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GABA$_B$ Receptors Couple to Gα$_q$ to Mediate Increases in Voltage-Dependent Calcium Current During Development

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Abstract: Metabotropic GABA$_B$ receptors are known to modulate the activity of voltage-dependent calcium channels. Previously, we have shown that GABA$_B$ receptors couple to a non-G$_{i/o}$ G-protein to enhance calcium influx through L-type calcium channels by activating protein kinase C in neonatal rat hippocampal neurons. In this study, the components of this signaling pathway were investigated further. Gα_q was knocked down using morpholino oligonucleotides prior to examining GABA$_B$-mediated enhancement of calcium influx. When Gα_q G-proteins were eliminated using morpholino-mediated knockdown, the enhancing effects of the GABA$_B$ receptor agonist baclofen

(10 μM) on calcium current or entry were eliminated. These data suggest that GABA$_B$ receptors couple to Gα$_q$ to regulate calcium influx. Confocal imaging analysis illustrating colocalization of GABA$_B$ receptors with Gα$_q$ supports this hypothesis. Furthermore, baclofen treatment caused translocation of PKCα (protein kinase C α) but not PKCβ or PKCε, suggesting that it is the α isoform of PKC that mediates calcium current enhancement. Inhibition of calcium/calmodulin-dependent kinase II did not affect the baclofen-mediated enhancement of calcium levels. In summary, activation of GABA$_B$ receptors during development leads to increased calcium in a subset of neurons through Gα$_q$ signaling and PKCα activation without the involvement of calcium/calmodulin-dependent kinase II.
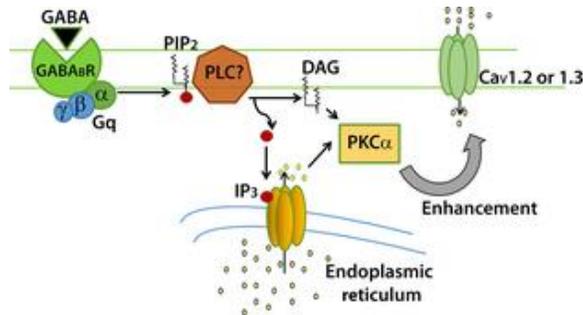
Activation of GABA$_B$ receptors in the neonatal rat hippocampus enhances voltage-dependent calcium currents independently of G$_{i/o}$. In this study, knockdown of Gα$_q$ with morpholino oligonucleotides abolished enhancement of calcium influx and protein kinase Cα was activated by GABA$_B$ receptors. Therefore, we hypothesize that GABA$_B$ receptors couple to G$_q$ to activate PKCα leading to enhancement of L-type calcium current.

Abbreviations used

Ant-AIP-II *Antennapodia* sequence-fused autocamtide-2 related inhibitory peptide II
CaMKII calcium/calmodulin-dependent protein kinase 2
CREB cAMP response element-binding protein
DAG diacylglycerol
ERK$_{1/2}$ extracellular signal-regulated kinase 1/2
GIRK G-protein coupled inwardly rectifying potassium channel
IIntide myristolated calmodulin kinase IINtide
IOD integrated optical density
IP$_3$ inositol trisphosphate
KCTD potassium channel tetramerization domain
PCC Pearson's correlation coefficient
PIP$_2$ phosphatidylinositol 4,5-bisphosphate
PKA protein kinase A
PKC protein kinase C
ROI region of interest

GABA$_B$ receptors are members of a large group of class C 7 transmembrane G-protein coupled receptors, sharing structural similarities with metabotropic glutamate receptors (Kaupmann *et al*. 1997). Uniquely, these receptors form heteromers, composed of a GABA$_{B1}$ and GABA$_{B2}$ subunit, which dimerize within the ER to be trafficked to the cell membrane. While there are allosteric interactions between the subunits during activation, the ligand binds the GABA$_{B1}$ subunit, and the G-protein interacts with GABA$_{B2}$. Functionally, GABA$_B$ receptors may be either pre- or post-synaptic, and their activation is considered inhibitory via coupling to G$\alpha_{i/o}$ G-proteins. There are three well-described mechanisms of this inhibition: first, the α subunit of G$\alpha_{i/o}$ signaling inhibits adenylyl cyclase, reducing cAMP levels and/or protein kinase A (PKA) activation (Nishikawa *et al*. 1997). Second, calcium channels are directly inhibited by the $\beta\gamma$ subunit. This reduces pre-synaptic neurotransmitter release and post-synaptic dendritic calcium spikes (Chalifoux and Carter 2011). Finally, GABA$_B$ receptor stimulation activates G-protein-coupled inwardly rectifying potassium channels, again through direct interaction with the $\beta\gamma$ subunit (Reuveny *et al*. 1994; Wickman *et al*. 1994). In this case, potassium efflux generates inhibitory post-synaptic potentials which hyperpolarize the cell.

While cases of GABA$_B$ receptor activation leading to inhibition have been well documented, there are reports demonstrating an enhancement of voltage-dependent calcium channel activity (Shen and Slaughter 1999; Carter and Mynlieff 2004; Bray and Mynlieff 2009, 2011; Park *et al*. 2010; Im and Rhim 2012). In the hippocampus, long-lasting (L-type) current is facilitated in a subset of cells when GABA$_B$ receptors were activated by the GABA$_B$ receptor-specific ligand baclofen (Carter and Mynlieff 2004). This phenomenon is observed early in development, peaking at postnatal day 6 (P6) to P8 in rats (Bray and Mynlieff 2009). Furthermore, this GABA$_B$ receptor-mediated enhancement of calcium current was not sensitive to pertussis toxin, a known inhibitor of G$\alpha_{i/o}$-mediated processes (Bray and Mynlieff 2011). Our laboratory demonstrated that this enhancement is dependent on protein kinase C (PKC) activation. Enhancement of calcium current was eliminated when global PKC inhibitors were used and treatment of cells with the PKC activator phorbol 12-myristate 13-acetate (PMA) mimicked the effect of activating GABA$_B$ receptors. However,

participants in the signaling pathway, such as specific G-proteins and isoforms of PKC, have not been previously investigated.

This study sought to identify components of the $GABA_B$ receptor mediated increase in calcium current. Because the effect of calcium current enhancement was not sensitive to pertussis toxin, we hypothesized that $GABA_B$ receptors may couple to another G-protein besides $G\alpha_{i/o}$. It is not likely a $G\alpha_s$ G-protein, because this pathway initiates PKA signaling. Instead, we hypothesized that the G-protein involved in this signaling pathway was a member of the $G\alpha_q$ family of G-proteins ($G\alpha_q$, $G\alpha_{11}$, $G\alpha_{14}$, $G\alpha_{15/16}$), which are known to activate PKCs through activation of phospholipase Cβ. To test this hypothesis, a morpholino-induced knockdown strategy was used to inhibit signaling of specific G-proteins.

Next, the identity of the specific isoform of PKC that is involved in the signal transduction pathway was investigated. There are at least 15 isoforms of PKC, separated into three families based on their requirements for activation. Conventional PKCs require calcium ions, diacylglycerol (DAG), and a phospholipid such as phosphotidylserine. Novel PKCs require DAG and a phospholipid, but not calcium ions; atypical PKCs require neither DAG nor calcium ions for activation. As PMA treatment mimics $GABA_B$ receptor activation, the isoform was not likely to be atypical. Furthermore, the highest proportion of neurons demonstrating current enhancement occurs at P7. Different PKC isoforms demonstrate different temporal expression, so those isoforms which are highly expressed at P7, and also not of the atypical family, were examined using translocation to the membrane as a measure of activation.

Finally, the involvement of calcium/calmodulin-dependent kinase II (CaMKII) as a component of the signaling cascade was investigated. It has been shown previously that CaMKII is required in other G-protein pathways which result in calcium current enhancement (O-Uchi *et al*. 2008). In addition, CaMKII is known to phosphorylate L-channels, thereby increasing current (Hudmon *et al*. 2005; Abiria and Colbran 2010). Thus, we hypothesized that PKC may activate CaMKII, which in turn phosphorylates the calcium channel. Results presented here provide evidence that the $GABA_B$ receptor couples to $G\alpha_q$ in neonatal hippocampal neurons, causing PKCα to translocate to the cell

membrane, where it putatively interacts with L-type calcium channels, bypassing the involvement of CaMKII.

Methods

Isolation of hippocampal neurons

All animal protocols were approved by the Marquette University Institutional Animal Care and Use Committee according to guidelines set forth by the National Research Council in the Guide for the Care and Use of Laboratory Animals. Hippocampal neurons were obtained from P6–P8 Sprague–Dawley rat pups of either sex (Onsite breeding colony, parents from Charles River Laboratories; Mynlieff 1997). Briefly, pups were anesthetized with 100% CO_2 and decapitated. Using sterile technique, the superior regions of the hippocampi were dissected, diced, and incubated in oxygenated PIPES-buffered saline with 0.5% Trypsin XI and 0.01% DNase I (Sigma-Aldrich, St. Louis MO, USA). After a 30 min incubation at 22°C the tissue was placed in a 35°C water bath for 60 min. After a series of rinses, the tissue was triturated and the cell suspension was plated onto poly-l-lysine coated dishes or glass coverslips (MW 38 500–60 000; Sigma Aldrich) and transferred to a 37°C 5% CO_2 water-jacketed incubator. Cultures were maintained a minimum of 20 h before experiments to allow recovery from enzymatic digestion.

Protein knockdown

Knockdown of $G\alpha_q$ G-proteins in neuronal cultures was performed with morpholinos delivered by the Endo-Porter delivery system (Gene Tools LLC, Philomath, OR, USA). Morpholino oligonucleotides were designed to sterically inhibit translation of $G\alpha_q$ mRNA (Entrez ID: 13591956 NM_031036, 5'ACGCCATGATGGACTCCAGAGTCAT); negative nonsense controls (5'AAACCCGGGTTTACG) were used to insure specificity of the target oligo. Morpholinos were synthesized with a 3' carboxyfluorescein tag to visualize delivery. Endo-Porter peptide (final concentration 4 μM) and morpholino oligos (final concentration 2 μM) were added one time to the Neurobasal-A media in the culture dishes immediately after dissociation of the cells. Delivery of oligos and cell viability was

assessed at 24, 48, and 96 h post delivery to insure cargo was delivered and cell health was not negatively impacted.

Western blot analysis

Whole hippocampal tissue or neurons from hippocampal cultures were obtained from rat pups age P6–P8. For whole tissue samples, hippocampi were dissected and homogenized in ice-cold buffer with protease inhibitors (250 mM sucrose, 10 mM Tris, 10 mM HEPES, 1 mM EDTA, 1 µg/mL pepstatin, 1 µg/mL leupeptin, 0.5 mg/mL Pefabloc; Sigma Aldrich; pH 7.2 with HCl) followed by centrifugation at 3287 g for 10 min at 4°C. The supernatant was centrifuged at 41473 g for 30 min at 4°C, and the pellet was re-suspended in buffer and stored at −80°C. For cultured cell samples, neurons were scraped from culture dishes in homogenization buffer with protease inhibitors. The sample was centrifuged at 25401 g for 10 min at 4°C, and the pellet was re-suspended in 5% of the original volume of buffer to concentrate the sample. The suspension was centrifuged at 3287 g for 10 min, the supernatant was retained, and centrifuged at 41473 g for 30 min. The pellet was re-suspended in 15% of the original volume of homogenization buffer and stored at −80°C. Protein concentrations were measured at 280 nm with a Biophotometer (Eppendorf, Enfield, CT, USA).

NuPAGE® lithium dodecyl sulfate sample buffer and reducing agent (Life Technologies, Carlsbad CA, USA) were added to protein samples and the mixture was heated at 70°C for 10 min. The protein samples were run on a NuPAGE® Novex 12% Bis-Tris minigel and transferred to a polyvinylidene difluoride membrane (0.45 µM pore size) using NuPAGE® transfer buffer (Life Technologies). Membranes were washed with phosphate-buffered saline (PBS, 134.4 mM NaCl, 4.36 mM KCl, 10.56 mM $NaHPO_4$, 1.66 mM NaH_2PO_4, pH to 7.4 with HCl) and blocked for 2 h in PBS containing 0.05% Tween, 5% non-fat dry milk, and 0.1% bovine serum albumin at 22°C. Proteins on membranes were labeled with polyclonal rabbit anti-$G\alpha_q$ (anti-GNAQ, 1 : 500-1 : 1000, GeneTex catalog# GTX114029, Irvine CA, USA) or polyclonal rabbit anti-$G\alpha_{11}$ (1 : 500, GeneTex catalog# GTX118876) in blocking solution. After a 90 min wash in PBS with 0.05% Tween, membranes were incubated with goat anti-rabbit horseradish

peroxidase-conjugated secondary antibody (1 : 1000-1 : 2500, Pierce, Rockford IL, USA). The SuperSignal West Dura Extended Duration chemiluminescent enhancement kit (Pierce) was used to visualize the protein bands. The amount of protein loaded was verified by labeling the membranes with anti-β-tubulin antibodies (1 : 2000, Cell Signaling, Danvers MA, USA). Quantification of bands was performed by measuring the integrated optical density (IOD) for each band using Labworks 4.6 software (UVP, Upland CA, USA). In experiments where expression was knocked down, data were normalized by dividing the IOD of the band labeled with anti-G-protein antibodies with the IOD of the band in the same lane visualized with anti-β-tubulin antibodies.

Electrophysiology

Calcium currents were measured in whole cell voltage clamp mode using a Dagan 3900A patch clamp amplifier (Dagan Corporation, Minneapolis MN, USA), Digidata 1322A acquisition setup, and pClamp 10.0 software (Molecular Devices, Sunnyvale CA, USA). Extracellular recording solution (pH 7.4 with CsOH, 310–320 mOsm/L) contained 10 mM $CaCl_2$, 145 mM tetraethylammonium chloride, 10 mM HEPES, and 1 μM tetrodotoxin (Tocris Bioscience, Ellisville MO, USA). Recording electrodes were pulled from borosilicate glass on a Flaming/Brown Micropipette Puller (model P87, Sutter Instrument Co., Novato CA, USA) to a resistance of 5–9 MΩ and filled with intracellular solution (140 mM Cs-aspartate, 5 mM $MgCl_2$, 10 mM Cs_2EGTA, 10 mM HEPES, 2 mM ATP-Na_2, and 0.1 mM guanosine triphosphate; pH 7.4 with CsOH, 300–310 mOsm/L). Cells were held at −80 mV and depolarized to +10 mV with a 300 ms pulse. Whole cell currents were electronically filtered at 1 kHz and digitized at 2 kHz. Linear components of leak current were subtracted *post hoc* by the passive resistance protocol in pClamp 10.0. The GABA$_B$ agonist (RS)-baclofen (Tocris Bioscience) was dissolved in HCl, diluted 1 : 1000 in recording solution to a final concentration of 10 μM and applied to neurons using a *U*-tube delivery system constructed with PE-10 polyethylene tubing housed in a glass tube. In experiments designed to test the effect of CaMKII inhibition, CaMKII inhibitors cell permeable autocamtide-2 related inhibitory peptide (Ant-AIP-II, IC$_{50}$ 4 nM, catalog#189485, EMD Millipore, Billerica, MA, USA; Ishida *et al.* 1998) or myristolated calmodulin kinase IINtide (IINtide, IC$_{50}$ 50 nM, catalog#208921, EMD

Millipore; Chang *et al*. 1998) were also added to the baclofen solution and delivered via *U*-tube.

Calcium current measurements were taken at the end of the 300 ms depolarizing pulse to maximize the contribution of L-type channels to the total current while minimizing the contribution of N- and P/Q-type channels. A graph of elicited sustained current magnitude versus time was plotted, and a linear regression with 95% confidence interval was constructed to account for run-up or run-down of calcium current (Carter and Mynlieff 2004; Bray and Mynlieff 2011). A cell that showed an increase or decrease in sustained calcium current in response to baclofen displayed a current magnitude that fell outside the confidence interval.

Calcium imaging

Cultured neurons were incubated for 1 h in the dark at 22°C in calcium imaging Ringer's solution (CIR, 154 mM NaCl, 5.6 mM KCl, 1 mM $MgCl_2$, 2 mM $CaCl_2$, 10 mM HEPES, 11 mM glucose, pH 7.4 with NaOH) with 5 µM Fura-2 acetoxymethyl ester (Fura-2 AM, Life Technologies). Cells were rinsed and incubated for 30 min at 22°C in CIR without FURA-2 AM to allow for de-esterification. Neurons were depolarized by perfusion of a high potassium solution (100 mM NaCl, 50 mM KCl, 2 mM $CaCl_2$, 1 mM $MgCl_2$, 10 mM HEPES, 11 mM glucose, pH to 7.4 with NaOH) to open voltage-gated calcium channels. Baclofen (10 µM) in CIR was perfused onto the cells for 15 s, followed by baclofen in high potassium solution for 30 s. CaMKII inhibitors were added to the CIR/baclofen solution for pre-treatment, then added to the high potassium/baclofen solution and applied via gravity. The fluorophore was excited at 340 and 380 nm, and emissions were measured at 510 nm using Slidebook 5.0 software (Intelligent Innovations, Denver CO, USA).

To determine inherent variability of the system, a group of control neurons were treated with high potassium solution three consecutive times ($N = 309$). Response #1 and #3 were averaged as the 'control' and the percent change with response #2 was calculated as the 'pseudo' drug response. The response to the second application of high potassium gave an average of 101.5 ± 13.20 (standard deviation) percent change when compared with the average of the

responses to application #1 and #3. During experiments, neurons were stimulated three times; the first and third stimulations were high potassium alone, and the middle stimulation was high potassium with another agent. To determine the effect of agent application during stimulation 2, its value was compared to the averaged value of stimulations 1 and 3 (high potassium control stimulations). The percent change with agent treatment when compared with the averaged before and after values was classified as an increase or decrease for a particular cell if the percent change with baclofen in 50 mM potassium solution was > 2 times the standard deviation (2 × 13.20) of the cellular response from the control neurons stimulated three times consecutively with high potassium solution alone.

Immunostaining

To analyze the colocalization of GABA$_B$ receptors and Gα_q, hippocampi from P6 to P8 rats were dissected, fixed, frozen and sliced into 20 μm sections. Sections were permeabilized with 0.5% Triton X-100 in PBS for 20 min and blocked with 10% goat serum (Invitrogen, Carlsbad, CA, USA) in PBS with 0.05% Triton X-100. The sections were incubated for 2 h with polyclonal rabbit anti-GNAQ (1 : 1000, Genetex catalog# GTX114029) and monoclonal mouse anti-GABABR1 (1 : 15, UC Davis Neuromab, catalog# 73-183, Davis, CA, USA) in PBS with 0.05% Triton X-100 and 0.1% goat serum. After rinsing, sections were incubated with Dylight® 488-conjugated goat anti-rabbit IgG and Dylight® 550-conjugated goat anti-mouse IgG (Thermo Scientific, Rockford, IL, USA) in PBS with 0.05% Triton X-100 and 0.1% goat serum. After rinsing, mounting medium with DAPI (4',6-diamidino-2-phenylindole; Vector Laboratories, Burlingame, CA, USA) was applied to stain nuclei; the sections were covered with a glass coverslip and sealed. Sections were imaged with a Nikon Perfect Focus Ti-E confocal microscope and NIS Elements imaging software (Nikon Instruments, Melville, NY, USA).

To quantify the colocalization of GABA$_B$ receptors and Gα_q G-proteins, regions of interest (ROI's) in the stratum oriens, pyramidal cell layer, and stratum radiatum were drawn for each image. A Pearson's correlation coefficient (PCC) was determined to quantify the

degree of colocalization of the two proteins in each ROI. The PCC measures overlap of pixels for the two fluorophores; the higher the PCC, the more likely that the two proteins are overlapping in that ROI. A PCC was determined for the 3 ROI's in each image (16 sections taken from three animals), then averaged across sections, such that there was a single PCC value for the stratum oriens, pyramidal cell layer, and stratum radiatum, respectively.

To analyze the translocation of PKC, neuronal cultures were treated with 1 μM phorbol-12-myristate-13 acetate (PMA, Calbiochem, La Jolla CA, USA) or 10 μM baclofen for 10 min prior to fixation. Cells were fixed in a solution of 4% paraformaldehyde in PBS for 30 min at 22°C, permeabilized in PBS with 0.5% Triton X-100 for 20 min and blocked with 10% goat serum in PBS with 0.05% Triton X-100 for 45 min. The cells were incubated with either monoclonal rabbit anti-PKCα (1 : 500 dilution, catalog# GTX61153, Genetex), polyclonal rabbit anti-PKCβ (1 : 200 dilution, catalog# E021184, EnoGene Biotech Co., Ltd., New York, NY, USA) or polyclonal rabbit anti-PKCε (1 : 1000 dilution, catalog# GTX 109028, Genetex) overnight at 4°C. Following two washes with PBS and 0.05% Triton X-100, cells were incubated with Dylight® 488-conjugated goat anti-rabbit secondary antibodies (1 : 500 dilution) for 1 h at 22°C. Mounting medium with DAPI was applied for visualization of nuclei, and cells were imaged on a Nikon Perfect Focus Ti-E confocal microscope and NIS Elements imaging software (Nikon Instruments).

Statistics

Comparisons of the number of cells that exhibited either enhancement of calcium entry with ratiometric imaging or enhancement of calcium current with electrophysiology in cells treated with morpholinos or CaMKII inhibitors were analyzed by a Chi-squared test. A one-way anova was performed on PCC values obtained for the different regions of the hippocampus using confocal image analysis of protein colocalization followed by Holm–Sidak pairwise comparisons. Differences in the number of cells demonstrating translocation of different PKC isoforms were analyzed with Fisher's exact test.

Results

Morpholino oligos inhibit Ga_q protein expression

To verify that morpholino oligos were able to penetrate the cell membrane and were not detrimental to cell viability, cultures were treated with Endo-Porter and fluorescein-labeled morpholinos and examined after 24, 48, and 96 h of incubation. Figure 1a shows cultures after 96 h treatment. Cells maintained their characteristic shapes and exhibited growing processes. Figure 1b illustrates that essentially every cell exhibited some level of cytoplasmic fluorescence. Although fluorescence levels vary, no punctate fluorescence is seen, which would indicate morpholinos unable to exit endosomes. Visible fluorescence requires ten-fold higher concentrations of morpholino than are necessary to produce significant protein knockdown (Gene-tools, 2015). At concentrations ≥ 4 µM of oligos or ≥ 6 µM Endo-Porter, morpholinos began to negatively affect growth and viability of neuronal cultures (data not shown). Thus, 2 µM morpholino and 4 µM Endo-Porter were used in all experiments. To determine the effectiveness of morpholino-induced protein knockdown, neurons were treated with Endo-Porter alone, nonsense morpholinos, or morpholinos against Ga_q and western blotting was performed at various time points after the treatment. Figures 1c and d show the timecourse of Ga_q knockdown. As demonstrated in Fig. 1d, preparations treated for 48 h with morpholinos against Ga_q appeared to have less Ga_q than preparations treated with nonsense morpholinos. Cultures treated with Endo-Porter alone or nonsense morpholinos demonstrated normal levels of Ga_q protein expression (e.g., see inset in Fig. 1d). Thus, using the Endo-Porter delivery system, Ga_q protein expression in neuronal cultures was reduced without negatively impacting cell health or viability. As a result of the relatively long half-life of G-proteins (Derrien *et al*. 1996), all electrophysiological and calcium imaging experiments were performed following 48 h incubation with Ga_q morpholinos to insure sufficient protein knockdown.
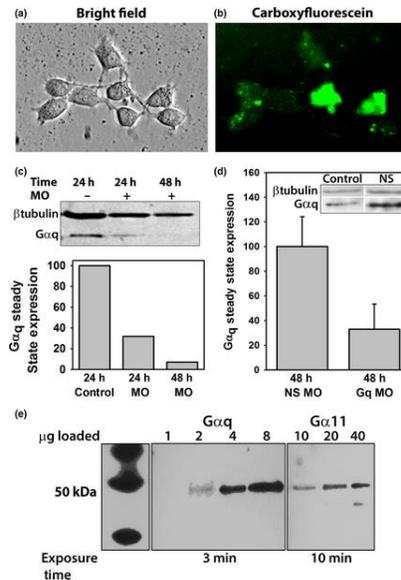
Figure 1. (a) Differential interference contrast image of a hippocampal culture from a postnatal day 4 rat pup treated with 10 μM control morpholino oligos and 2 μM Endo-Porter peptide, and maintained in culture for 96 h. (b) Fluorescent image of (a); oligos were tagged with carboxyfluorescein for visualization. (c) Western blot of proteins isolated from either a control culture (lane 1), a culture treated for 24 h (lane 2) or 48 h (lane 3) with 2 μM $G\alpha_q$ oligos. Protein bands were visualized with $G\alpha_q$ antibodies and β-tubulin antibodies as a loading control. $G\alpha_q$ band densities were calculated by dividing the integrated optical density (IOD) of the $G\alpha_q$ band by the IOD of the β tubulin band to control for loading and expressed as percent of control. (d) Western blot analysis of six different preparations from cultured neurons treated for 48 h with nonsense oligos ($N = 3$, NS MO) or $G\alpha_q$ oligos ($N = 3$, Gq MO). The data were analyzed as in (c), with the average of the nonsense oligo-treated preparations defined as 100%. Data are expressed as mean ± SEM. The inset is an example of $G\alpha_q$ expression in control tissue compared to cells treated with nonsense morpholinos to demonstrate that the nonsense morpholinos do not cause non-specific knockdown of $G\alpha_q$. (e) Western blot analysis of $G\alpha_q$ and $G\alpha_{11}$ G-protein in the neonatal hippocampus. Analysis was performed using tissue from the superior region of the hippocampus and antibodies against either $G\alpha_q$ or $G\alpha_{11}$ proteins. The left panel shows molecular weight markers (same experiment but exposed to film for 1 s). To visualize bands labeled with $G\alpha_{11}$ antibody, 10 times more protein was loaded (right panel) than for lanes labeled with $G\alpha_q$ antibodies (center panel) and the exposure time was increased to 10 min, compared with 3 min exposure for bands labeled with $G\alpha_q$ antibodies.

$G\alpha_q$ protein is more highly expressed than $G\alpha_{11}$ in neonatal hippocampus

$G\alpha_{11}$ shares 90% sequence similarity with $G\alpha_q$, and both G-proteins have a ubiquitous distribution pattern (Mizuno and Itoh 2009). Therefore, it was important to determine whether there could be non-specificity in the morpholino knockdown of $G\alpha_q$ that may decrease $G\alpha_{11}$ as well. Protein preparations from cultured cells treated

with $G\alpha_q$ or nonsense morpholinos were analyzed by western blotting using $G\alpha_{11}$ antibodies. If the morpholinos were specific to $G\alpha_q$, there would be no change in the level of $G\alpha_{11}$. However, no $G\alpha_{11}$ signal was detected, even in control cultures not treated with morpholinos or Endo-Porter (data not shown). This result was suggestive that either the basal level of $G\alpha_{11}$ was too low to detect with western blotting in preparations of cultured neurons, or that the $G\alpha_{11}$ antibody did not recognize its target. To determine if the $G\alpha_{11}$ antibody could recognize $G\alpha_{11}$ G-protein, protein samples were prepared from the superior region of fresh whole hippocampus so that the amount of protein loaded on the gel could be increased. The $G\alpha_{11}$ antibody recognizes its target, but only if 10 times the amount of protein was loaded and the membrane was exposed to film for a longer period of time (Fig. 1e). When a lane loaded with 2 µg of protein was probed with $G\alpha_q$ antibody, the IOD was 7.2. When a lane loaded with 20 µg of protein was probed with $G\alpha_{11}$ antibody, the IOD was 5.0 on the same film at the same exposure. These data suggest that the $G\alpha_q$ antibody recognizes its protein target without detecting any $G\alpha_{11}$ protein. Thus, while it cannot be ruled out that the $G\alpha_q$ morpholino may non-specifically knock down $G\alpha_{11}$ G-protein, a more likely explanation is that while both G-proteins are present, only $G\alpha_q$ G-protein is knocked down.

Inhibiting $G\alpha_q$ expression abolishes baclofen-mediated enhancement of voltage-dependent calcium entry or current

Despite a lack of precedence for the $GABA_B$ receptor coupling to a $G\alpha_q$ family member, downstream actions, including PKC activation, were consistent with the actions of a $G\alpha_q$ type G-protein. Therefore, we chose to knock down $G\alpha_q$ and assess whether application of the $GABA_B$ receptor agonist baclofen (10 µM) could still lead to calcium current enhancement. Hippocampal neurons were treated with either nonsense or $G\alpha_q$ morpholinos, and enhancement of calcium entry or current was assessed with calcium imaging and electrophysiology. In the nonsense morpholino-treated cells, 6 of 64 cells (9.37%) demonstrated an enhancement of high potassium induced calcium entry with baclofen treatment using calcium imaging (Fig. 2a). Of the 207 cells treated with $G\alpha_q$ morpholinos, no cells demonstrated an

enhancement of high potassium-induced calcium entry with baclofen (Fig. 2c, Chi square, $p < 0.001$). Electrophysiology verified this result; in control cells treated with nonsense oligos, 3 of 25 cells demonstrated an enhancement of sustained calcium current when baclofen was applied (Fig. 2b). In contrast, no cells of 15 treated with $G\alpha_q$ morpholino oliogos for 48 h showed any calcium current enhancement (Fig. 2d). This set of experiments demonstrates that knockdown of the $G\alpha_q$ G-protein abolished enhancement of calcium current or entry upon $GABA_B$ receptor activation, linking a $G\alpha_q$ family member to this pathway and thus the $GABA_B$ receptor itself.
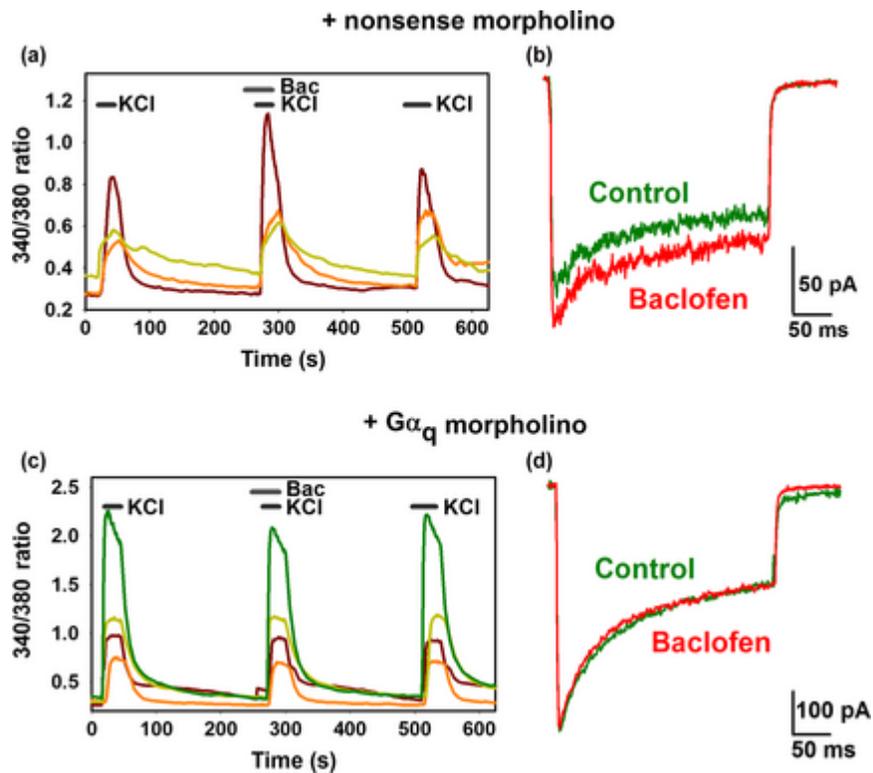
Figure 2. Effect of 48 treatment with $G\alpha_q$ morpholino oligos on calcium entry in hippocampal neurons. Neurons were treated for 48 h with nonsense morpholino oligos (a and b) or $G\alpha_q$ morpholino oligos (c and d). (a and c) Increases in intracellular calcium were measured with ratiometric calcium imaging and reported as the 340/380 ratio. Neurons were stimulated with high potassium, followed by a pre-treatment with 10 µM baclofen and baclofen in high potassium (see 'Methods'). Each colored line represents a single cell. (b and d) Calcium currents were elicited by a 300 ms depolarizing pulse to +10 mV from a holding potential of −80 mV in the absence (green trace) and presence of 10 µM baclofen (red trace).

GABA_B receptors colocalize with Ga_q G-proteins

When hippocampal sections were labeled with anti-GABA_B receptor antibodies (red), nearly all areas were labeled, with the exception of the granule cells in the dentate gyrus (Fig. 3a). At high magnification, there was an absence of nuclear labeling with GABA_B antibodies, with a clear delineation between the nuclei and the labeling (Fig. 3e and h). Antibodies against Ga_q labeled cells within both the pyramidal cell layer and the granule cell layer (Fig. 3b and f). In addition, there appeared to be individual cell bodies labeled within the stratum oriens and stratum radiatum without any diffuse labeling throughout these layers, as seen with GABA_B antibodies. At low magnification, colocalization of the two fluorphores was most evident in the pyramidal cell layer and is absent from the dentate gyrus granule cell layer (see white arrows indicating pyramidal cell layer and granule cell layer; Fig. 3c and d). Quantitative analysis done at high magnification in the superior region of the hippocampus supports qualitative evidence seen at low magnification (Fig. 3c and g). The average Pearson's Correlation Coefficient (PCC), used to determine colocalization, was 0.125 ± 0.03 (SEM) in the stratum oriens. The PCC was 0.476 ± 0.03 in the pyramidal cell layer and 0.102 ± 0.01 in the stratum radiatum. The colocalization of GABA_B receptors and Ga_q is highest in the pyramidal cell layer, as suggested by the PCC value; the PCC is significantly higher in the pyramidal cell layer than either the stratum oriens or stratum radiatum (Fig. 3g; One-way anova followed by Holm–Sidak pairwise comparison, $p < 0.001$). A small number of neurons in both the stratum radiatum and the stratum oriens were labeled by antibodies against Ga_q, and some of these neurons also appeared to be labeled with GABA_B receptor antibodies (Fig. 3i, arrowhead). These neurons had a relatively high (0.5–0.75) PCC value. The functional significance or physiological identity of these neurons is unknown.
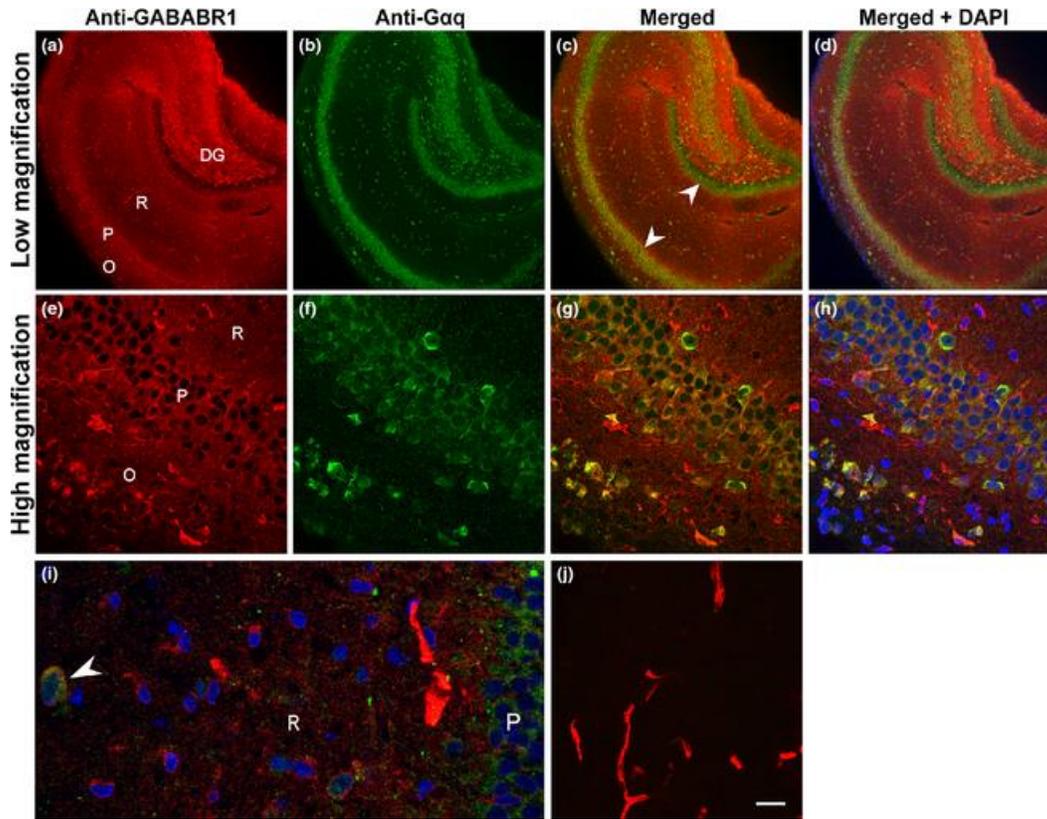
Figure 3. GABA$_B$ receptors colocalize with Gα$_q$ G-proteins in rat hippocampus. Hippocampal sections were labeled with polyclonal rabbit anti-GNAQ and monoclonal mouse anti-GABABR1 followed by Dylight® 488-conjugated goat anti-rabbit IgG (green) and Dylight® 550-conjugated goat anti-mouse IgG (red) for visualization. Nuclei were stained blue with DAPI. Colocalization of the red and green fluorophore appears yellow in the merged images (c, d, g, h, and i). (a–d) Low magnification images showing the whole section of hippocampus. (e–h) High magnification images showing the pyramidal cell layer (P), the stratum oriens (O), or the stratum radiatum (R). A small number of cells in both the stratum oriens and stratum radiatum appeared to show colocalization of GABA$_B$ and Gα$_q$ with a high Pearson's correlation coefficient (PCC) value. (i) Expansion of a high magnification image demonstrating colocalization(white arrowhead). (j) control sections (high magnification) processed without primary antibodies demonstrate autofluorescence of blood vessels. DG dentate gyrus. Scale bar in panel (high magnification) *J* = 10 µm.

CaMKII activity is not involved in GABA$_B$ receptor-mediated increases in intracellular calcium

Cultured neurons were depolarized with 50 mM potassium solution, followed by a 50 mM potassium solution containing both 10 µM baclofen and a cell permeable CaMKII inhibitor, Ant-AIP-II (50 nM). Both baclofen and Ant-AIP-II were applied during a 15 s pre-treatment period as well as during the high potassium application.

Twenty-two of 255 (8.63%) cells imaged continued to demonstrate an increased 340/380 ratio, despite the presence of the inhibitor. When the inhibitor/baclofen solution was washed off, the response returned to original levels (Fig. 4a). Because there have been reports of non-specific actions of CaMKII inhibitors on voltage-dependent calcium channels (Gao *et al*. 2006; Karls and Mynlieff 2013), the result was confirmed using a second cell permeable inhibitor, IINtide (250 nM). A similar result was obtained, where 47 of 432 (10.88%) neurons showed a reversible increase in 340/380 ratio when baclofen was co-applied with IINtide (Fig. 4b). These values were not significantly different from control cells treated with baclofen alone, where 46 of 392 (11.73%) of cells showed increases in the high potassium-stimulated 340/380 ratio (Fig. 4c, Chi square). Electrophysiological recordings were performed in the presence and absence of 50 nM Ant-AIP-II and 10 µM baclofen. The calcium current enhancement displayed when Ant-AIP-II was co-applied with baclofen was not significantly different than that seen with baclofen alone (Fig. 4d). In these experiments, 4 of 23 (17.39%) cells treated with Ant-AIP-II and baclofen displayed a significant increase in calcium current. In control cells, 6 of 27 (22.22%) displayed a significant increase when treated with baclofen alone. Thus, since neither inhibitor blocked the enhancement of calcium entry or current, it is likely that CaMKII is not involved in the pathway where activation of GABA$_B$ receptors results in increased calcium entry via L-type channels.
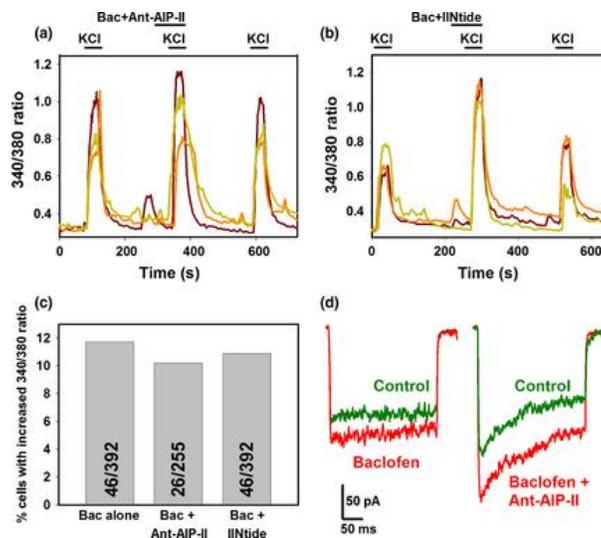

Figure 4. The increase in calcium entry as a result of GABA$_B$ receptor activation with baclofen is not affected by calcium/calmodulin-dependent protein kinase 2 (CaMKII)

inhibitors. Neurons were stimulated with high potassium, followed by a pre-treatment with 10 µM baclofen with or without a CaMKII inhibitor followed by baclofen and CaMKII inhibitor in high potassium for ratiometric calcium experiments. Each colored line in (a and b) represents a single cell. Enhancement of calcium influx by baclofen persists in the presence of CaMKII inhibitor *Antennapodia* sequence-fused autocamtide-2 related inhibitory peptide II (Ant-AIP-II) (a, 50 nM) or myristolated CaMKII inhibitor IINtide (b, 250 nM). Concentrations chosen were 5–10 fold higher than the reported IC_{50} values. (c) There is no significant difference in number of cells demonstrating a baclofen-mediated increase in calcium influx when either CaMKII inhibitor is present in comparison to control cultures without (Chi square, total N is inset in each bar). (d) Electrophysiological recording shows persistent calcium current enhancement when baclofen (left) or baclofen and Ant-AIP-II (right) are present (red line) compared to a control trace (green line).

PKCa translocates to the membrane upon GABA_B receptor activation

Analysis of immunohistochemical images with confocal microscopy was utilized to determine whether PKCα, PKCβ, or PKCε was translocated from the cytosol to the plasma membrane followed by 10 min treatment of cultured hippocampal neurons with baclofen (10 µM). A previous study demonstrated that activation of PKC by phorbol esters mimicked the effect of GABA_B receptor stimulation on calcium currents in cultured hippocampal neurons (Bray and Mynlieff 2011). Therefore, only phorbol ester-sensitive isoforms of PKC that are expressed in the early neonatal period were tested (Tanaka and Nishizuka 1994; Roisin and Barbin 1997). Cells were either treated with no drugs (unstimulated) or with the phorbol ester PMA (1 µM) to verify that the translocation to the membrane was apparent in confocal images. Fig. 5a demonstrates that both treatment with PMA and baclofen caused translocation of PKCα from the cytosol to the plasma membrane. A small percent of cells in each culture demonstrated spontaneous translocation of all three isoforms of PKC examined in unstimulated cultures (Fig. 5b; 2.99–7.69%, *N* = 104–568). All three isoforms translocated to the plasma membrane upon exposure to 10 min of 1 µM PMA in a subset of cells (50.17% of 291 cells for PKCα, 20.34% of 188 cells for PKCβ, 27.59% of 203 cells for PKCε). Only the percentage of cells demonstrating translocation of PKCα with baclofen treatment (9.83%, *N* = 478) was significantly higher than the percent of cells demonstrating spontaneous translocation in unstimulated conditions (2.99%, *N* = 568; Fisher's exact test, *p* < 0.0001).
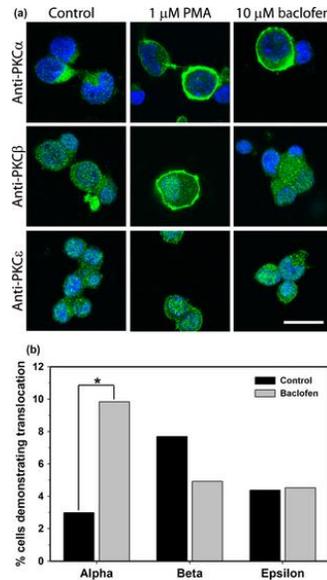
Figure 5. Activation of GABA$_B$ receptors causes translocation of PKCα. (a) Cultured hippocampal neurons were treated with control media, the phorbol ester phorbol 12-myristate 13-acetate (PMA) (1 μM) in media, or baclofen (10 μM) in media for 10 min before immunostaining. The scale bar in the lower right corner is 20 μm. (b) The percent cells demonstrating translocation of PKCα, PKCβ, and PKCε in control cultures (black bars) was compared to the percent cells demonstrating translocation of PKCα, PKCβ, and PKCε following stimulation with baclofen (gray bars). N = 568, 478, 104, 183, 183, and 177 for the PKCα control, PKCα baclofen, PKCβ control, PKCβ baclofen, PKCε control, and PKCε baclofen, respectively. *$p < 0.001$, Fisher's exact test.

Discussion

Previous findings in our lab have shown that in neonatal hippocampus, GABA$_B$ receptor activation decreases calcium current in a subset of neurons via Gα$_{i/o}$ and enhances L-type calcium current in other neurons via a non-G$_{i/o}$ G-protein (Carter and Mynlieff 2004; Bray and Mynlieff 2011). Despite a variation in percentage of neurons demonstrating calcium current enhancement (approximately 10% of neurons in calcium imaging versus 25% in electrophysiology), knocking down Gα$_q$ produced a consistent result – that enhanced calcium entry could be abolished. The variation in number of cells which demonstrate enhancement is likely because of the sampling of every cell within a field during calcium imaging experiments, whereas sampling in electrophysiology allows the experimenter to pick only the healthiest, best looking cells. While this percentage may at first appear low, the hippocampus is composed of no less than 37 different types of inhibitory interneurons (Ascoli 2013); thus a small percentage could encompass one or more of these 37 types, many of which have clearly

delineated functions of their own. While the subset of cells demonstrating a reduction in calcium current has been attributed to inhibition of calcium channels by $G\alpha_{i/o}$ G-proteins, the pathway describing calcium current enhancement upon GABA$_B$ receptor activation has not previously been explored. The data obtained in this study support a model in which GABA$_B$ receptors couple to $G\alpha_q$ activating PKCα to cause enhancement of voltage-dependent calcium current without the involvement of CaMKII (Fig. 6).
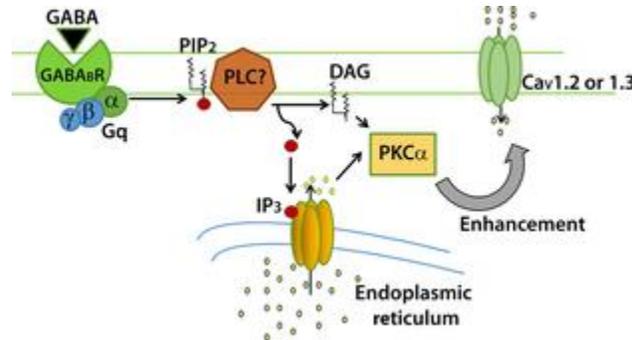
Figure 6. The current model for the pathway which begins with GABA$_B$ receptor activation and results in enhanced L-type calcium current. Binding of ligand to the GABA$_B$ receptor leads to activation of a $G\alpha_q$ G-protein, which putatively activates phospholipase C. The activity of PLC hydrolyses PIP$_2$ into diacylglycerol (DAG) and IP$_3$, which can activate PKCα. PKCα bypasses calcium/calmodulin-dependent protein kinase 2 (CaMKII) to lead to changes of the L-type calcium channel such that current is enhanced when the channel opens.

We chose to determine the involvement of $G\alpha_q$ in the pathway because a similar bidirectional pathway involving both $G\alpha_{q/11}$ (mediating calcium current increase) and $G\alpha_{i/o}$ (mediating calcium current decrease) was observed in cardiac myocytes (O-Uchi *et al*. 2008). In addition, activation of $G\alpha_q$ family members ($G\alpha_q$, $G\alpha_{11}$, $G\alpha_{14}$, $G\alpha_{15/16}$) are known to activate PKC by stimulating β isoforms of phospholipase C that hydrolyze phosphatidylinositol bisphosphate (PIP$_2$), forming inositol tris-phosphate (IP$_3$) and DAG (for review see (Rhee 2001). DAG and free calcium ions activate PKC, which then translocates from the cytosol to the cell membrane. Here, PKC may potentially phosphorylate the L-type calcium channel, either directly or indirectly through another second messenger. The phosphorylated channel putatively allows more calcium to enter when the cell is depolarized; biophysical changes to the channel have not yet been identified.

To test the model that $G\alpha_q$ is the G-protein that couples to the $GABA_B$ receptor, the translation of $G\alpha_q$ was inhibited by morpholinos, synthetic oligonucleotides that bind near the start codon of 5' mRNA to inhibit binding of the ribosome. Morpholinos have several advantages over siRNA for knockdown of protein expression. siRNA are sensitive to intracellular enzymes, can trigger innate immune responses, and change the methylation state of DNA (Bayne and Allshire 2005; Judge *et al*. 2005; Kawasaki and Taira 2005; Marques and Williams 2005). None of these things occur with morpholinos (Hudziak *et al*. 1996) and they are easily taken up into cultured cells through endocytosis stimulated by the Endo-Porter peptide. The lack of enzymatic degradation makes morpholinos particularly useful to knock down proteins with a relatively long half-life, such as G-proteins. Despite these advantages, there is only one report of Endo-Porter mediated morpholino knockdown in mammalian neurons (Chih *et al*. 2006). Validation of protein knockdown demonstrated here highlights the usefulness of this technique.

Western blot analysis indicated that the morpholinos were efficient in knocking down $G\alpha_q$ in primary hippocampal cultures, but it was important to verify the specificity of this knockdown. The most likely candidate for non-specific knockdown would be $G\alpha_{11}$ because of high sequence homology with $G\alpha_q$. The first 25 coding amino acids (the site of morpholino binding) are 92% identical. Data shown here suggest significantly higher expression of $G\alpha_q$ than $G\alpha_{11}$ in neonatal hippocampus, consistent with other reports (Milligan 1993; Ihnatovych *et al*. 2002). However, caution must be used in interpreting these results. Because different antibodies were used to detect each protein, it is possible that $G\alpha_{11}$ antibodies simply give a weaker signal than $G\alpha_q$ antibodies, even if there is abundant protein. Despite this, we believe that $G\alpha_q$ is expressed to a relatively higher degree than $G\alpha_{11}$, both because of previous expression data, and the temporal expression pattern of $G\alpha_q$. The high expression of $G\alpha_q$ relative to $G\alpha_{11}$ makes it the most likely target of the morpholinos. It is also noteworthy that $G\alpha_q$ is most highly expressed during the first postnatal week (Ihnatovych *et al*. 2002), which corresponds to the time when the highest percentage of neurons demonstrates calcium current enhancement with baclofen (Bray and Mynlieff 2009).

Knockdown of $G\alpha_q$ eliminated the enhancement of calcium current by $GABA_B$ receptors supporting the hypothesis that $G\alpha_q$ is the G-protein that couples to the $GABA_B$ receptor. In addition, confocal imaging data demonstrated colocalization of $G\alpha_q$ and $GABA_BRs$. Mannoury La Cour *et al*. (2008) used an antibody-capture/scintillation proximity assay to show a lack of coupling between $G\alpha_{q/11}$ and $GABA_BRs$ (2008). However, this work reported the EC_{50} for baclofen at approximately 50 μM for $G\alpha_{i/o}$ coupling. These values are much higher than those used by others examining $G\alpha_{i/o}$ activation by $GABA_BRs$ (Dolphin and Scott 1986; Sodickson and Bean 1996). Furthermore, the enhancing effect of baclofen is seen at high nanomolar concentrations while attenuation requires greater values (Shen and Slaughter 1999). Thus, it is possible that the assay used by Mannoury La Cour *et al*. (2008) is not sensitive enough to show $G\alpha_q/GABA_BR$ coupling at low agonist concentrations.

The molecular interaction between the $GABA_{B2}$ receptor subunit and $G\alpha_{i/o}$ involves both the second and third intracellular loops of $GABA_{B2}$ (Duthey *et al*. 2002; Havlickova *et al*. 2002). This interaction occurs with the extreme C-terminus of the G-protein (Franek *et al*. 1999). The lack of sequence homology between $G\alpha_{i/o}$ and $G\alpha_q$ suggest that $G\alpha_q$ must couple to the $GABA_R$ at sites unique to those examined. Identifying the sites of direct interaction between the $GABA_BR$ and $G\alpha_q$ will support the physiological and colocalization data presented here. Although this is the first example of $G\alpha_q$ coupling to $GABA_B$ receptors, coupling to more than one G-protein has been shown for other G-protein coupled receptors, including those that couple to both $G\alpha_q$ and $G\alpha_{i/o}$ (Offermanns and Simon 1995; Hawes *et al*. 2000; Macfarlane *et al*. 2001). We believe $GABA_B$ receptors to have the same ability, coupling to both $G\alpha_{i/o}$ to mediate classic inhibitory responses and $G\alpha_q$ to mediate calcium current enhancement.

Enhancement of current by $GABA_B$ receptors may be mediated through a number of different mechanisms. It is possible that activation of $GABA_B$ receptors alone is sufficient to open L-type channels, as shown by Kuczewski *et al*. (2011). This report showed that application of 50 μM baclofen without depolarization caused an increase in calcium entry through L-type channels on the cell surface; however, we were unable to replicate this effect using the same concentration of baclofen on our P6–P8 cultured hippocampal neurons

(data not shown). $GABA_B$ receptors have been demonstrated to both increase and decrease potassium currents by interacting with different combinations of auxiliary potassium channel tetramerization domain-containing subunits (Schwenk *et al*. 2010; Hayasaki *et al*. 2012). Hayasaki *et al*. point out that while these auxiliary subunits are most likely responsible for the bidirectional response observed, several factors, including G-protein diversity, may lead to differential effects in $GABA_B$ receptor signaling. Based on data presented here, we believe $GABA_B$-mediated enhancement of L-type calcium current is because of its coupling to a $G\alpha_q$ G-protein through a novel pathway, rather than the canonical $G\alpha_{i/o}$ pathway. There are, however, other possible mechanisms for the observed effect. In airway smooth muscle, $GABA_B$ receptor stimulation led to activation of $G\alpha_i$ G-proteins and subsequent enhancement of calcium current; this study demonstrated that it was the βγ subunit of the G-protein which activated the same PLC/PKC pathway as $G\alpha_q$ (Mizuta *et al*. 2011). However, inhibiting $G\alpha_{i/o}$ signaling with pertussis toxin did not eliminate the observed enhancement in the hippocampus (Bray and Mynlieff 2009). It is also unlikely that the $G\alpha_q$ βγ subunit may be mediating this effect, because βγ signaling requires a functional α subunit interaction for proper assembly (Smrcka 2008). Therefore, while it is possible another mechanism exists in the $GABA_B$ receptor – L-type channel pathway, the most likely mechanism is $GABA_B$ coupling to $G\alpha_q$.

$GABA_B$-receptor-mediated activation of PKC is not the only possible pathway involved. Previous studies in our laboratory examined if calcium current enhancement could be mediated by PKA (Bray and Mynlieff 2011). In this case, $G\alpha_s$ would likely couple to the $GABA_B$ receptor, leading to a cascade that would result in PKA, either in addition to or instead of PKC, phosphorylating the calcium channel. However, because enhancement was so clearly eliminated by knockdown of $G\alpha_q$, and because baclofen caused PKC translocation, this G-protein is involved in mediating current enhancement. Furthermore, results of experiments designed to inhibit PKA with H-89 were ambiguous in determining a role for PKA in the pathway. Thus, while PKA (and therefore $G\alpha_s$) may be involved, we believe $G\alpha_q$ is responsible for $GABA_B$-mediated calcium current enhancement.

The regulation of L-type calcium channels by PKC has been reported to both increase and decrease calcium current, and the effect

on current seems to depend on the isoform of PKC involved. For example, phosphorylation by PKCε on the N terminus of $Ca_V1.2$ L-type calcium channels inhibits calcium current in cardiac cells (Yue *et al*. 2004). However, phosphorylation of the C terminus of $Ca_V1.2$ by multiple PKC isoforms in HEK293 cells, including PKCα, led to an increase in L-type channel activity (Yang *et al*. 2009). Stimulation of PKCβII and PKCε with PMA decreased activity of $Ca_v1.3$ L-type calcium channels (Baroudi *et al*. 2006). Thus, identifying the PKC isoform in $GABA_B$-mediated L-type current enhancement provides more detailed information regarding isoform-specific regulation of calcium channels.

We have previously shown that activation of a phorbol ester-sensitive PKC is required for $GABA_B$-receptor-mediated enhancement of calcium current (Bray and Mynlieff 2011) and thus, here we sought to identify which isoform was involved. Of the 15 PKC isoforms identified only four have been shown to be robustly expressed in the early neonatal period and only three of these (α, β, and ε) fall into the categories of conventional or atypical PKCs that respond to phorbol ester stimulation. All three isoforms showed some basal level of translocation to the membrane (a measure of activation); however, only PKCα showed a significant increase in number of neurons showing translocation when treated with baclofen. Notably, the proportion of neurons that demonstrated this baclofen-mediated translocation of PKCα is approximately the same as the proportion that undergoes current enhancement.

In an effort to describe as complete a pathway as possible, we examined the involvement of CaMKII in the signal transduction cascade. Evidence of CaMKII activation by PKC and phosphorylation of L-type channels in cardiac myocytes (O-Uchi *et al*. 2008) suggested that CaMKII may be a component here; however, when neurons were treated with CaMKII inhibitors Ant-AIP-II (50 nM) or myristolated CaMKIIntide (250 nM) plus baclofen, a subset of cells still showed calcium enhancement when depolarized. Although we cannot be sure that the incubation period was sufficient for the inhibitors to penetrate the cells at the applied concentration, the data suggest that CaMKII is

not part of the signaling pathway.

Acknowledgments and conflict of interest disclosure

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29