent inhibition of Ca<sub>v</sub>2.2-e37a (Gray et al., 2007; Raingo et al., 2007). The Ca<sub>v</sub>2.2-e37a splice variant is predominantly expressed in nociceptive DRG neurons where it is implicated in nociception transmission. The more prevalent Ca<sub>v</sub>2.2-e37b variant is widely distributed in the central nervous system (Altier et al., 2007). Interestingly, the one

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known underlying cause of decreased morphine efficacy is thought to be due to altered  $Ca_v2.2$ -e37a to  $Ca_v2.2$ -e37b ratios in DRG neurons (Andrade et al., 2010). However, Vc1.1 affects both  $Ca_v2.2$  splice variants as inhibition was observed in both the stable  $Ca_v2.2$  cell line (expressing e37b) and in cells transiently co-transfected with  $Ca_v2.2$ -e37a. This rules out a role for tyrosine kinase phosphorylation at exon 37a in Vc1.1 inhibition. Another potential mechanism of action is  $GABA_B$ R-activated internalisation of the  $GABA_B$ R- $Ca_v2.2$  complex. However, it was recently shown that  $\beta$ -arrestin, which is important for receptor internalisation, is not bound to  $GABA_B$ R (Sudo et al., 2012). Furthermore, surface biotinylation showed that  $GABA_B$ R cell surface expression was not affected by exposure to Vc1.1 (Cuny et al., 2012).

However, one common feature of GABA/baclofen- and Vc1.1-dependent inhibition is the requirement for  $G_{i/o}$  G proteins. Pre-treatment with PTX abolished Vc1.1 inhibition of  $Ca_v2.2$  channels, indicating that  $G_{i/o}$  proteins may be involved. Moreover, we showed previously that GDP- $\beta$ -S also prevented Vc1.1 inhibition of HVA calcium channel currents (Callaghan et al., 2008; Berecki et al., 2014). However, Vc1.1 inhibited  $Ca_v2.2$  channels in cells co-expressing  $Ca_v2.2$  and  $GABA_B$ R constructs with known GABA/baclofen binding sites removed ( $GABA_{B1}$ -S270A,  $GABA_{B1}$ -S246A) or that reduce activation of G proteins ( $GABA_{B2}$ -R576D). Thus, we conclude that PTX-sensitive G proteins are required for Vc1.1 inhibition but that Vc1.1 appears to act as an allosteric modulator, similar to the biased signaling mechanisms described for other receptors (Wootten et al., 2013; Wisler et al., 2014).

***Vc1.1 inhibition of  $Ca_v2.2$  via  $GABA_B$ R activation is dependent on the  $GABA_{B1a}$  subunit PCT domain***

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Our findings raise the question of how Vc1.1 activates GABA<sub>B</sub>R to inhibit Ca<sub>v</sub>2.2 channels without activating G proteins. One explanation may be direct contact between the GABA<sub>B</sub>R and Ca<sub>v</sub>2.2 and such interaction could involve G proteins. GABA<sub>B</sub>R physically interacts with the GIRK channel to modulate its function via G proteins (Fowler et al., 2007; Ciruela et al., 2010). However, Vc1.1 does not appear to activate GIRK channels (T. Huynh and P. Slesinger, unpublished observation; see also McIntosh et al., 2009).

Laviv et al. (2011) showed functional FRET coupling of GABA<sub>B</sub>R with Ca<sub>v</sub>2.2 via G proteins. This was dependent on the PCT domain of the GABA<sub>B1a</sub> subunit (Laviv et al., 2011). Also, heterodimeric GABA<sub>B</sub> receptors can form higher-ordered oligomers that may regulate GABA<sub>B</sub>R function (Comps-Agrar et al., 2011). We hypothesize that GABA<sub>B</sub>R and Ca<sub>v</sub>2.2 form a complex with G proteins and this interaction is dependent on the GABA<sub>B1a</sub> PCT domain (Fig. 9). Interestingly, deleting the GABA<sub>B1a</sub> PCT domain reduced Vc1.1, but not GABA/baclofen, inhibition of Ca<sub>v</sub>2.2 channels. This suggests the PCT domain may couple GABA<sub>B</sub>R and Ca<sub>v</sub>2.2 during Vc1.1 activation of GABA<sub>B</sub>Rs. The PCT domain may act as the anchoring site for the channel and if so, its deletion would prevent the formation of a GABA<sub>B</sub>R – Ca<sub>v</sub>2.2 channel complex. In hippocampal neurons, the PCT domain of the GABA<sub>B</sub> receptor was essential for formation of a Ca<sub>v</sub>2.2 and G protein complex (Laviv et al., 2011). We speculate the PCT domain serves the same role for enabling Vc1.1 inhibition. It will be interesting to investigate whether Vc1.1 produces a decrease in FRET between GABA<sub>B</sub>R PCT and Ca<sub>v</sub>2.2, indicating coupling and physical contact between GPCR and channel.

### ***Physiological significance of bias to GABA<sub>B</sub>R signaling***

Our results show the PCT domain of the GABA<sub>B1a</sub> subunit is integral to  $\alpha$ -conotoxin Vc1.1 activation of GABA<sub>B</sub>R and inhibition of Ca<sub>v</sub>2.2 channels. In contrast, the PCT is not required

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for GABA/baclofen  $G\beta\gamma$ -facilitated channel inhibition.  $GABA_B$ R-biased signaling upon Vc1.1 activation is a newly identified mechanism for presynaptic inhibition of neurotransmitter release at central synapses. A comparison of baclofen and Vc1.1-mediated inhibition of  $Ca_v2.2$  via  $GABA_B$ R activation is summarized in Table 2. It is also distinct from that recently reported for  $Ca_v2.3$  inhibition by  $\alpha$ -conotoxin Vc1.1.  $Ca_v2.3$  inhibition is voltage-independent and mediated by specific c-Src kinase-dependent phosphorylation sites in the C-terminus of the channel (Berecki et al., 2014).

$GABA_B$ Rs are widely distributed in the central and peripheral nervous systems. Baclofen, a selective  $GABA_B$ R agonist, has been used to treat various neurological disorders, including spasticity, chronic pain and alcoholism (Hudgson and Weightman, 1971; Hering-Hanit, 1999; Balerio and Rubio, 2002; Garbutt and Flannery, 2007). However, it causes numerous side effects due to its indiscriminate activation of multiple  $GABA_B$ R signaling pathways (Bowery, 2007). In contrast,  $\alpha$ -conotoxin Vc1.1 activates a distinct voltage-independent  $GABA_B$ R- $Ca_v2.2$  signaling pathway that requires the  $GABA_{B1a}$  PCT domain to couple  $GABA_B$ R to  $Ca_v2.2$  ultimately inhibiting  $Ca_v2.2$  channels. This  $GABA_B$ R-biased signaling mechanism likely contributes to the superior selectivity and analgesic properties of Vc1.1 (Klimis et al., 2011).

Our enhanced understanding of the molecular mechanism of Vc1.1 inhibition of  $Ca_v2.2$  channels and its analgesic properties, along with the fact that it did not cause observable side effects in animal models of chronic pain, provides a platform from which analgesic conotoxins could be developed into safe and effective drugs (Satkunanathan et al., 2005; Vincler et al., 2006; Klimis et al., 2011).

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### **Authorship Contributions**

*Participated in research design:* Adams, Slesinger, Huynh, Cuny

*Conducted experiments:* Huynh, Cuny

*Contributed new reagents or analytical tools:* Adams, Slesinger

*Performed data analysis:* Huynh, Cuny, Adams

*Wrote or contributed to the writing of the manuscript:* Huynh, Cuny, Slesinger, Adams

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## Footnotes

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- b)** Huynh, T.G. (2013) *Mechanism of  $\alpha$ -conotoxin Vc1.1 inhibition of Cav2.2 channel*. PhD Thesis, Queensland Brain Institute, The University of Queensland.
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- e)** Conflict of Interest: The authors have no conflicting financial interests.

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## Figure Legends

**Figure 1.  $\alpha$ -Conotoxin Vc1.1 inhibits  $Ca_v2.2$  channels by activating  $GABA_B$ Rs in HEK293 cells.** (A) Representative superimposed current traces from HEK293 cells co-expressing human  $GABA_B$ R and  $Ca_v2.2$  channels in the absence (a) and presence of Vc1.1 (200 nM) (b) and baclofen (50  $\mu$ M) (c). (B) Peak current amplitude plotted as a function of time. Bars indicate duration of drug application. Lower case letters represent superimposed current traces shown in (A). (C) Bar graph of inhibition of peak current amplitude by 200 nM Vc1.1 ( $41.0 \pm 5.3\%$ ), 50  $\mu$ M baclofen ( $61.5 \pm 5.7\%$ ), 10  $\mu$ M GABA ( $69.6 \pm 2.3\%$ ), and 200 nM Vc1.1 + 50  $\mu$ M baclofen ( $78.1 \pm 3.6\%$ ). Number of experiments is indicated in parenthesis. Data represent mean  $\pm$  SEM (\*\* $P < 0.01$ , \*\*\* $P < 0.001$  versus Vc1.1, one-way ANOVA). (D) Concentration–response relationship for Vc1.1 inhibition of peak current in HEK293 cells co-expressing  $GABA_B$ R and  $Ca_v2.2$  channels with an  $IC_{50}$  of 19.1 nM and Hill slope of  $-1$  ( $n = 6-7$  cells per data point).

**Figure 2. Mutation of the GABA/Baclofen binding site does not affect Vc1.1 inhibition of  $Ca_v2.2$ .** (A) Schematic diagram of wild-type and mutated  $GABA_{B1a}$  subunits. (B) Representative superimposed current traces obtained from HEK293T cells co-expressing human  $GABA_B$ R and  $Ca_v2.2$  channels in the absence (a) and presence of Vc1.1 (200 nM) (b) and baclofen (50  $\mu$ M) (c). (C) Peak current amplitude plotted as a function of time. Bars indicate duration of drug application. Lower case letters represent time at which the superimposed current traces were obtained. (D) Bar graph comparing Vc1.1 and baclofen inhibition of peak current amplitude. Vc1.1 (200 nM) inhibition of peak current was not affected by the mutation ( $52.3 \pm 8.6\%$  compared with control  $47.1 \pm 14.4\%$ ,  $P = 0.75$ ). However, baclofen (50  $\mu$ M) inhibition was significantly reduced by the mutation ( $14.7 \pm 3.0$

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% compared with control  $43.3 \pm 8.6$  %). Number of experiments is indicated in parenthesis.

Data represent mean  $\pm$  SEM (\*\* $P < 0.01$ ).

**Figure 3. Differential effects of Vc1.1 and baclofen on the voltage-dependence of Ca<sub>v</sub>2.2 channel activation and inactivation in HEK293 cells co-expressing human Ca<sub>v</sub>2.2 and GABA<sub>B</sub>R.**

**(A)** Upper panel: Current-voltage relationships obtained in the absence ( $n = 7$ ) and presence of baclofen ( $n = 7$ ) and Vc1.1 ( $n = 6$ ). Lower panel: Voltage-dependence of Ca<sub>v</sub>2.2 activation in the absence (control) and presence of Vc1.1 (200 nM) and baclofen (50  $\mu$ M). Ca<sub>v</sub>2.2-mediated currents were evoked by step depolarization from a holding potential of  $-80$  mV to  $+50$  mV in 5 mV increments at 0.1 Hz. Baclofen shifted the  $V_{0.5}$  activation to more depolarized potentials ( $+3.7 \pm 1.5$  mV,  $n = 7$ ), whereas Vc1.1 did not change  $V_{0.5}$  activation ( $-5.1 \pm 0.9$  mV,  $n = 8$ ) compared to control ( $-6.3 \pm 0.7$  mV,  $n = 8$ ). Data represent mean  $\pm$  SEM. **(B)** Analysis of Ca<sub>v</sub>2.2 activation using a tail current protocol. Upper panel: representative superimposed whole-cell currents obtained in the absence and presence of baclofen from a cell expressing Ca<sub>v</sub>2.2 and GABA<sub>B</sub>R. Tail currents were measured upon repolarization to  $-60$  mV, immediately following depolarizing test pulses from  $-40$  mV to  $+55$  mV in 5 mV increments. Lower panel: Ca<sub>v</sub>2.2 activation curves obtained from tail currents were fitted with the sum of two Boltzmann functions. Baclofen shifted both  $V_{0.5}$  and  $k$ , whereas Vc1.1 did not. The parameters of the major Boltzmann function obtained from the best fit to the data were  $V_{0.5, \text{act}} = +8.9 \pm 2.6$  mV and  $k = 5.8 \pm 1.2$  mV ( $n = 8$ ) under control conditions,  $V_{0.5, \text{act}} = +7.8 \pm 2.1$  mV and  $k = 4.5 \pm 2.4$  mV ( $n = 6$ ) in the presence of Vc1.1 and  $V_{0.5, \text{act}} = +17.4 \pm 3.8$  mV and  $k = 8.4 \pm 1.5$  mV ( $n = 4$ ) in the presence of baclofen. **(C)** Ca<sub>v</sub>2.2 steady-state inactivation (SSI) curves obtained in the absence and presence of Vc1.1 (200 nM) and baclofen (50  $\mu$ M). Peak current amplitudes following a 10 s prepulse at varying potentials were normalized to the maximum peak current amplitude recorded at  $+5$  mV with a

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prepulse of  $-120$  mV.  $V_{0.5, \text{inact}}$  was unchanged in the presence of baclofen ( $-64.3 \pm 1.8$  mV,  $n = 7$ ) compared to control ( $-65.5 \pm 0.6$  mV,  $n = 8$ ), whereas Vc1.1 shifted  $V_{0.5, \text{inact}}$  to  $-78.8 \pm 0.5$  mV ( $n = 10$ ) which was significantly different to control ( $P < 0.05$ , one-way ANOVA). Data represent mean  $\pm$  SEM. **(D)** SSI of  $\text{Ca}_v2.2$  channels was further investigated in individual cells in the absence (control) and presence of Vc1.1 (200 nM). Upper left panel: voltage protocol to evaluate SSI of  $\text{Ca}_v2.2$ . Upper right panel: ratio of peak current amplitudes elicited by depolarizing test pulses to  $+10$  mV separated by a 10 s prepulse at  $-110$ ,  $-80$  and  $-50$  mV (P1/P2) in the absence (control) and presence of Vc1.1. Holding potential  $-80$  mV. In the presence of Vc1.1 and at a prepulse potential of  $-110$  mV, P2/P1 was reduced from  $1.33 \pm 0.07$  to  $1.01 \pm 0.01$  ( $n = 11$ ;  $P < 0.01$ , paired  $t$ -test) and at  $-50$  mV from  $0.55 \pm 0.03$  to  $0.43 \pm 0.03$  ( $n = 11$ ;  $P < 0.01$ ). Data represent mean  $\pm$  SEM. Lower panel: representative superimposed current traces evoked by step depolarization to  $+10$  mV (P2) following 10 s pre-pulses to  $-110$ ,  $-80$  and  $-50$  mV in the absence ( $\circ$ ) and presence of Vc1.1 ( $\blacksquare$ ).

**Figure 4. Baclofen and Vc1.1 effects on the  $\text{Ca}_v2.2$  channel activation rate.**

Representative superimposed whole-cell  $\text{Ba}^{2+}$  currents from HEK293 cells expressing  $\text{Ca}_v2.2$  channels and  $\text{GABA}_B$ Rs obtained in the absence and presence of 50  $\mu\text{M}$  baclofen **(A)** and 200 nM Vc1.1 **(B)**. The superimposed red trace represents a single exponential function fitted to the time course of current activation. The superimposed grey trace represents the current recorded in the presence of baclofen and Vc1.1 normalized to control. **(C)** Data sets for activation time constants of  $\text{Ca}_v2.2$  obtained in the absence ( $\tau_{\text{control}}$ :  $1.63 \pm 0.18$  ms) and presence of baclofen ( $\tau_{\text{baclofen}}$ :  $2.13 \pm 0.36$  ms) ( $P < 0.05$ , paired  $t$ -test), and absence ( $\tau_{\text{control}}$ :  $1.69 \pm 0.25$  ms) and presence of Vc1.1 ( $\tau_{\text{Vc1.1}}$ :  $1.26 \pm 0.23$  ms) ( $P < 0.05$ , paired  $t$ -test). The

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time constants of activation of  $I_{Ba}$  obtained in the presence of baclofen and Vc1.1 were significantly different ( $P < 0.05$ , unpaired  $t$ -test). Data represent mean  $\pm$  SEM.

**Figure 5. Pre-pulse relief of GABA but not Vc1.1 inhibition of  $Ca_v2.2$ .** (A) Prepulse protocol and representative superimposed traces of  $Ca_v2.2$  currents in HEK293 cells obtained in the absence (control) and presence of GABA (B) and Vc1.1 (C). The depolarizing prepulse to +120 mV relieved GABA inhibition of peak current amplitude, but did not significantly affect inhibition by Vc1.1. The outward current in response to the 50 ms prepulse to +120 mV has been blanked to better display the inward current. (D) Bar graph of GABA's inhibition of average peak current amplitude in the absence ( $64.0 \pm 4.0\%$  [-pp]) and presence ( $4.0 \pm 5.0\%$  [+pp]) of prepulse. The prepulse significantly relieved peak current amplitude ( $***P < 0.001$ , unpaired  $t$ -test) in the presence of GABA, but did not change Vc1.1 inhibition of peak current amplitude ( $50.0 \pm 11.0$  [-pp] to  $36.0 \pm 6.0\%$  [+pp]). Number of experiments is indicated in parenthesis. Data represent mean  $\pm$  SEM. (E) Peak inward current amplitude in response to a depolarizing test pulse to +5 mV plotted as a function of time in the absence and presence of Vc1.1 and GABA as indicated. Closed symbols ( $\bullet$ , -pp) represent peak current amplitude without a preceding prepulse whereas open symbols ( $\circ$ , +pp) represent peak current amplitude following prepulse.

**Figure 6. PTX prevents  $\alpha$ -conotoxin Vc1.1 inhibition of  $Ca_v2.2$  channels.** (A) Representative superimposed current traces obtained in the absence (a) and presence of Vc1.1 (200 nM) (b) and baclofen (50  $\mu$ M) (c) in HEK293 cells pre-incubated in PTX (3  $\mu$ g/ml) for at least 16 hr. (B) Peak current amplitude plotted as a function of time. Bars indicate duration of drug application. Lower case letters represent time at which superimposed current traces were obtained. (C) Bar graph of Vc1.1 and baclofen inhibition of peak current amplitude with

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or without PTX pre-treatment. Vc1.1 inhibition was significantly less in cells pre-treated with PTX ( $0.9 \pm 3.5\%$ ) than in untreated cells ( $42.0 \pm 5.7\%$ ). Similarly, baclofen inhibition was significantly less in cells pre-treated with PTX ( $5.0 \pm 2.3\%$ ) than in untreated cells ( $70.7 \pm 2.3\%$ ). Number of experiments is indicated in parenthesis. Data represent mean  $\pm$  SEM ( $***P < 0.001$ , unpaired *t*-test).

**Figure 7. Point mutation of arginine 576 to aspartic acid on the GABA<sub>B2</sub> subunit does not affect Vc1.1 inhibition of Ca<sub>v</sub>2.2 currents.** Currents were recorded in HEK293T cells co-expressing Ca<sub>v</sub>2.2 with either wild-type GABA<sub>B</sub>R or GABA<sub>B1a</sub>-WT/GABA<sub>B2</sub>-R576D and in the presence of Vc1.1 or baclofen. **(A)** Schematic diagram of wild type and mutated GABA<sub>B2</sub> subunits. **(B)** Representative superimposed current traces obtained in the absence (a) and presence of Vc1.1 (200 nM) (b) and baclofen (50  $\mu$ M) (c). **(C)** Peak current amplitude plotted as a function of time. Bars indicate duration of drug application. Lower case letters represent times at which the superimposed current traces were obtained. **(D)** Bar graph comparing Vc1.1 and baclofen inhibition of peak current amplitude with co-expressed wild-type GABA<sub>B</sub>R and mutated GABA<sub>B</sub>R subunits. Vc1.1 inhibition of peak current was not affected by the mutation (GABA<sub>B2</sub>-R576D,  $52.9 \pm 8.9\%$ ; control  $41.1 \pm 6.1\%$ ) but baclofen inhibition of peak current was significantly reduced (GABA<sub>B2</sub>-R576D,  $11.2 \pm 4.3\%$ ; control  $64.8 \pm 4.0\%$ ). Number of experiments is indicated in parenthesis. Data represent mean  $\pm$  SEM ( $***P < 0.001$ , unpaired *t*-test).

**Figure 8. Vc1.1 inhibition of Ca<sub>v</sub>2.2 currents involves the PCT domain of the GABA<sub>B1a</sub> subunit.** **(A)** Schematic diagram of wild-type (WT) and mutant GABA<sub>B1a</sub> subunits. Inward Ba<sup>2+</sup> currents were recorded from HEK293T cells co-expressing Ca<sub>v</sub>2.2 channels with GABA<sub>B1a</sub>- $\Delta$ 887/GABA<sub>B2</sub>-WT **(B)**, GABA<sub>B1a</sub>- $\Delta$ 863/GABA<sub>B2</sub>-WT **(C)** or GABA<sub>B1a</sub>-

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$\Delta$ PCT/GABA<sub>B2</sub>-WT (**D**). Left panel: representative superimposed current traces obtained in the absence (a) and presence of Vc1.1 (200 nM) (b) and baclofen (50  $\mu$ M) (c). Right panel: peak current amplitude plotted as a function of time. Bars indicate duration of drug application. Lower case letters represent times at which the superimposed current traces were obtained. (**E**) Vc1.1 inhibition of peak current in HEK293T cells expressing GABA<sub>B1a</sub>- $\Delta$ 887/GABA<sub>B2</sub>-WT ( $24.9 \pm 6.9\%$ ,  $n = 7$ ) was not significantly different from that in wild-type GABA<sub>B</sub>R expressing cells ( $37.9 \pm 5.6\%$ ,  $n = 11$ ). Vc1.1 inhibition of peak current in HEK293T cells expressing GABA<sub>B1a</sub>- $\Delta$ 863/GABA<sub>B2</sub>-WT ( $9.5 \pm 4.0\%$ ,  $n = 12$ ) or GABA<sub>B1a</sub>- $\Delta$ PCT/GABA<sub>B2</sub> ( $5.3 \pm 3.9\%$ ,  $n = 5$ ) was significantly different from that in wild-type GABA<sub>B</sub>R expressing cells ( $37.9 \pm 5.6\%$ ,  $n = 11$ ,  $P < 0.05$ ). Baclofen inhibition of peak currents was the same in GABA<sub>B1a</sub>- $\Delta$ 887/GABA<sub>B2</sub>-WT ( $62.0 \pm 7.3\%$ ,  $n = 7$ ), GABA<sub>B1a</sub>- $\Delta$ 863/GABA<sub>B2</sub>-WT ( $56.6 \pm 3.5\%$ ,  $n = 10$ ) and GABA<sub>B1a</sub>- $\Delta$ PCT/GABA<sub>B2</sub>-WT expressing cells ( $60.7 \pm 2.7\%$ ,  $n = 5$ ). Data represent mean  $\pm$  SEM (\*\* $P < 0.01$ , one-way ANOVA).

**Figure 9. Schematic diagram of Vc1.1 and baclofen signaling pathways.** Baclofen (blue) inhibits Ca<sub>v</sub>2.2 channels by binding to the Venus Fly Trap on the GABA<sub>B1</sub> subunit. This activates the G protein complex in a signaling process involving R576 on the GABA<sub>B2</sub> subunit and results in G $\beta\gamma$  binding to the Ca<sub>v</sub>2.2 channel. Vc1.1 (red) inhibits Ca<sub>v</sub>2.2 channels by binding to an as yet unidentified site on the GABA<sub>B</sub>R triggering inhibition of Ca<sub>v</sub>2.2 channels by non-activated G proteins. Vc1.1 inhibition of Ca<sub>v</sub>2.2 involves the PCT domain of the GABA<sub>B1a</sub> subunit.

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## Tables

**Table 1. Voltage-dependence of Ca<sub>v</sub>2.2 channels in HEK293 cells co-expressing GABA<sub>B</sub>Rs in the absence (control) and presence of Vc1.1 or baclofen.**

	Activation G-V		Activation I <sub>tail</sub> -V		Steady-state Inactivation	
	V <sub>0.5, act</sub>	<i>k</i>	V <sub>0.5, act</sub> <sup>a</sup>	<i>k</i> <sup>a</sup>	V <sub>0.5, inact</sub>	<i>k</i>
Control	-6.3 ± 0.7 (8)	4.4 ± 0.6	8.9 ± 2.6 (8)	5.8 ± 1.2	-65.5 ± 0.6 (8)	-8.0 ± 0.5
Baclofen	3.7 ± 1.5 (7) <sup>b</sup>	8.6 ± 1.4 <sup>b</sup>	17.4 ± 3.8 (4)	8.4 ± 1.5	-64.3 ± 1.8 (7)	-11.0 ± 1.8
Vc1.1	-5.1 ± 0.9 (8)	6.1 ± 0.8	7.8 ± 2.1 (6)	4.5 ± 2.4	-78.8 ± 0.5 (10) <sup>c</sup>	-9.1 ± 0.5

Data represent mean ± SEM. Number of experiments is indicated in parenthesis. V<sub>0.5, act</sub> is half-maximum activation (mV), V<sub>0.5, inact</sub> is half-maximum inactivation (mV); and *k* is the slope factor.

<sup>a</sup>These parameters were obtained from the major component of the double Boltzmann fit of I<sub>tail</sub>-V.

<sup>b</sup>V<sub>0.5, act</sub> and *k* obtained in the presence of baclofen are statistically different from control (*P* < 0.05, one-way ANOVA).

<sup>c</sup>V<sub>0.5, inact</sub> obtained in the presence of Vc1.1 is statistically different from control (*P* < 0.05, one-way ANOVA).

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**Table 2. Comparison of baclofen- and Vc1.1-mediated inhibition of Ca<sub>v</sub>2.2 via GABA<sub>B</sub>R activation.**

<b>Property</b>	<b>Baclofen</b>	<b>Vc1.1</b>
Onset	Fast (ms)	Slow (s)
V <sub>0.5</sub> for activation	+10 mV shift	No change
V <sub>0.5</sub> for inactivation	No change	-13 mV shift
Activation kinetics	Slower	Faster
Prepulse sensitivity	Yes	No
Frequency dependence	Yes	No
Involvement of PCT	No	Yes
Pertussis toxin sensitivity	Yes	Yes
GDP-β-S sensitivity <sup>a</sup>	Yes	Yes

<sup>a</sup>Intracellular GDP-β-S antagonized the inhibitory effect of baclofen and Vc1.1 on HVA calcium channel currents (Callaghan et al., 2008; Berecki et al., 2014).

Figure 1

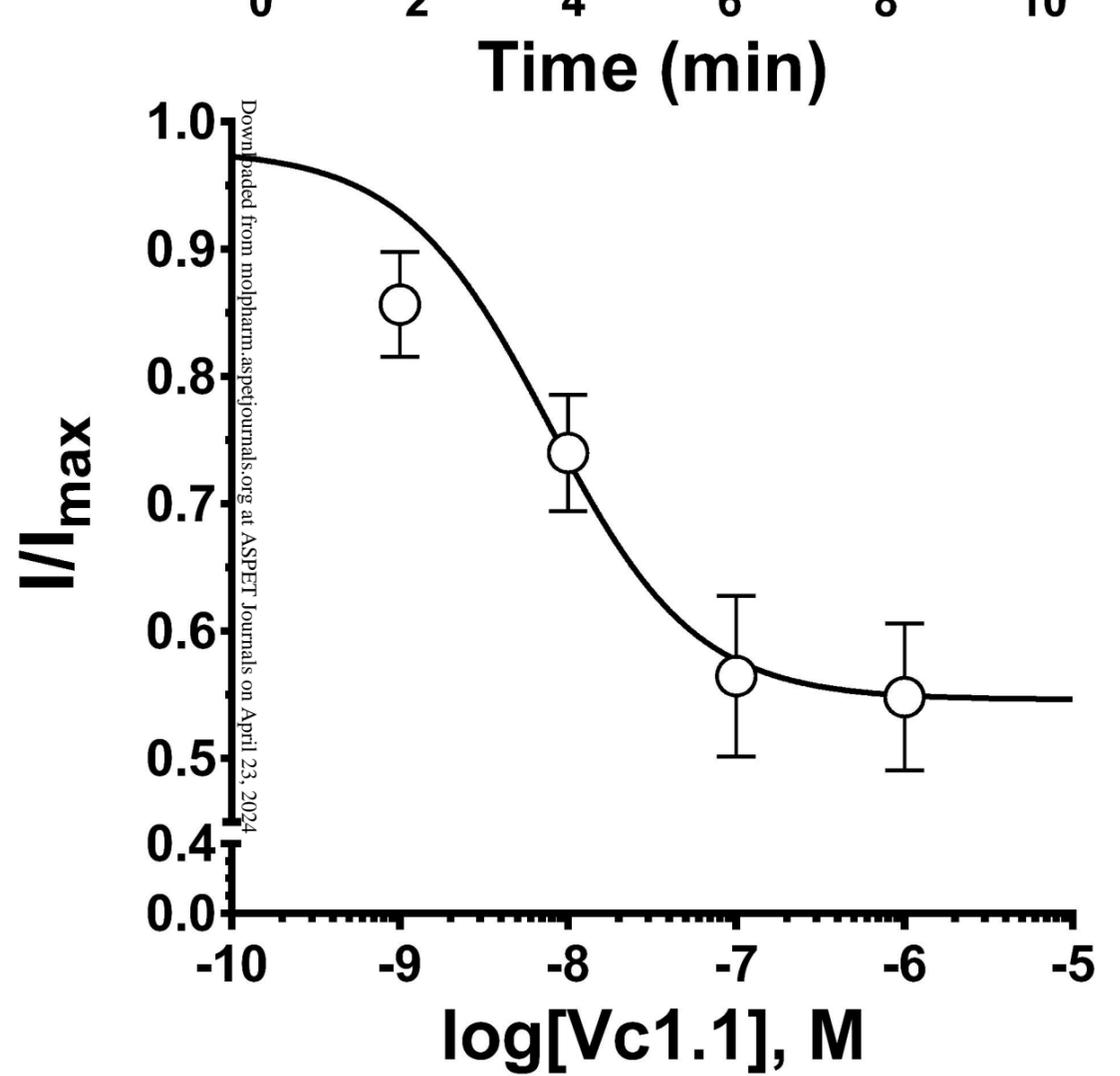
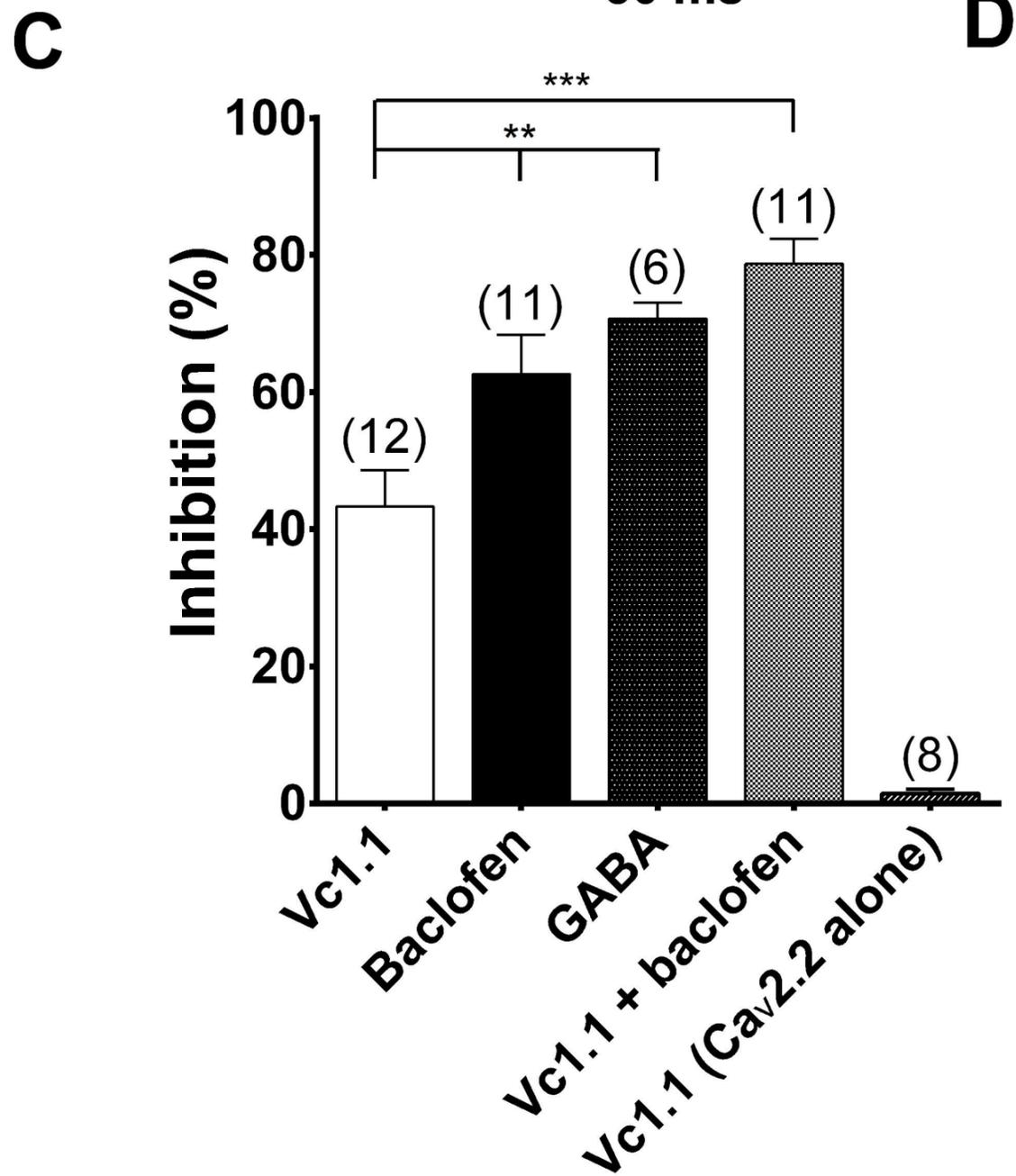
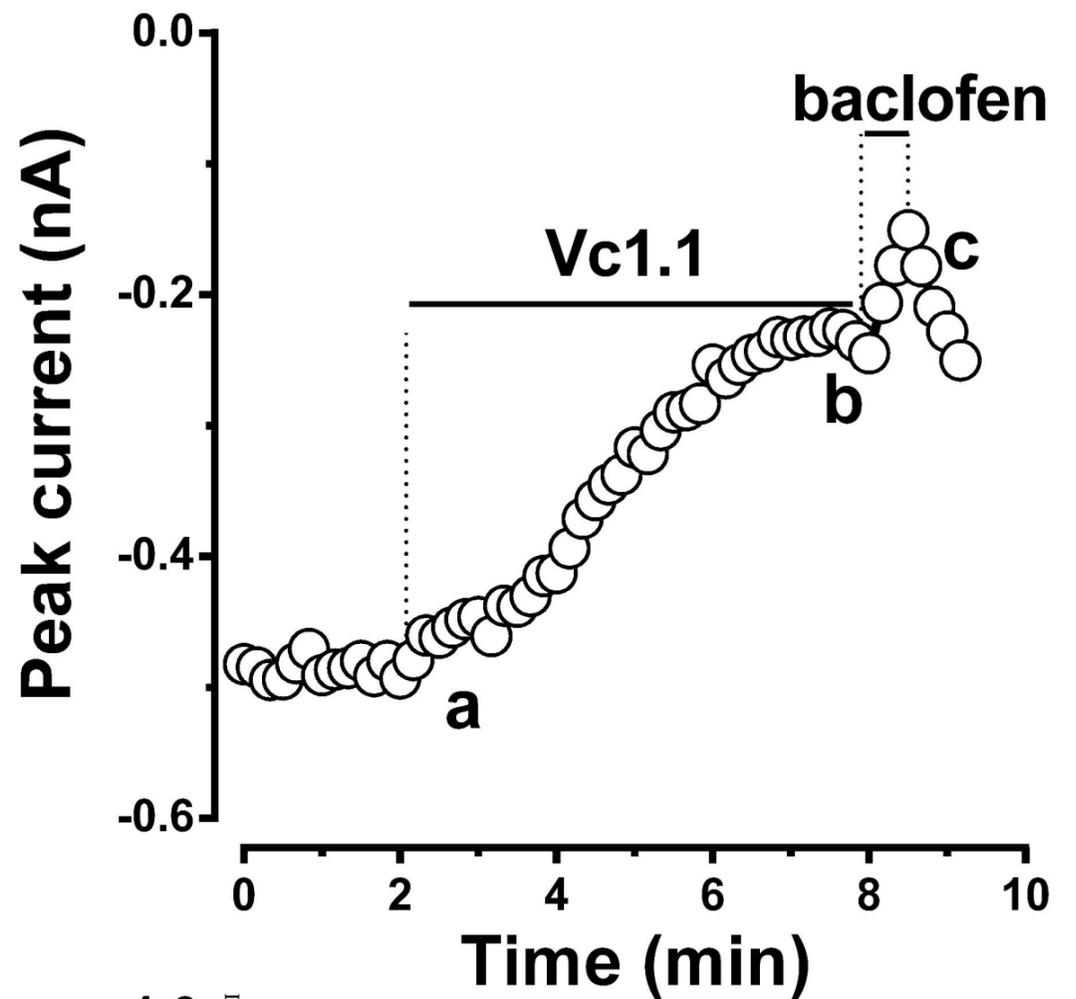
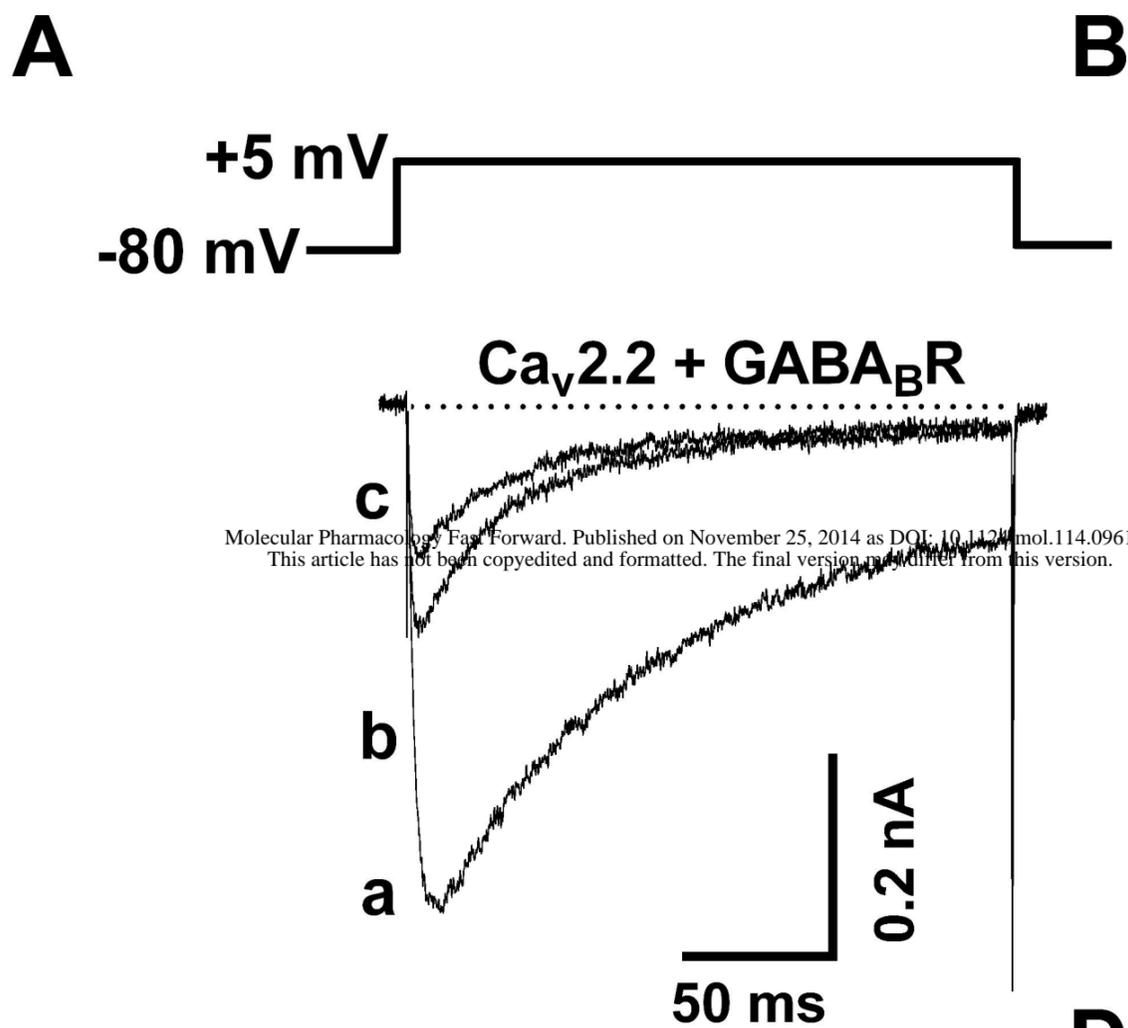
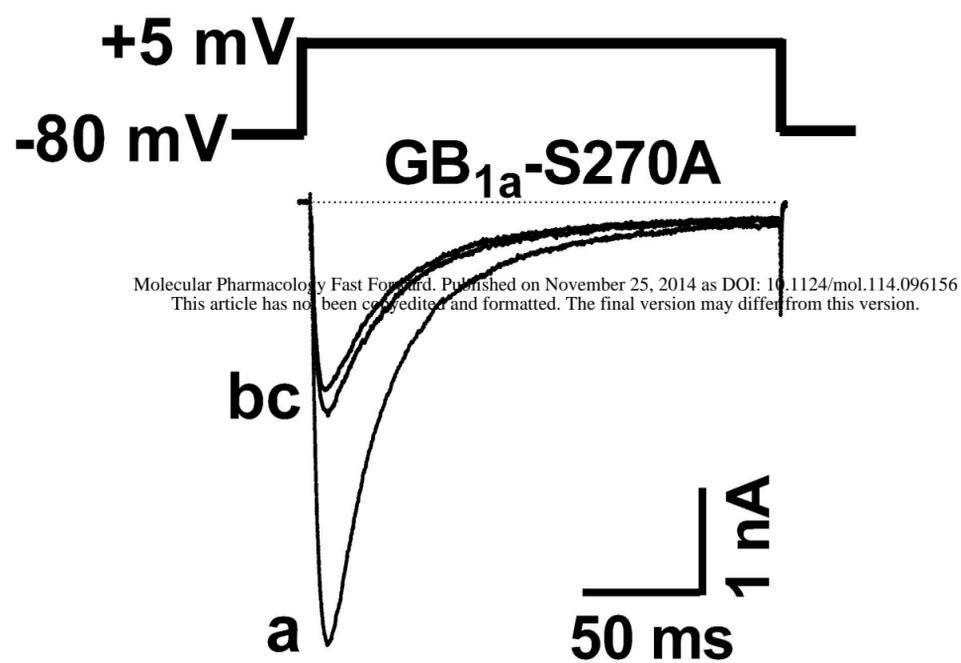


Figure 2

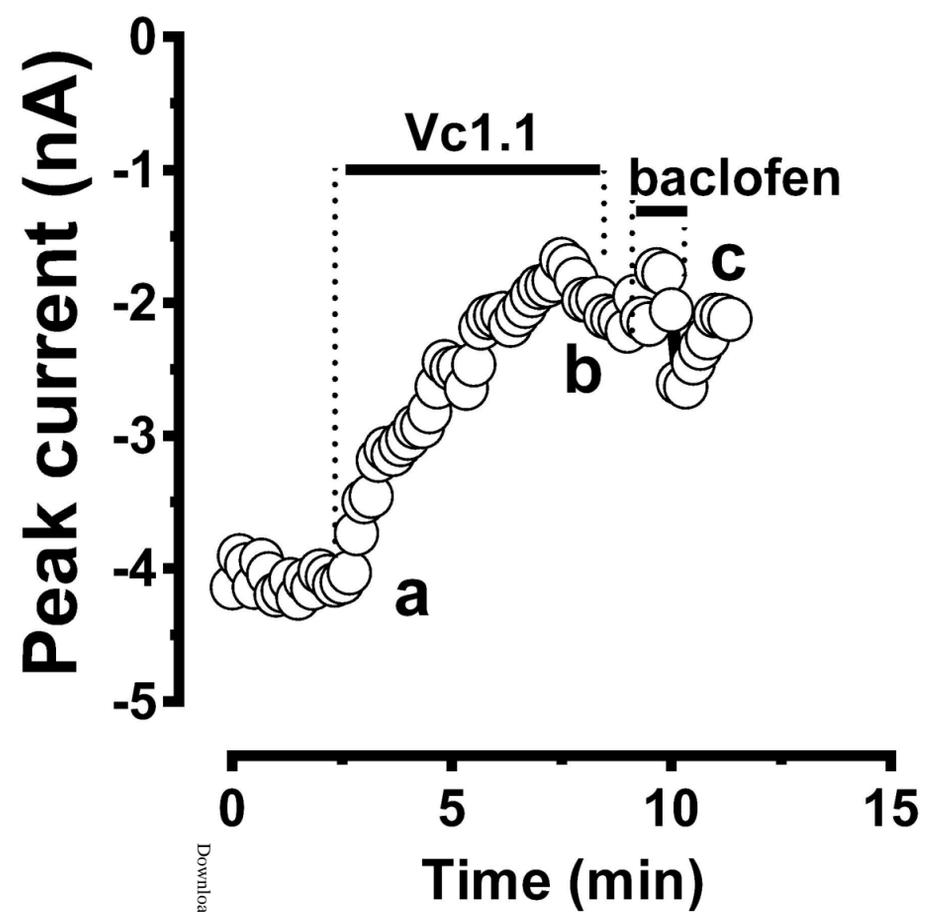
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B



C



D

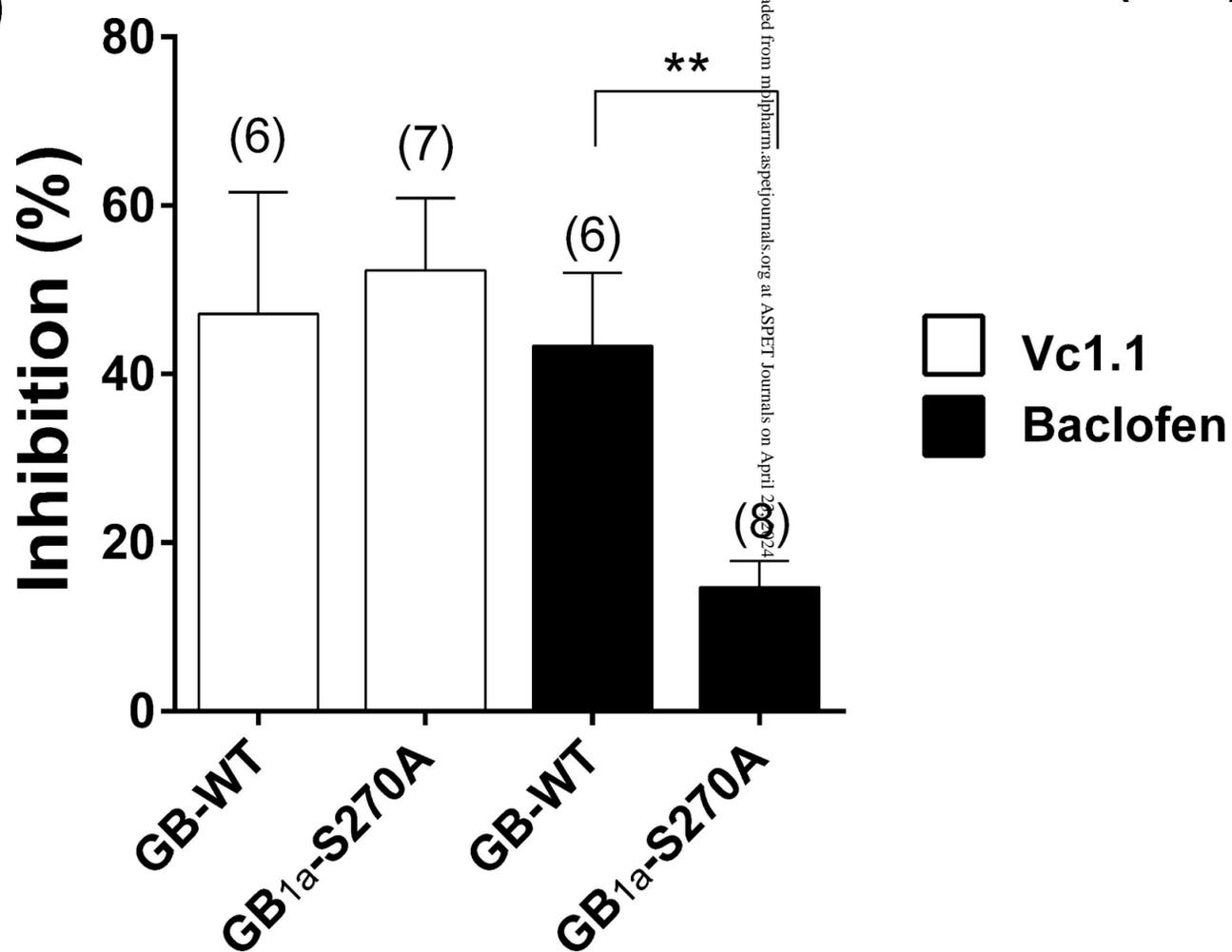


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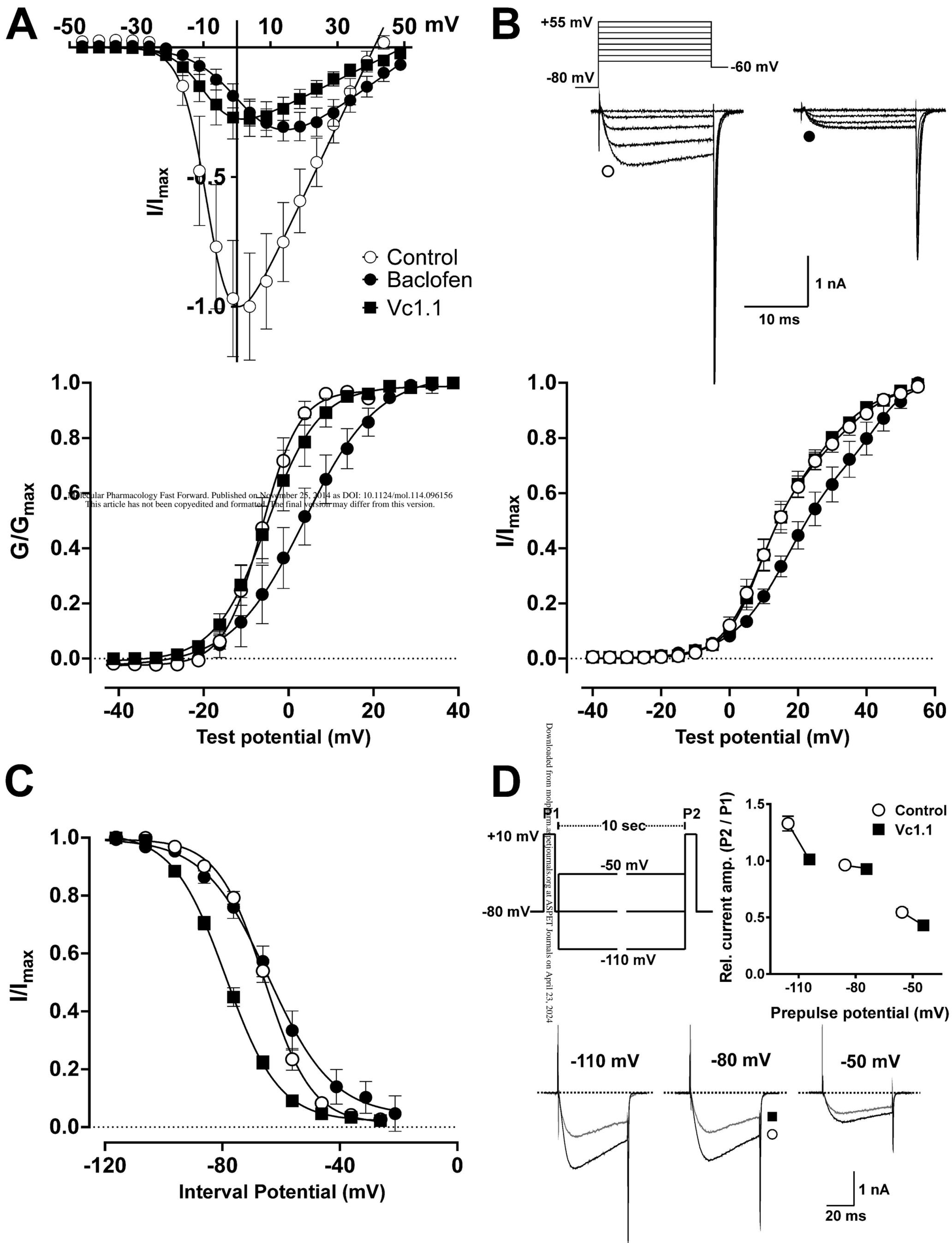
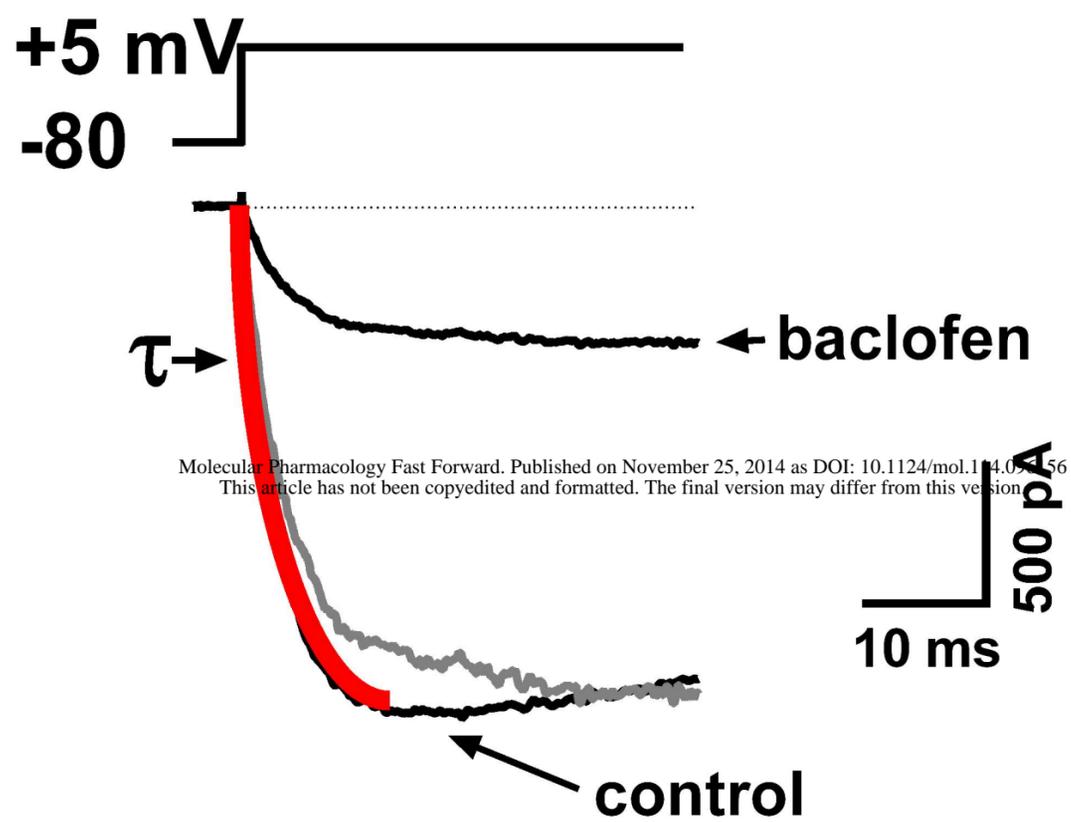
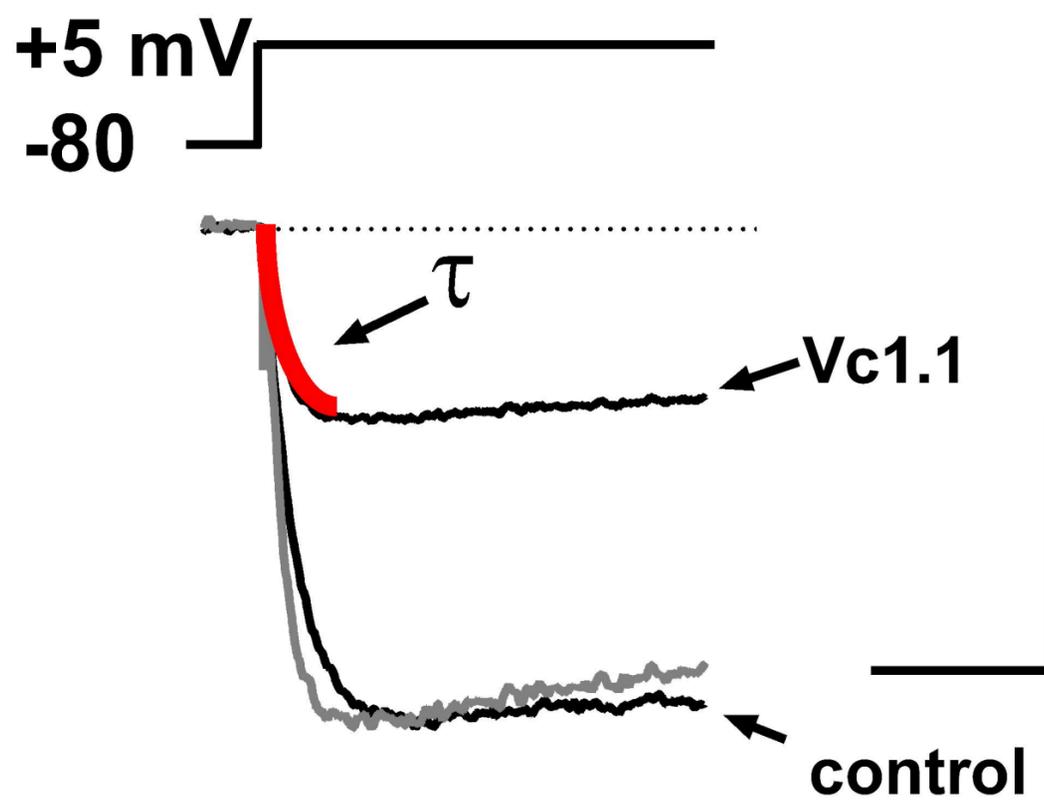


Figure 4

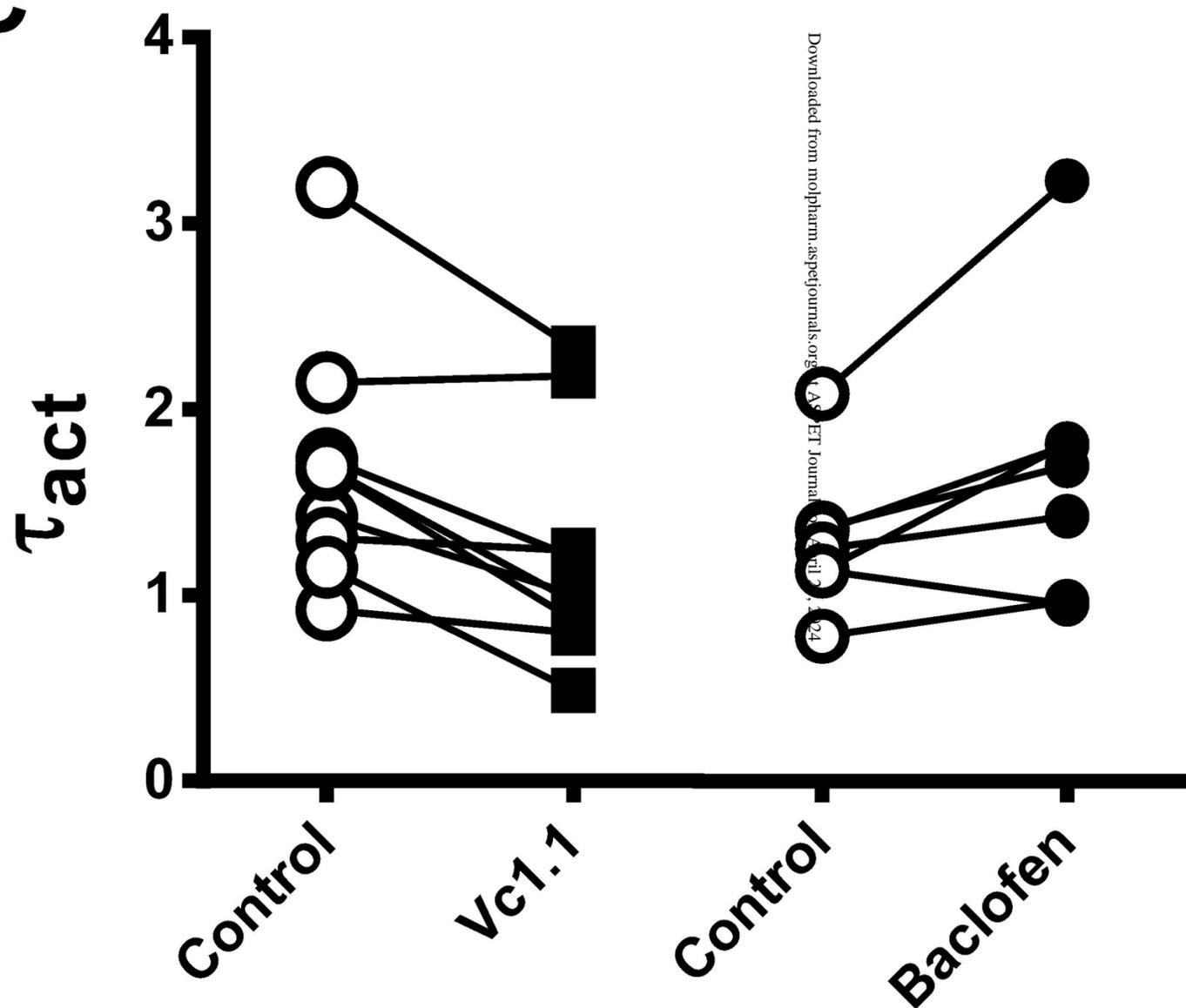
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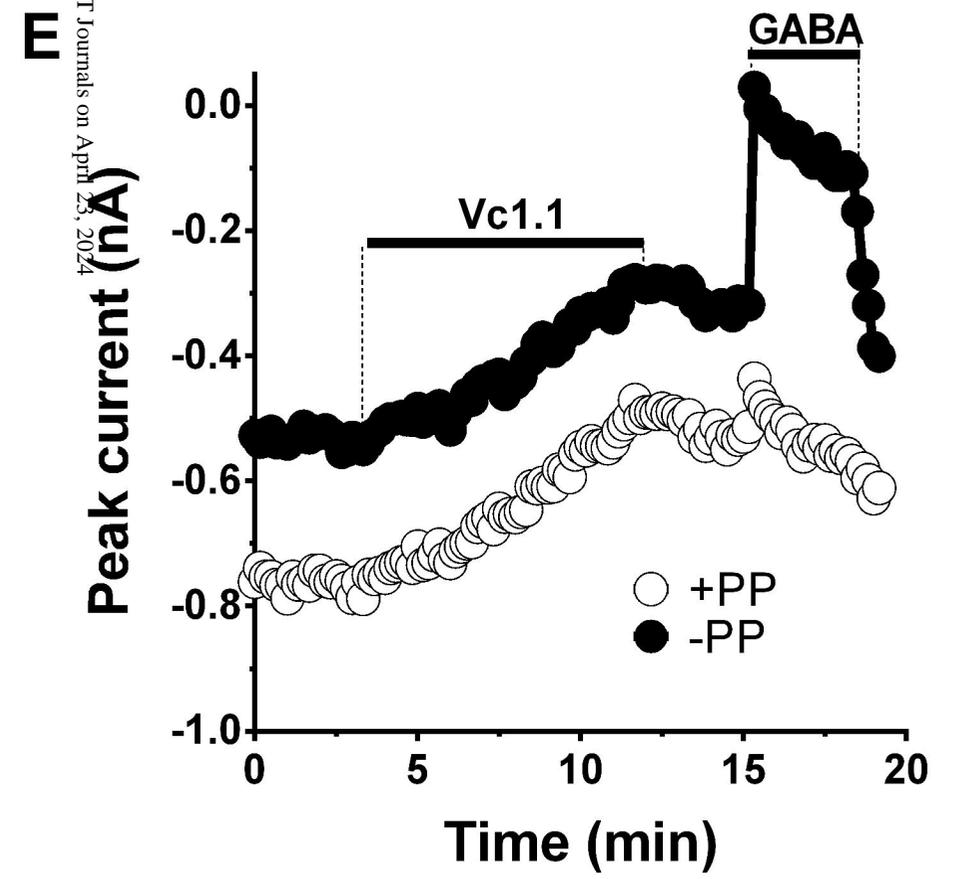
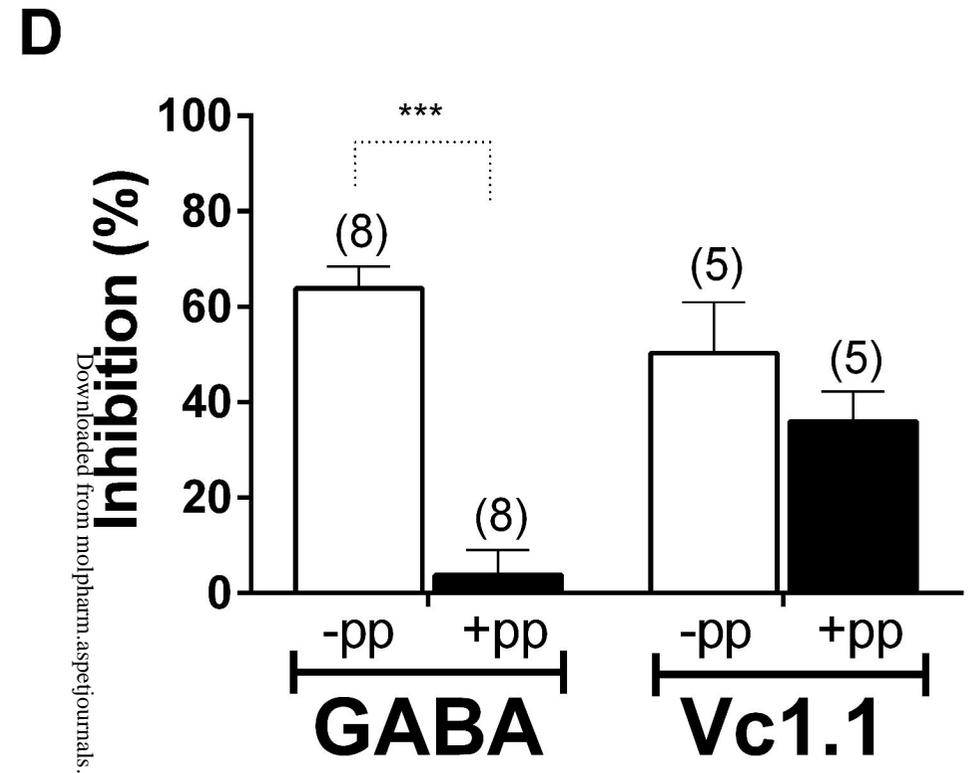
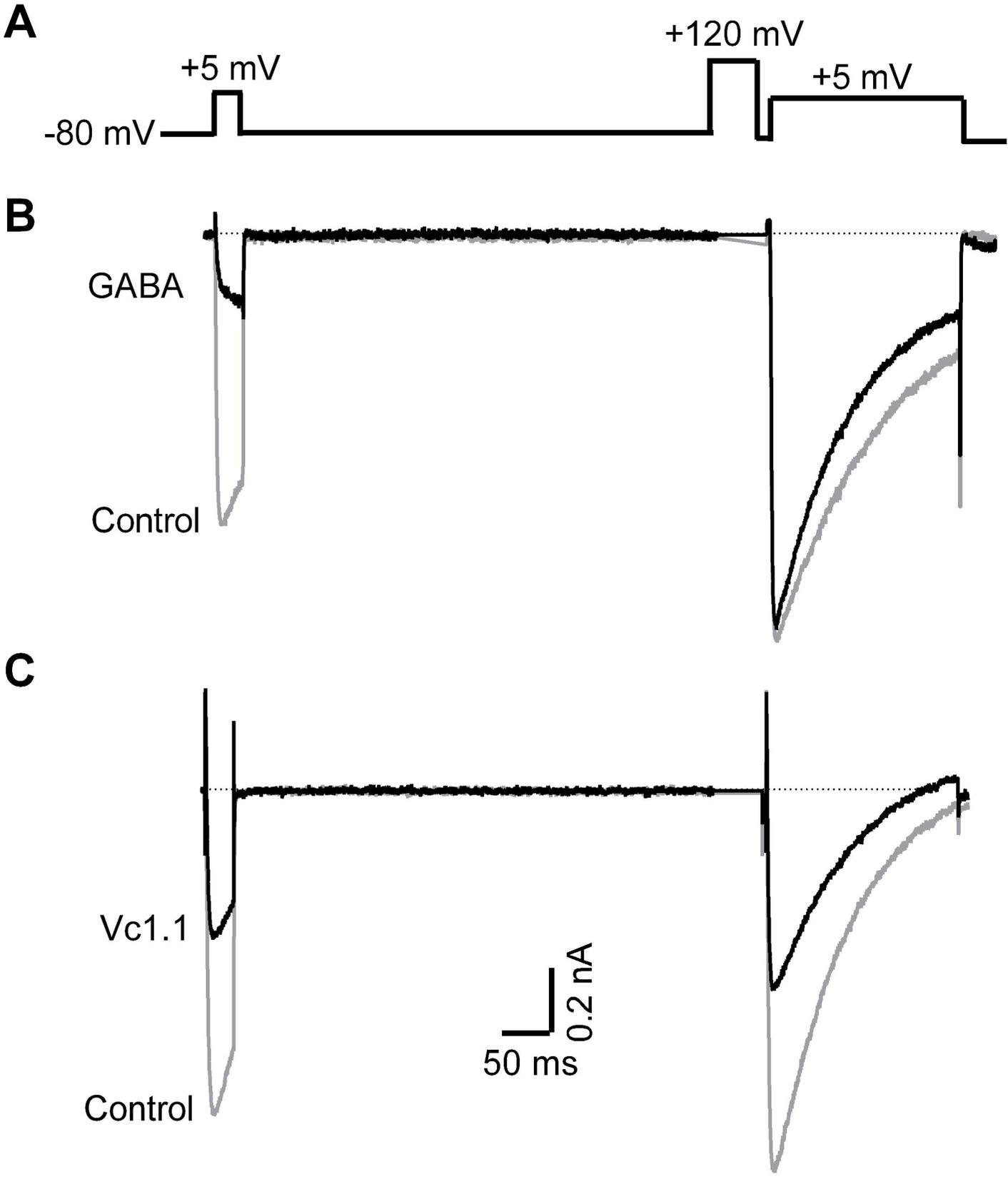
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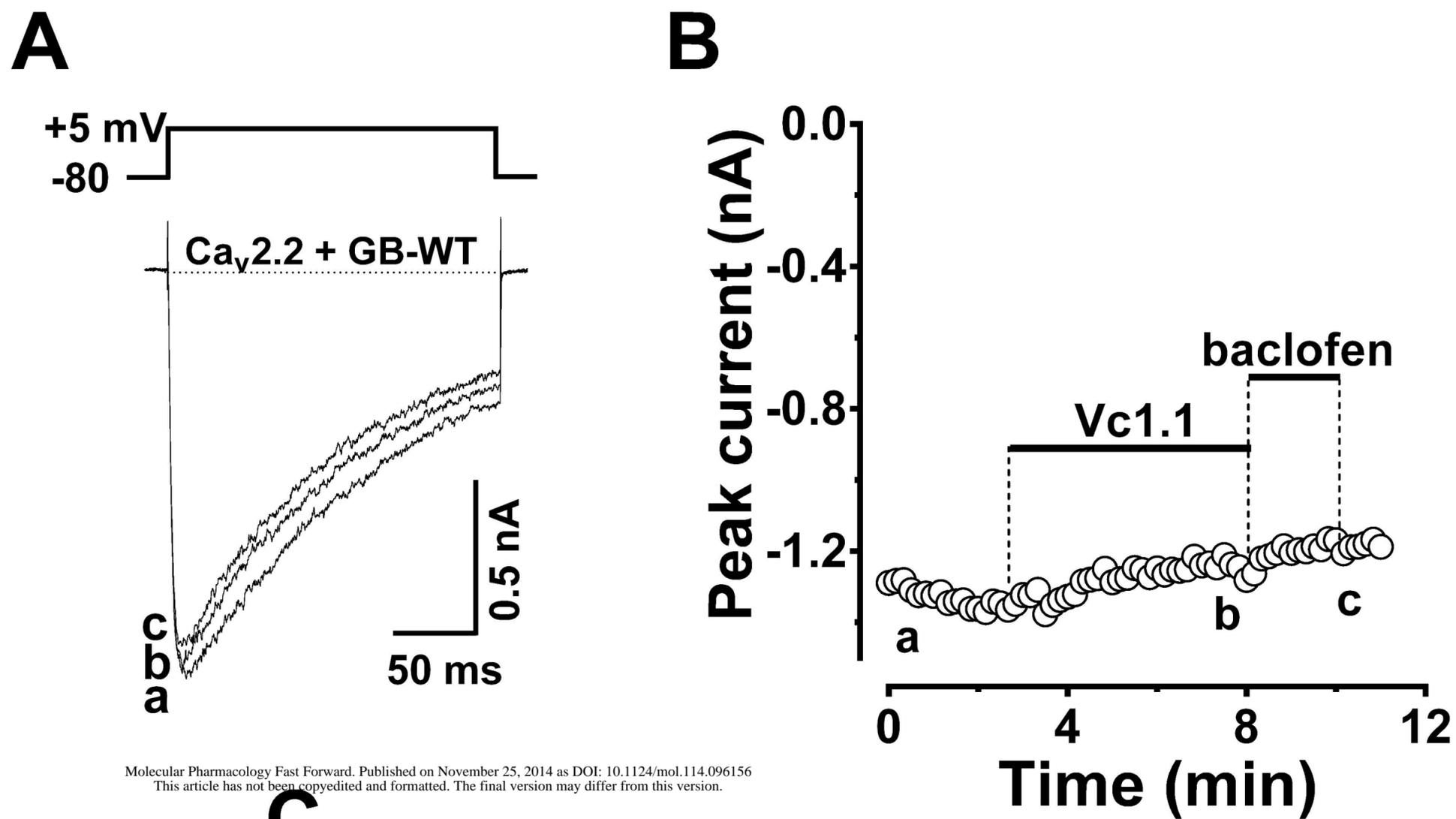


**Figure 5**



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Figure 6



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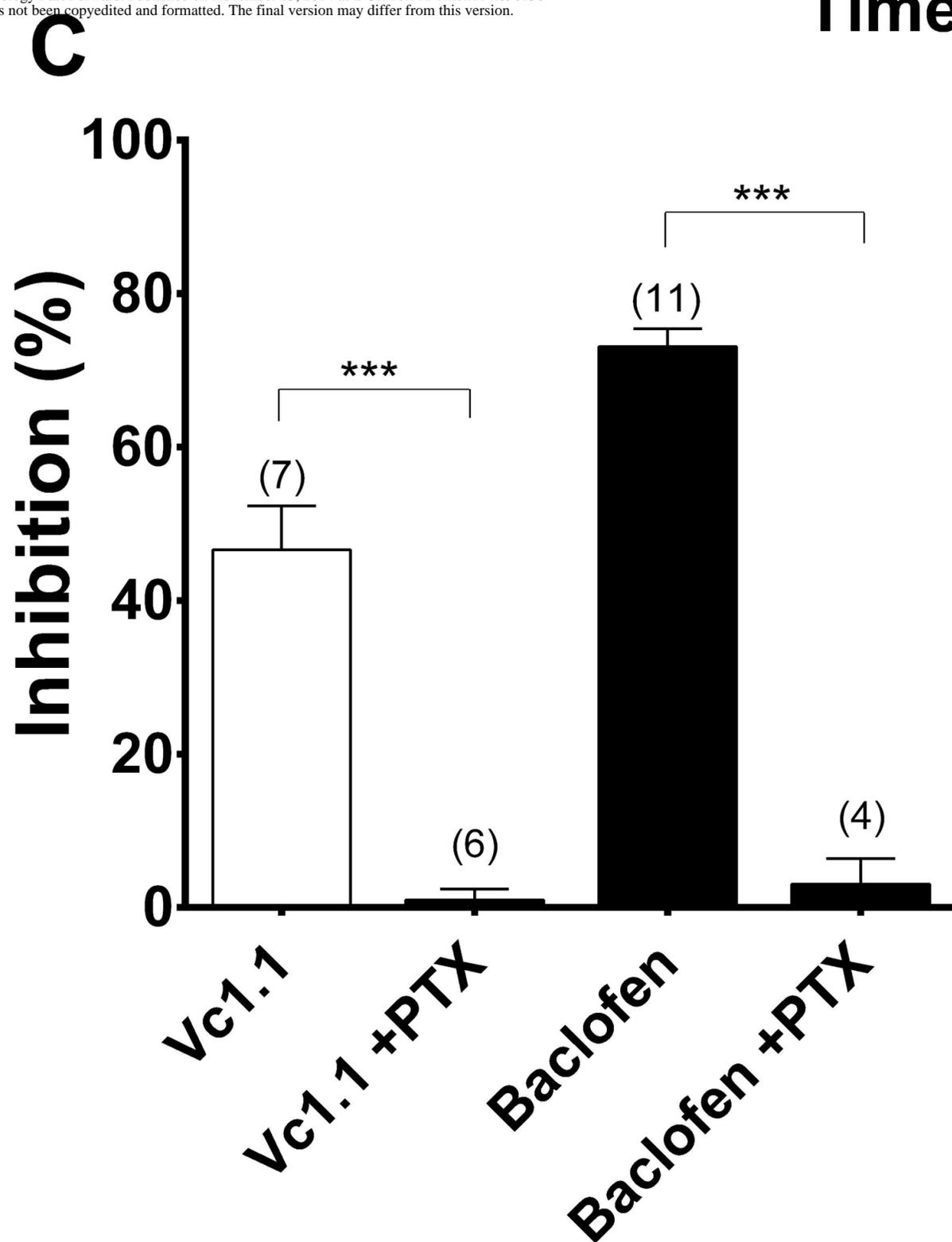
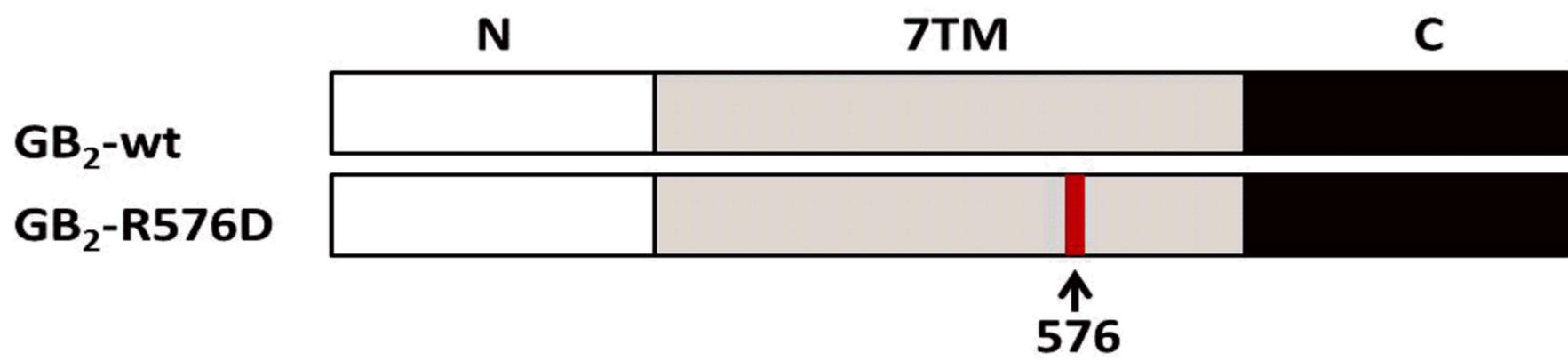
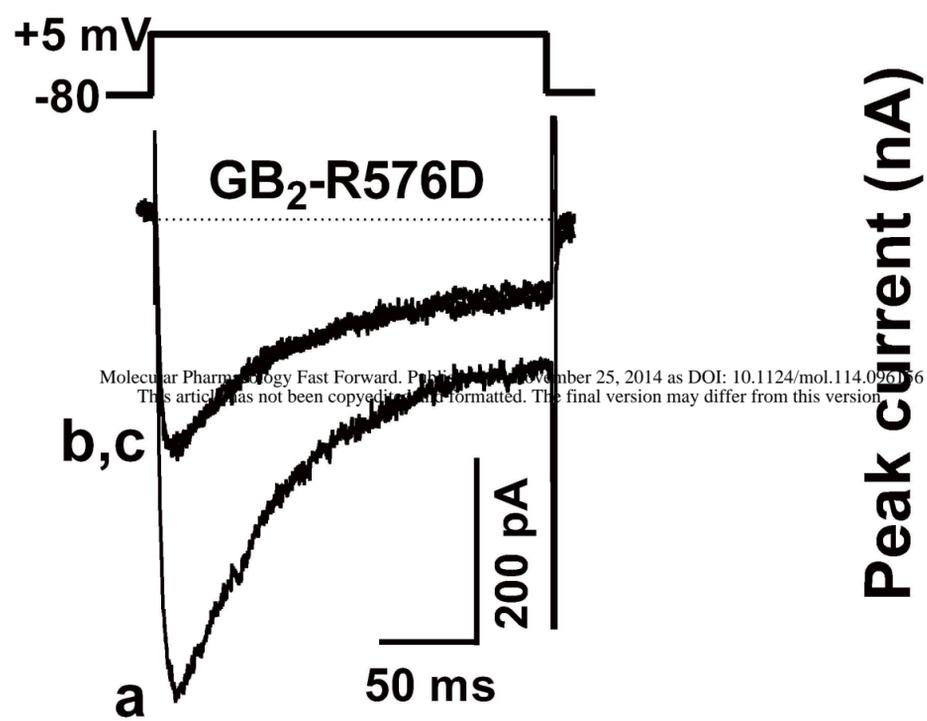


Figure 7

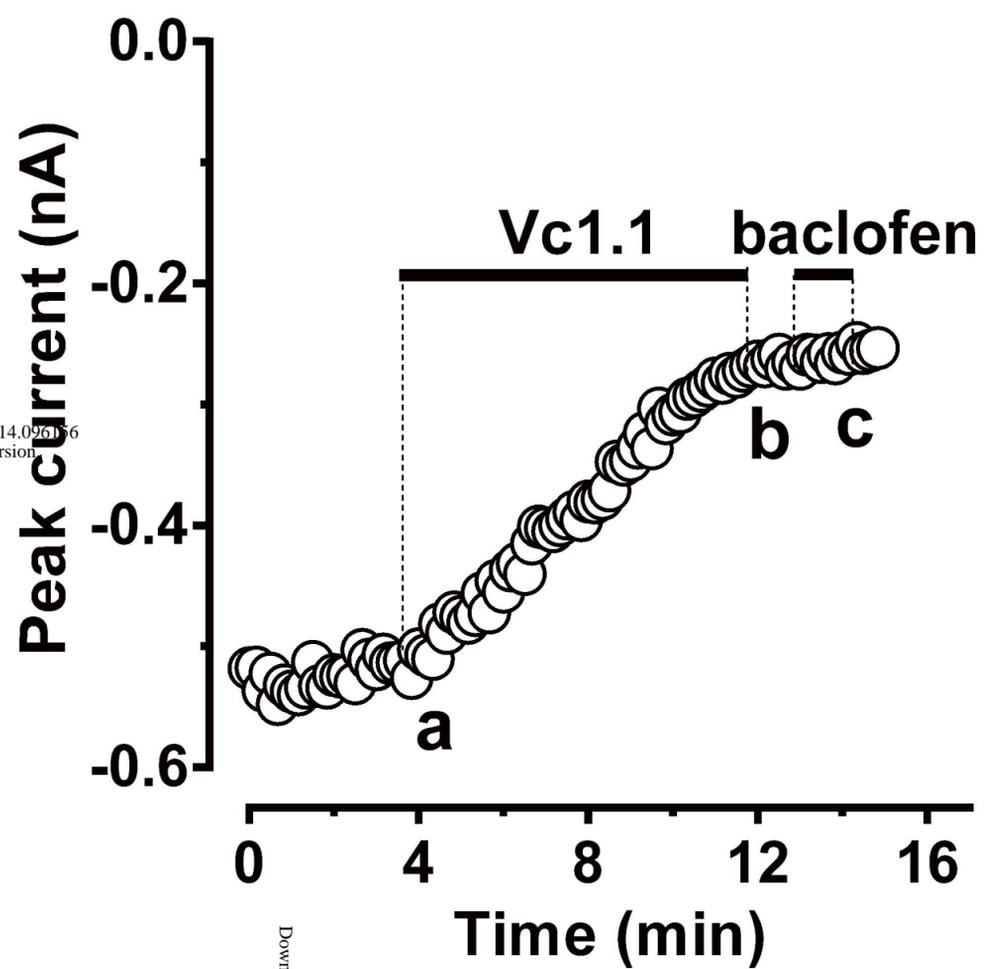
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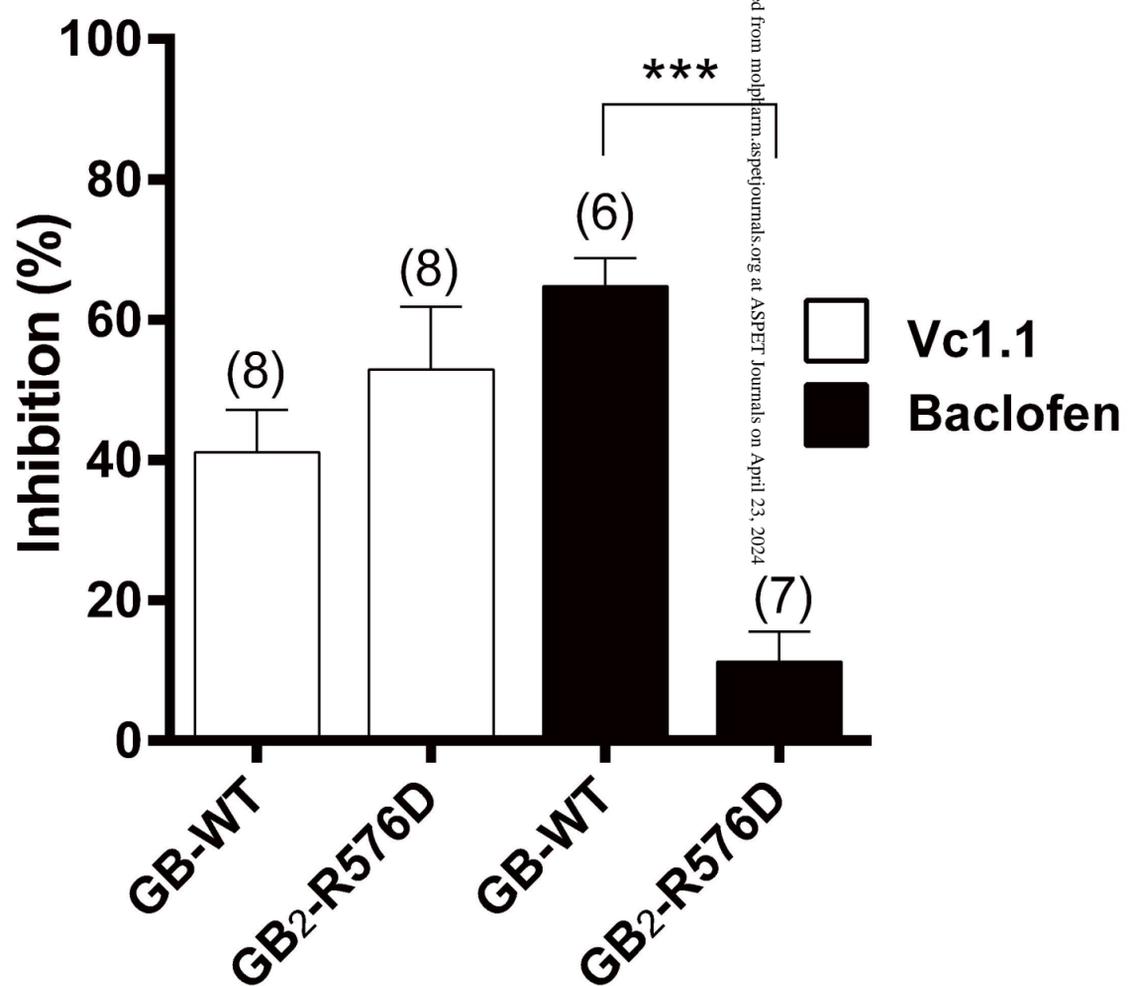
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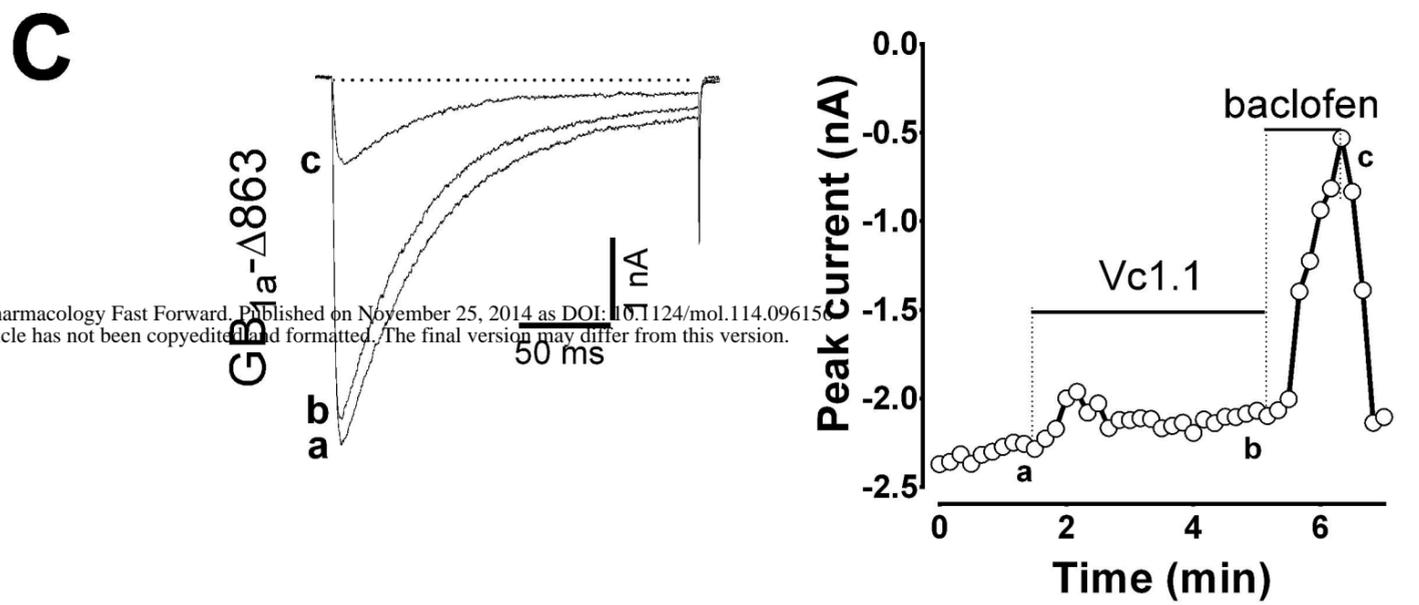
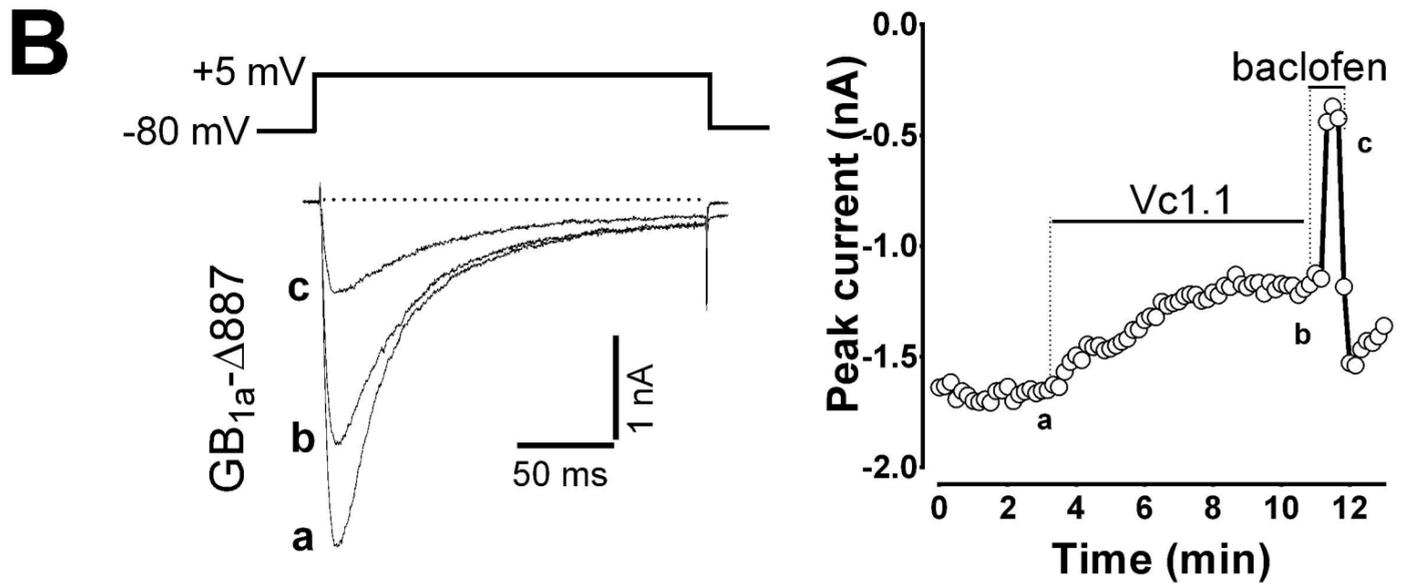
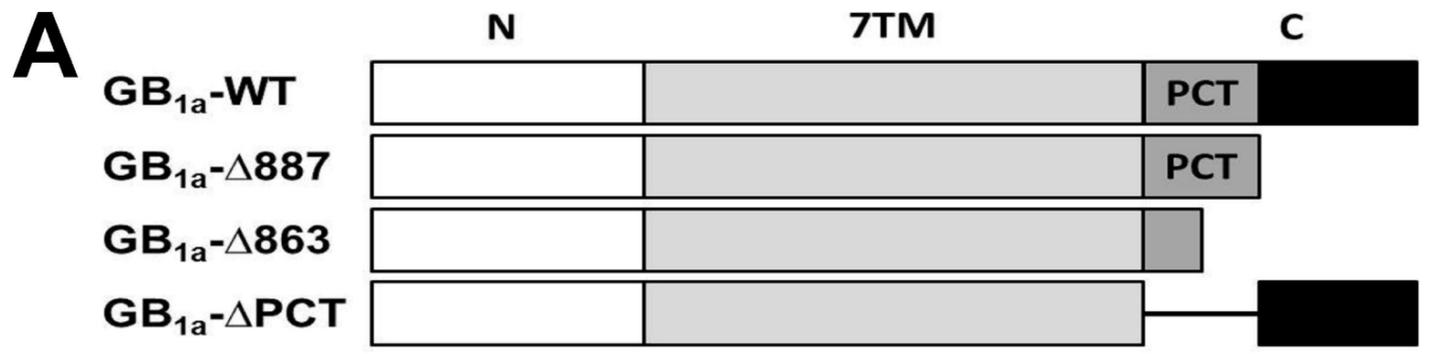
**C**



**D**



**Figure 8**



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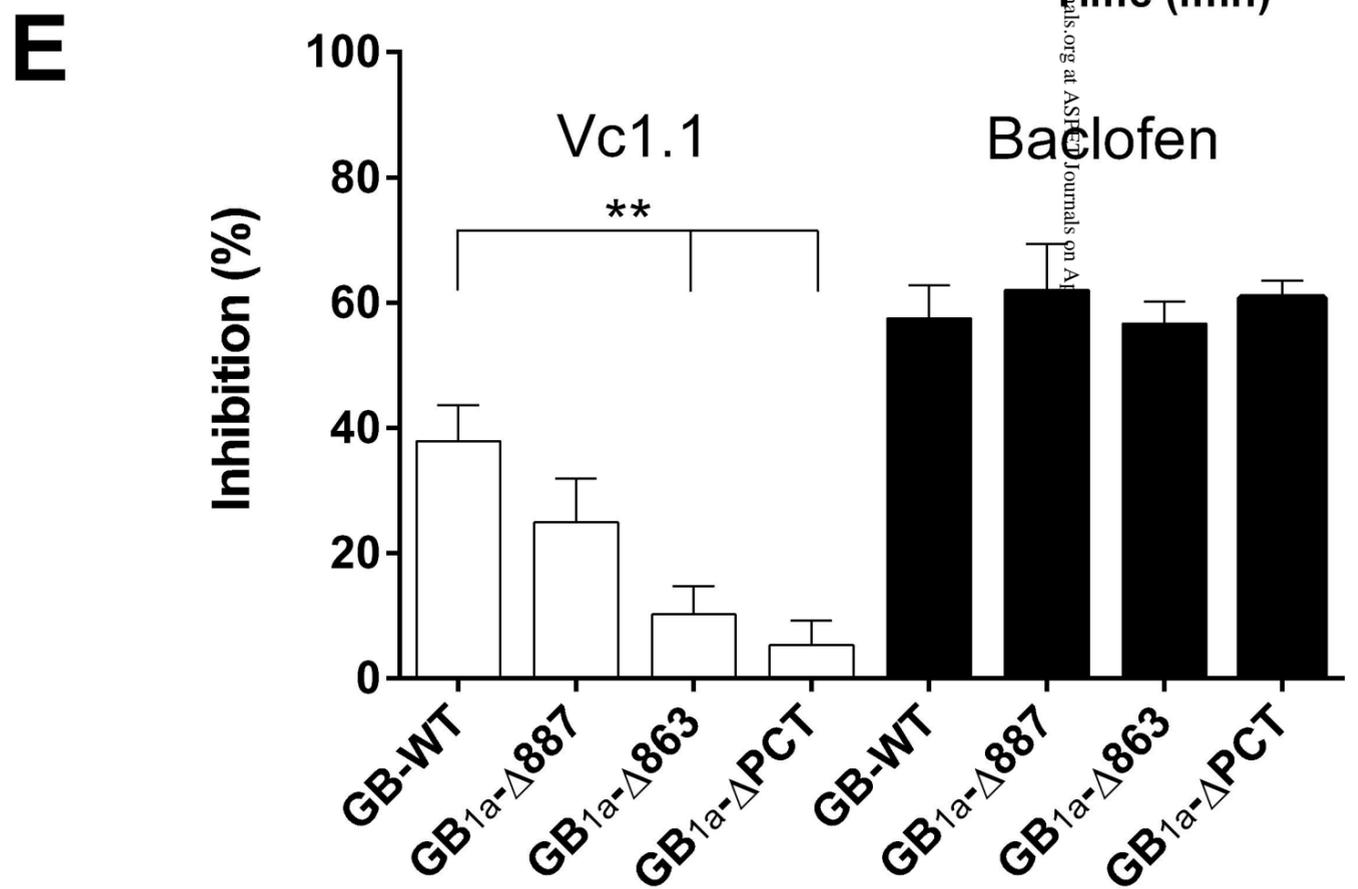
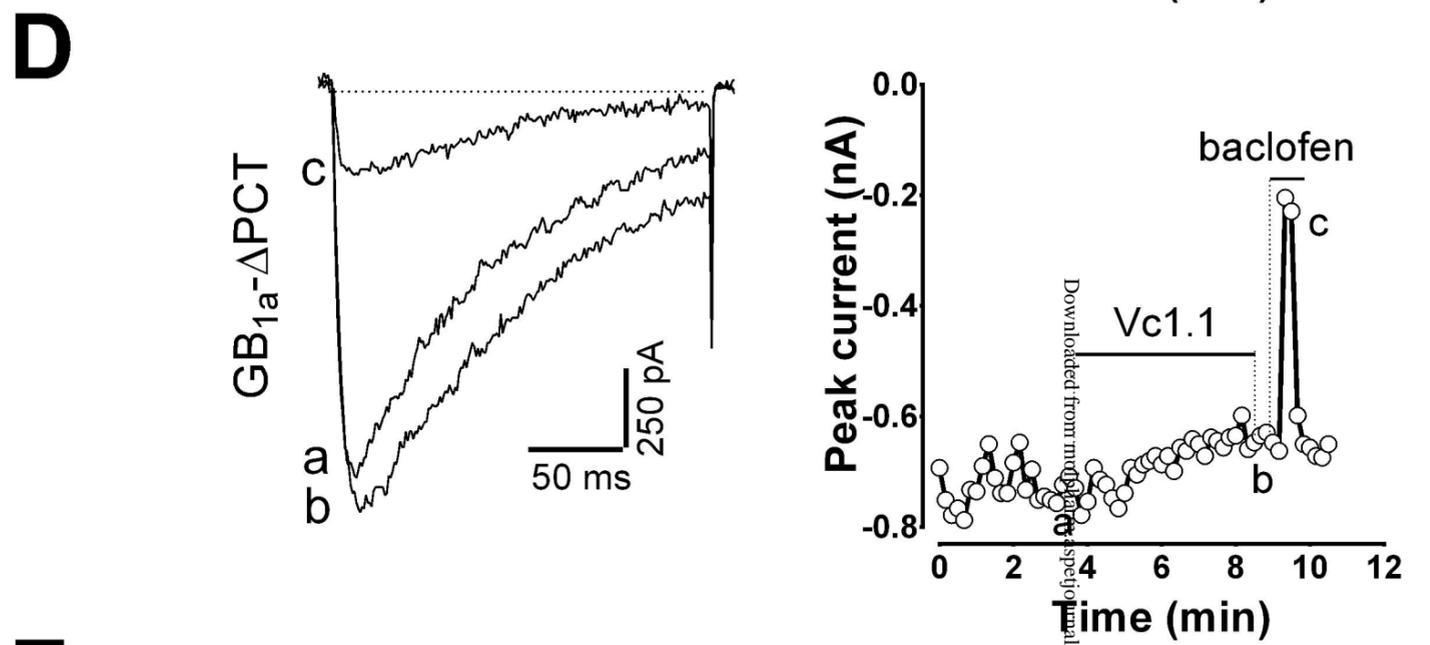


Figure 9

Baclofen

Vc1.1

