**N-Arachidonyl Glycine Does Not Activate G Protein–Coupled Receptor 18 Signaling via Canonical Pathways**

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**ABSTRACT**

Recent studies propose that N-arachidonyl glycine (NAGly), a carboxylic analogue of anandamide, is an endogenous ligand of the Gαi/o protein–coupled receptor 18 (GPR18). However, a high-throughput β-arrestin–based screen failed to detect activation of GPR18 by NAGly (Yin et al., 2009; JBC, 18:12328). To address this inconsistency, this study investigated GPR18 activation of GPR18 by NAGly (Yin et al., 2009; JBC, 18:12328). Other proposed agonists of GPR18, including anandamide and abnormal cannabidiol, also failed to induce inhibition of calcium currents. Mutants of GPR18, designed to constitutively activate receptors, did not tonically inhibit calcium currents, indicating a lack of GPR18 activation or coupling to endogenous G proteins. Other downstream effectors of Gαi/o–coupled receptors, G protein–coupled inwardly rectifying potassium channels and adenylyl cyclase, were not modulated by GPR18 signaling. Furthermore, GPR18 did not couple to other G proteins tested: Gαs, Gαζ, and Gα16. These results suggest NAGly is not an agonist for GPR18 or that GPR18 signaling involves noncanonical pathways not examined in these studies.

**Introduction**

Seven-transmembrane G protein–coupled receptors (GPCRs) are the single largest family of receptors localized to the cell surface and the most common target for currently available therapeutics (Jacoby, 2006). These receptors are defined by their ability to activate heterotrimeric guanine nucleotide binding proteins upon agonist stimulation. GPCRs without an identified endogenous ligand are considered “orphan receptors” and represent novel therapeutic targets. One such orphan receptor, G protein–coupled receptor 18 (GPR18), is found at high levels in the testis, small intestines and cells associated with the immune system including lymphocytes, thymus and spleen (Gantz et al., 1997).

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**ABBREVIATIONS:** Abn-Cbd, abnormal cannabidiol; ADRA2A, α2A-adrenergic receptor; AEA, anandamide, N-arachidonyl ethanolamide; ANOVA, analysis of variance; BRET, bioluminescence resonance energy transfer; BSA, bovine serum albumin; CAMYEL, cAMP sensor using YFP-Epac; CTX, cholera toxin; DPBS, Dulbecco’s phosphate buffered saline; EGFP, enhanced green fluorescent protein; FAH, fatty acid amide hydrolase; FR, facilitation ratio; GPR18, G protein–coupled receptor 18; GPCR, G protein–coupled receptors; HEK, human embryonic kidney; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; HRP, horseradish peroxidase; HVA, high voltage activated; i/o proteins; MEM, minimum essential medium; NAGly, N-arachidonyl glycine; NE, norepinephrine; PEI, polyethyleneimine; PTX, pertussis toxin; Qdot655, quantum dot 655; SCG, superior cervical ganglion; TBS-T, Tris buffered saline with 0.05% Tween-20; TEA-OH, tetraethylammonium hydroxide; TTX, tetrodotoxin.

Recently, N-arachidonyl glycine (NAGly) was identified as the endogenous ligand for GPR18 (Kohno et al., 2006). NAGly is a lipoamino acid found most abundantly in the spinal cord and brain (Huang et al., 2001). The chemical structure of NAGly is similar to that of anandamide (N-arachidonyl ethanolamide, AEA), but NAGly shows no activity at the two identified cannabinoid receptors, CB1 and CB2 (Huang et al., 2001). Anandamide and abnormal cannabidiol (Abn-Cbd), a synthetic cannabinoid, show agonist activity in GPR18-expressing cells (McHugh et al., 2010, 2012). Of note, anandamide exerts vasodilatory effects independent of CB1− and CB2R activity (Jarai et al., 1999), suggesting the existence of a third member of the cannabinoid receptor family, possibly GPR18 (McHugh et al., 2010).

GPR18 coupling to pertussis toxin–sensitive Gαi/o proteins has been suggested in studies using cell line expression systems (Kohno et al., 2006; McHugh et al., 2010, 2012; Takenouchi et al., 2012). However, a high-throughput screening of orphan GPCRs and lipid ligands failed to detect activation of GPR18 by NAGly (Yin et al., 2009). This
discrepancy could arise from the different assays used to detect G protein activation. The PathHunter assay used by Yang's group assesses G protein–coupling using β-arrestin–mediated internalization, which is a common but not universal desensitization pathway for GPCRs. The mitogen-activated protein kinase phosphorylation assay used by Bradshaw's group is a distant downstream effector of GPCR activation that can also be activated by multiple types of receptors (e.g., tyrosine kinase receptors). Here, we further examine the G protein signaling pathways of GPR18. We used rat superior cervical ganglion neurons to heterologously express GPR18 for study and measured inhibition of Ca²⁺ current as an assay for G protein activation. Ga₁₆ protein–coupled receptor modulation of Ca²⁺ current is mediated directly by liberated Gβγ (Herlitze et al., 1996; Ikeda, 1996) and occurs within a time course that can be observed during an electrophysiology experiment. The system is robust—to our knowledge, all Ga₁₆-coupled GPCRs that traffic to the plasma membrane produce a Ca²⁺ current inhibition when activated with a cognate agonist.

Surprisingly, we found a lack of GPR18 activation by NAGly and other proposed agonists, including anandamide activated with a cognate agonist. Other commonly associated NAGly and other proposed agonists, including anandamide activated with a cognate agonist, and abnormal cannabidiol. To examine the G protein signaling pathways of GPR18. We used receptors (e.g., tyrosine kinase receptors). Here, we further

Materials and Methods

**Molecular Cloning and Mutagenesis.** Oligonucleotide primers for cloning were designed on the basis of NM_182806.1 (Mus musculus GPR18 ReiSeq accession number) and commercially synthesized (IDT, Coralville, IA). GPR18 was amplified from marathon-ready mouse brain cDNA (Clontech, Mountain View, CA) using polymerase chain reaction and PhuUltra DNA polymerase (Stratagene, La Jolla, CA). To subclone into the mammalian expression vector pC1 (Promega, Madison, WI), we used the following primers: forward 5′-GATCGAATCTATCCAGCGCTTGGGTCATGATGTT–3′ and reverse 5′-GATCGATCAGGCCTCCTCAAAGATCTCAGTGCCTGTTGC-3′ (NotI site underlined), EcoRV site italicized), were used to return the expression primers, forward 5′-GCAATCTGAAGATCTCAGTGCCTGTTGC-3′ and reverse 5′-GATCGATCAGGCCTCCTCAAAGATCTCAGTGCCTGTTGC-3′ (NotI site underlined), EcoRV and NotI restriction enzymes. A C-terminal 3xHA-tagged construct of GPR18, GPR18-3xHA, was produced by cutting and subcloning from the GPR18-EGFP construct with HindIII and EcoRV. Point mutations of GPR18 and the α₂A-adrenergic receptor (ADRA2A) were generated using QuikChange site-directed mutagenesis and the primers listed in Table 1. We used all generated constructs were confirmed by DNA sequencing (Supplemental Fig. 1, A and B; Macrogen, Rockville, MD).

**Live- and Fixed-Cell Staining.** HeLa cells (ATCC, Manassas, VA) were cultured (2.0 × 10⁶ cells per ml) in minimal essential medium supplemented with 10% fetal bovine serum, 100 U/ml penicillin, and 100 μg/ml streptomycin (MEM+/+; Gibco, Grand Island, NY) on poly-L-lysine coated glass-bottomed dishes (MatTek, Ashland, MA). Cells were transfected with a mixture of 0.5 μg cDNA and 7 μl fully decylated polyethyleneimine (PEI) at 7.5 μM in 100 μl MEM+/+ overnight. For live-cell staining, dishes were gently washed with Dulbecco’s phosphate-buffered saline (DPBS) with Ca²⁺ and Mg²⁺ (DPBS+/+) to remove culture medium before a blocking solution, 2% bovine serum albumin (BSA) in DPBS+/+, was added for 30 minutes at 37°C. HeLa cells were then incubated with primary antibody (biotin-labeled anti-HA.11 clone 16B12, 1:200; Covance, Berkeley, CA) in blocking solution for 1 hour at 37°C. Cells were washed with blocking solution for 10 minutes and then exchanged for DPBS+/+ before imaging.

For immunocytochemistry, dishes were gently washed with DPBS+/+ before fixing with 4% paraformaldehyde for 20 minutes at room temperature. After washing out fixative with DPBS+/+, HeLa cells were permeabilized with 0.5% Tween-20 in DPBS+/+ for 30 minutes at room temperature. Following solution exchange with DPBS+/+, a blocking solution, 2% BSA in Tris buffered saline with 0.05% Tween-20 (TBS-T; 10 mM Tris base, 250 mM NaCl, pH 7.5), was added for 1 hour at room temperature. Primary antibody (biotin anti-HA, 1:500; Covance) in blocking solution was incubated with cells overnight at 4°C. After cells were washed with TBS-T for 10 minutes, secondary antibody (streptavidin Qdot655, 1:500; Molecular Probes) incubation was for 2 hours at room temperature. Cells were washed with TBS-T for 10 minutes and then exchanged for DPBS+/+ before imaging.

**Table 1**

<table>
<thead>
<tr>
<th>Point Mutation</th>
<th>Forward Primer (5′–3′ End)</th>
<th>Reverse Primer (5′–3′ End)</th>
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<tr>
<td>GPR18 A108N</td>
<td>GCGGGTCTTACCACCGAATCACTCGCTGGTGTCTTCCG</td>
<td>GCCAAAGCAGCAATGGGCTCGTGGGTAACACACCAC</td>
</tr>
<tr>
<td>GPR18 N40A</td>
<td>GCTGTGCTTGGTCTACGTGCTGTCG</td>
<td>CCCACCCACCCAGCAGCAGCAGCAGCACCC</td>
</tr>
<tr>
<td>GPR18 D118A</td>
<td>GCTCTGCTGTTGCGAGAGTCACTCGCTGGTG</td>
<td>CGGTGGCTGTTGCTGTTGCTGGGTGTAACACACCAC</td>
</tr>
<tr>
<td>GPR18 D118T</td>
<td>GCTCTGCTGTTGCGAGAGTCACTCGCTGGTG</td>
<td>CGGTGGCTGTTGCTGTTGCTGGGTGTAACACACCAC</td>
</tr>
<tr>
<td>GPR18 I231E</td>
<td>GCTGTGCTGTTGCGAGAGTCACTCGCTGGTG</td>
<td>CGGTGGCTGTTGCTGTTGCTGGGTGTAACACACCAC</td>
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<tr>
<td>ADRA2A N51A</td>
<td>GCTGTGCTGTTGCGAGAGTCACTCGCTGGTG</td>
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<td>ADRA2A D130A</td>
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<tr>
<td>ADRA2A T373E</td>
<td>GCGGGTCTTACCACCGAATCACTCGCTGGTGTCTTCCG</td>
<td>GCCAAAGCAGCAATGGGCTCGTGGGTAACACACCAC</td>
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All staining experiments included parallel negative controls, which received the same treatment except without primary antibody incubation.

**Imaging.** HeLa cells and neurons expressing EGFP-constructs were imaged using a 63× (1.2 numerical aperture) or 40× (1.2 numerical aperture) objective mounted on a Zeiss LSM510 Meta confocal microscope with ZEN 2008 acquisition software (Carl Zeiss, Jena, Germany). For EGFP fluorescence, the excitation wavelength was 488 nm and emission wavelength was band-pass filtered between 500 and 550 nm. Qdot655 fluorescent images were acquired with 488-nm excitation and a 650- and 710-nm band-pass–filtered emission.

**Western Blotting.** Unless otherwise indicated, all reagents for Western blotting were from Thermo Scientific (Rockford, IL). Transfected HeLa cells were lysed with mammalian protein extraction reagent with protease inhibitors for 5 minutes at room temperature. A reducing SDS loading buffer was added to lysates and heated, 85°C for 5 minutes, before electrophoresing samples on a 4–15% Tris-glycine precast gel (Bio-Rad, Hercules, CA) with Laemmli running buffer (Laemmli, 1970). Proteins were transferred to a polyvinylidene difluoride membrane at 280 mA for 1 hour. The membrane was blocked with 5% BSA in TBS-T for 3 hours before incubating overnight with a mouse anti-GFP antibody (1:2000; UC Davis/NIH NeuroMab Facility, Davis, CA) with a 4°C. A goat anti-mouse horseradish peroxidase (HRP)–conjugated secondary antibody (1:1000, 1 hour) in blocking solution was applied before chemiluminescent detection of blots with SuperSignal West Femto substrate and a Kodak Image Station 4400R (Carestream Molecular Imaging, Woodbridge, CT). Membranes were stripped with Restore PLUS stripping buffer and reprobed for loading controls. Rabbit anti-tubulin (1:2000; overnight; Cell Signaling, Boston, MA) and rabbit anti–cyclophysin B (1:5000; overnight; Abcam, Cambridge, MA) primary antibodies were used, followed by a goat anti-rabbit HRP-conjugated secondary antibody (1:1000, 1 hour) and chemiluminescent detection.

**Superior Cervical Ganglion Neuron Dissociation and Intra-Nuclear Microinjection of cDNA.** All animal studies were conducted in accordance with the National Institutes of Health’s Guide for the Care and Use of Laboratory Animals.

Superior cervical ganglion (SCG) neurons from adult (6–12 weeks old) male Wistar rats were disected and dissociated as described previously (Ikeda, 2004; Ikeda and Jeong, 2004). Briefly, animals were anesthetized by CO2 inhalation and decapitated before dissection. Two SCGs per rat were removed, desheathed, cut into small pieces, and incubated in modified Earles’ balanced salt solution containing 2 mg/mL collagenase (CLS4; Worthington Biochemical, Lakewood, NJ), 0.6 mg/mL trypsin (Worthington Biochemical) and 0.1 mg/mL DNase I at 36°C for 1 hour in a water bath shaker oscillating at 110 rpm. The Earles’ balanced salt solution was supplemented with 3.6 g/L glucose and 10 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES).

After incubation, neurons were mechanically dissociated by vigorously shaking the flask for 10 seconds. Neurons were centrifuged at 570 rpm for 6 minutes and resuspended in MEM+/- twice before being plated on pol-y-L-lysine–coated tissue culture dishes. Cells were maintained in a humidified 95% air/5% CO2 incubator at 37°C.

Three to 6 hours after dissociation, plasmid constructs were injected directly into the nucleus of SCG neurons as described previously (Ikeda, 2004; Ikeda and Jeong, 2004; Lu et al., 2009). Briefly, cDNA was injected with a FemtoJet microinjector and 5171 micromanipulator (Eppendorf, Hamburg, Germany) using an injection pressure and duration of 140–160 hPa and 0.3 second, respectively. Injected plasmids were diluted in elution buffer (10 mM Tris-HCl, pH 8.5) and centrifuged in capillary tubes at 10000 rpm for at least 30 minutes. GFP18 constructs were injected at a concentration of 50–100 ng/µl, and ADRA2A mutants were injected at a lower concentration (10 ng/µl). To identify successfully injected neurons, pEGFP-N1 cDNA (Clontech) was coinjected at a concentration of 5 ng/µl. After injections, neurons were incubated overnight at 37°C and electrophysiological experiments were the following day.

**Electrophysiology.** Ca2+-channel currents (ICa) and G protein–coupled inwardly rectifying K+ currents (IKr) were recorded using conventional whole-cell patch-clamp techniques (Hamill et al., 1981). Patch electrodes were pulled from borosilicate glass capillaries (1.65 mm outer diameter, 1.20 mm inner diameter; King Precision Glass, Claremont, CA) using a Model P-97 micropipette puller (Sutter Instrument, Novato, CA). The patch electrodes were coated with silicone elastomer (Sylgard 184; Dow Corning, Midland, MI) and firepolished. An Ag/AgCl pellet connected to the bath solution via a 0.15 M NaCl/agar bridge was used as a ground. The cell membrane capacitance was canceled and series resistance was compensated (>85% prediction and correction; lag set to 5 microseconds) with a patch-clamp amplifier (Axopatch 200A/B; Molecular Devices, Sunnyvale, CA). Voltage protocol generation and data acquisition were performed using custom-designed software (SS) on a Macintosh G4 computer (Apple, Cupertino, CA). Current traces were filtered at 2 kHz (~3 dB; four-pole Bessel), digitized at 10 kHz with a 16-bit analog-to-digital converter board (ITC-18, HEKA, Bellmore, NY) and stored on the computer for later analyses.

For recording ICa, patch pipettes were filled with an internal solution containing (in mM) 120 N-methyl-D-glucamine, 20 tetrathylammonium hydroxide (TEA-OH), 11 ethylene glycol tetraacetic acid (EGTA), 10 HEPES, 10 sucrose, 1 CaCl2, 14 Tris-creatine phosphate, 4 MgATP and 0.3 NaGTP, pH 7.2 with methanesulfonic acid. External ICa recording solution consisted of (in mM) 140 methanesulfonic acid, 145 TEA-OH, 10 HEPES, 10 glucose, 1 CaCl2 and 0.0003 tetrodotoxin (TTX), pH 7.4 with TEA-OH. A Tris-based external ICa solution was also tested containing (in mM) 155 Tris-base, 20 HEPES, 10 glucose, 10 CaCl2 and 0.0003 TTX, pH 7.4 with methanesulfonic acid.

To measure G protein modulation of Ca2+-channels, a double-pulse protocol consisting of two 25-millisecond test pulses to +10 mV separated by a 50-millisecond conditioning pulse to +80 mV (Elmslie et al., 1990) was evoked every 10 seconds from a holding potential of −80 mV. To measure low- and high-voltage activated (LVA and HVA)-ICa in the same cell, two 25-millisecond pulses, the first to −40 mV and the second to +10 mV, separated by a 60-millisecond pulse to −60 mV, to inactivate LVA-ICa, was applied every 10 seconds from a holding potential of −80 mV. ICa–voltage relationships were studied by applying a series of 70-millisecond depolarizing voltage steps from a holding potential of −80 mV.

For recording IKr, patch pipettes were filled with an internal solution containing (in mM) 135 KCl, 11 EGTA, 10 HEPES, 2 MgCl2, 1 CaCl2, 4 MgATP, and 0.3 NaGTP, pH 7.2 with KOH. External IKr recording solution consisted of (in mM) 140 NaCl, 5.4 KCl, 10 HEPES, 15 glucose, 15 sucrose, 2 CaCl2, 0.8 MgCl2 and 0.0003 TTX, pH 7.4 with NaOH.

IKr were elicited from 200-millisecond voltage ramps from −140 to −40 mV and the holding potential was set to −60 mV.

**Live-Cell Bioluminescence Resonance Energy Transfer-based Assay for cAMP.** Human embryonic kidney (HEK-)293 cells (ATCC) were plated (5.0 × 105 cells per mL) on 24-well plates in MEM+/- Cells were transfected overnight with a mixture of 150 ng cAMP sensor using YFP-Epac-RLuc (CAMYEL) cDNA (Jiang et al., 2007), 100 ng empty vector or selected G protein–coupled receptor cDNA and 4 µl PEI in 50 µl MEM−/− per well. Approximately 16 hours after transfection, HEK cells were removed from 24-well plates with TrypLE Express (Gibco) and washed twice in DPBS+/− before being loading onto black 96-well microplates (Berthold, Bad Wildbad, Germany).

Light intensity was measured using a Tristar LB941 luminometer (Berthold) controlled by MikroWin 2000 acquisition software (Berthold). Net bioluminescence resonance energy transfer (BRET) was calculated from the light intensity measured alternately from donor and acceptor channels in 1-second intervals using the emission filters 460/60 nm and 542/27 nm, respectively (Semrock, Rochester, NY). For testing the kinetic responses of GPCR s coupled to Gαi3 pathways, 14 seconds of baseline recording, followed by injection of 5 µM

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h-coelenterazine (Nanolight Technology, Pinetop, AZ) substrate, 120 seconds of recording, injection of 1 μM forskolin, 240 seconds of recording, injection of agonist (10 μM NAGly or 100 μM glutamate), and 540 seconds of recording. For testing the kinetic responses of GPCRs coupled to Gαs pathways, 14 seconds of baseline recording, followed by injection of 5 μM h-coelenterazine, 120 seconds of recording, injection of agonist (10 μM NAGly or 10 μM dopamine), 240 seconds of recordings, injection of 10 μM forskolin, and 240 seconds of recording.

Drugs and Chemicals. Unless otherwise stated, all chemicals were purchased from Sigma-Aldrich. TTX was purchased from Alomone Laboratories (Jerusalem, Israel); pertussis toxin (PTX) and cholera toxin (CTX) were purchased from List Biologic Laboratories (Campbell, CA); NAGly, N-arachidonoyl-L-serine, AEA, Abn-Cbd, and O-1602 were purchased from Cayman Chemical (Ann Arbor, MI); h-coelenterazine was purchased from Nanolight Technology (Pinetop, AZ), and dopamine hydrochloride was purchased from Tocris (Bristol, UK). PTX or CTX was added (0.5 mg/ml) to the culture medium, bathing SCG neurons or HEK cells during overnight incubation (>16 hours).

Drugs for electrophysiologic experiments were diluted to final concentrations from stock solutions on the day of experiment and applied directly onto neurons using a custom-made gravity-fed perfusion system with separate perfusion lines feeding into a four-bore glass capillary tube (VitroCom, Mountain Lakes, NJ) connected to a fused silica capillary tube. A constant flow of external solution was applied onto cells during baseline recordings and switched to a drug solution during drug applications to avoid flow-induced artifacts. All recordings were performed at room temperature (20–24°C).

For BRET experiments, drugs were injected into each well by the luminometer’s injection system. Before the start of experiments, all lines were primed with distilled water and then reprimed with the drug solution to be injected. Chemicals were made the day of experiments at a concentration so the total volume (injected volume + volume in well) would produce the final desired concentration.

Data Analysis and Statistical Testing. ImageJ software, version 1.45i (National Institutes of Health, Bethesda, MD), was used to analyze and adjust contrast of images for presentation in figures. Igor Pro, version 6 (WaveMetrics, Portland, OR), was used to analyze current traces. I_{Ca} amplitude was measured isochronally 10 milliseconds after the initiation of a test pulse to 10 mV or at the maximum peak I_{Ca} during test pulses to −40 mV. The facilitation ratio (FR) was determined as the ratio of postpulse to prepulse I_{Ca}.

The peak I_{HK} was taken 3 milliseconds after the start of the ramp. Drug responses were normalized to baseline I_{Ca} or I_{HK} using the equation I_{drug}/I_{baseline} > 100, where I_{drug} and I_{baseline} are the current amplitudes during and before drug application, respectively. Net BRET was calculated as A/D − d, where A is the acceptor channel intensity, D is the donor channel intensity, and d is the background or spectral overlap (calculated previously as the A/D for donor, RLuc8, alone). A single net BRET value, 4 minutes after injection of agonist, was used for comparison of net BRET values between groups.

Statistical tests were performed with GraphPad Prism 5 for Mac OS X (GraphPad Software, La Jolla, CA). Individual data points were represented on graphs with the mean ± S.E.M. Statistical significance between two groups was determined using an unpaired t test. To compare three or more groups, a one-way analysis of variance (ANOVA) test followed by Newman-Keuls post-test was performed. P ≤ 0.05 was considered to represent a statistically significant finding, except when a multiple comparison Bonferroni correction was applied as indicated.

Results

Heterologous Expression of Full-length GPR18 at the Membrane. To determine the cellular localization of heterologously expressed receptor, GPR18-EGFP cDNA was injected into SCG neurons and compared with injected EGFP and EGFP-KRas tail cDNA expression, which label the cytoplasm and plasma membrane of the cell, respectively. The GPR18 construct displayed a “rim-like” fluorescence (Fig. 1A, top panel). This pattern was unlike that of cytoplasmic EGFP (Fig. 1B, top panel) but was similar to the membrane-bound EGFP-KRas tail (Fig. 1C, top panel). Line plots of fluorescence intensity (Fig. 1, A–C, top panel insets) show the highest intensity values along the edge of neurons injected with GPR18-EGFP or EGFP-KRas tail, whereas fluorescence intensities of EGFP-injected SCG neurons were uniform across the cell. HeLa cells were also transfected with EGFP-labeled constructs to compare expression in a cell line expression system (Fig. 1, A–C, bottom panels). In expressing HeLa cells, GPR18-EGFP and EGFP-KRas tail displayed a “rim-like” fluorescence pattern and EGFP displayed fluorescence throughout the cell, similar to SCG neuron expression.

Western blotting was used to confirm expression of full-length GPR18. An antibody against GFP was used to detect EGFP-tagged receptor because commercially available antibodies for GPR18 have not been validated against a GPR18-knockout animal. An approximately 80-kDa band was detected in the GPR18-EGFP lane (Fig. 1D). This band is larger than the predicted size of the protein, 66 kDa, which suggests post-translational modification of the receptor. The GFP antibody also detected EGFP and EGFP-KRas tail constructs, and their bands corresponded to the predicted protein mass, 27 and 29 kDa, respectively. The same blot was reprobed for loading controls α-tubulin and cyclophilin B, which corresponded to bands of 51 and 19 kDa, respectively (Fig. 1E).

To confirm GPR18 expression in the plasma membrane, live-cell staining of epitope-tagged GPR18 was performed. The perimeter of HeLa cells expressing the external epitope-tagged version of GPR18, 3xHA-GPR18, was stained after the live-cell staining procedure (see Materials and Methods and Fig. 2A), whereas staining was absent in HeLa cells expressing the internal epitope-tagged version of GPR18, GPR18-3xHA (Fig. 2B). To confirm expression of receptors, GPR18-3xHA-expressing cells were stained after fixation and permeabilization to allow antibody access to the interior of cells. Fixed and permeabilized GPR18-3xHA cells stained positive for the HA-epitope along the edge and within HeLa cells (Fig. 2C). Staining was absent in negative controls (data not shown).

Taken together, heterologously expressed GPR18 inserts with appropriate topology into the plasma membrane, where it is accessible to agonists and G proteins.

Direct Potentiation of HVA-Ca^{2+} Channels by NAGly but No GPR18-Mediated Inhibition of I_{Ca} by NAGly. To study coupling of GPR18 to G proteins, we used sympathetic neurons as a heterologous expression system. We have expressed non-native GPCRs in SCG neurons by intraneural microinjection (Ikedå, 2004; Ikeda and Jeong, 2004; Lu et al., 2009) or RNA transfection (Williams et al., 2010) and reliably recapitulated endogenous G protein signaling pathways for study (Ikeda et al., 1995; Guo and Ikeda, 2004, 2005Guo et al., 2008b). Furthermore, we can examine G protein activity in SCG neurons by measuring GPCR-mediated inhibition of endogenous Ca^{2+} current (I_{Ca}). We have previously shown
that Ca\textsuperscript{2+}-channels in SCG neurons are modulated by GPCRs coupled through various G protein families: G\textsubscript{ai/o} (Ikeda et al., 1987, 1995; Ikeda, 1992; Zhu and Ikeda, 1993; Guo and Ikeda, 2004, 2005), including G\textsubscript{az} (Jeong and Ikeda, 1998), G\textsubscript{as} (Zhu and Ikeda, 1994), and G\textsubscript{aq/11} (Kammermeier et al., 2000).

We first examined the effect of NAGly application on IC\textsubscript{a} recorded from uninjected neurons. IC\textsubscript{a} was evoked at 0.1 Hz using a double-pulse voltage protocol (Fig. 3Ai, inset) in solutions designed to isolate IC\textsubscript{a}. The facilitation ratio (FR, ⊗), defined as the ratio of IC\textsubscript{a} evoked in the second test pulse (postpulse, Fig. 3, d) to IC\textsubscript{a} in the first test pulse (prepulse, Fig. 3, s), was used as a measure of Gbg-mediated IC\textsubscript{a} modulation. NAGly (10 \mu M) potentiated IC\textsubscript{a} (Fig. 3Ai), increasing both pre- and postpulse IC\textsubscript{a} and resulting in no change in the FR (Fig. 3, Aii and Aiii). A lower concentration of NAGly (1 \mu M) did not produce a change in IC\textsubscript{a}. As a positive control for G protein activation, norepinephrine (NE; 10 \mu M) was applied to the same uninjected neuron. NE, activating endogenous \alpha\textsubscript{2}-adrenergic receptors, produced a robust decrease in IC\textsubscript{a} and an increase in the FR (Fig. 3, Aii and Aiii).

Our laboratory has previously documented a direct effect of lipoamino acids on voltage-gated Ca\textsuperscript{2+} channels (Guo et al., 2008a). Here, we reproduced the result of enhanced IC\textsubscript{a}, in a voltage-dependent manner and a hyperpolarized IC\textsubscript{a}-voltage curve with NAGly (Fig. 3B). NAGly had an effect on untagged GPR18-expressing SCG neurons similar to that of un.injected controls (Fig. 3C). A lower dose of NAGly (1 \mu M) produced no change in IC\textsubscript{a}, and a higher dose increased IC\textsubscript{a} (Fig. 3Ci) both pre- and postpulse currents (Fig. 3Ciii) with no change in FR (Fig. 3Cii). NE-mediated inhibition of IC\textsubscript{a} persisted in untagged GPR18-expressing neurons (Fig. 3, Cii and Ciii). Drug responses, expressed as IC\textsubscript{a} amplitude during drug application normalized to baseline IC\textsubscript{a}, were compared between both groups in Figure 3D. Responses to low or high concentrations of NAGly were not significantly different between uninjected controls and untagged GPR18-expressing neurons (unpaired t test, P > 0.05). NE responses were also not significantly different between uninjected controls and untagged GPR18-expressing neurons (unpaired t test; P > 0.05). In every cell tested, both concentrations of

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**Fig. 1.** Expression and localization of heterologously expressed GPR18-EGFP. Confocal images of (A) GPR18-EGFP, (B) EGFP, and (C) EGFP-KRas tail constructs expressed in SCG neurons (top panels) or HeLa cells (bottom panels). Insets of top panels: line plots of fluorescence intensity from dashed line across injected SCG neuron. Note the “rim-like” fluorescence of the GPR18-EGFP construct, similar to the membrane-bound EGFP-KRas tail-expressing cells and different from cytosolic EGFP-expressing cells. Scale bar is 20 \mu m. (D) Sample Western blot of EGFP-tagged constructs. Primary anti-GFP antibody (1:2000, NeuroMab) and secondary anti-mouse HRP-conjugated antibody (1:1000, Thermo) were used. Band in GPR18-EGFP lane at approximately 80 kDa, band in EGFP lane at 27 kDa, and band in EGFP-KRas tail lane at 29 kDa. (E) Stripped and reprobed blot for loading controls \alpha\textsubscript{tubulin (1:2000, Cell Signaling) and cyclophilin B (1:5000, Abcam) corresponding to bands at 51 and 19 kDa, respectively. MW, molecular weight.

**Fig. 2.** Live-cell staining and imaging of hemagglutinin-tagged GPR18. Confocal images of stained HeLa cells transfected with (A) 3xHA-GPR18 or external HA-tagged GPR18, (B) GPR18-3xHA or internal HA-tagged GPR18. EGFP was cotransfected to label transfected cells. (A and B) Live-cell staining with biotin-labeled anti-HA antibody (1:200; Covance) and streptavidin conjugated Qdot655 secondary antibody (1:200; Molecular Probes). (C) HA staining followed fixation and permeabilization of HeLa cells. First panels are images obtained from the GFP channel (500- to 550-nm emission band-pass filter), second panels are fluorescent images from the far-red channel (650- to 710-nm emission band-pass filter), and the last panels are merged images pseudo-colored green and purple. Scale bar is 20 \mu m.
NAGly failed to inhibit I_{Ca}, whereas all exhibited NE-mediated inhibition of I_{Ca}. The enhancement of I_{Ca} by NAGly was not mediated by endogenous G_{q/11} protein–coupled receptors because overnight incubation of un.injected and untagged GPR18-injected SCG neurons with PTX, which uncouples G_{q/11} protein signaling from GPCRs, did not affect NAGly responses. After PTX treatment, NAGly still potentiated I_{Ca} in uninject ed SCG neurons (106.9% ± 3.2% baseline I_{Ca}, n = 8) and untagged GPR18-expressing SCG neurons (112.4% ± 9.7% baseline I_{Ca}, n = 3).

NAGly and NE responses were also tested in N- and C-terminal tagged versions of GPR18. I_{Ca} were potentiated by NAGly 114.6% ± 5.7% (n = 7), 105.1% ± 1.6% (n = 7), and 105.1% ± 2.8% (n = 5) from baseline I_{Ca} in GPR18-EGFP–3xHA-GPR18–, and GPR18-3xHA–expressing neurons, respectively. NE inhibited I_{Ca} 41.3% ± 3.7%, 43.1% ± 4.5%, and 42.9% ± 5.1% of baseline I_{Ca} in GPR18-EGFP–3xHA-GPR18–, and GPR18-3xHA–injected neurons, respectively. No significant difference in NAGly or NE responses was observed between untagged and tagged versions of GPR18 (one-way ANOVA, P > 0.05). Untagged GPR18 was heterologously expressed in cells for the rest of this study, unless otherwise stated.

Inhibition of LVA Ca^{2+} channels by NAGly (Barbara et al., 2009) was used as a positive control for agonist activity. SCG neurons do not endogenously express T-type Ca^{2+} channels (Fig. 4A), so we injected cDNA encoding Ca_{v}3.1 or Ca_{v}3.2 into cells. LVA-I_{Ca} elicited by a test pulse to –40 mV (Fig. 4A, inset) was transiently activated showing fast inactivation during the 25-millisecond pulse (Fig. 4B). Currents were also reversibly inhibited by application of 100 μM Ni^{2+} (Ni^{2+}– inhibition of Ca_{v}3.1-expressing neurons, 17% ± 4.5% baseline I_{Ca}, n = 5; Ni^{2+}– inhibition of Ca_{v}3.2-expressing neurons, 9.0% ± 1.1% baseline I_{Ca}, n = 5). NAGly applied to Ca_{v}3.1-injected SCG neurons inhibited LVA-I_{Ca} while potentiating HVA-I_{Ca} (Fig. 4B). In SCG neurons injected with Ca_{v}3.2, NAGly–induced inhibition of LVA-I_{Ca} (64.1% ± 4.8% baseline I_{Ca}, n = 3) and potentiated HVA-I_{Ca} (106.1% ± 10.6% baseline I_{Ca}, n = 3). In SCG neurons co-injected with Ca_{v}3.1 and GPR18, NAGly inhibited LVA-I_{Ca} and potentiated HVA-I_{Ca} (Fig. 4C). In cells injected with Ca_{v}3.2 and GPR18, NAGly inhibited LVA-I_{Ca} (52.1% ± 7.6% baseline I_{Ca}, n = 8) and potentiated HVA-I_{Ca} (109.3% ± 7.8% baseline I_{Ca}, n = 8). No significant difference in NAGly inhibition of LVA-I_{Ca} was observed between Ca_{v}3.1-expressing neurons with or without GPR18 coexpressed (Fig. 4D; unpaired t test, P > 0.05). NAGly enhancement of Ca^{2+} currents and the hyperpolarizing shift in the IV curve peak. (Cii) Sample superimposed I_{Ca} traces evoked from an untagged GPR18-injected SCG neuron using the double-pulse I_{Ca} protocol, shown as inset. Two 25-millisecond test pulses to 10 mV from a holding potential of –80 mV, separated by a 50-millisecond conditioning pulse to 80 mV. For both sample traces displayed in the figure, solid black trace is the baseline I_{Ca}, solid gray trace is the I_{Ca} during application of 10 μM NAGly, y-axis scale bar is 0.5 nA, and x-axis scale bar is 10 milliseconds. (Cii) Time course of I_{Ca} amplitude in an uninject ed neuron during exposure to 1 and 10 μM NAGly (solid gray line) and 10 μM norepinephrine (NE, dashed black line). ○ represents the prepulse I_{Ca}, ● represents the postpulse I_{Ca}, (Ciii) Time course of the FR of the same sample cell in (Cii) during exposure to 1 and 10 μM NAGly and 10 μM NE. (B) Ca^{2+} current–voltage relationship of uninjected neuron before (○) and during 10 μM NAGly (●), and during 10 μM NE. (D) Changes in I_{Ca} amplitude produced by NAGly (1 or 10 μM) and NE (10 μM) from each cell are represented as individual points in the dot plot graph. Drug responses were normalized to baseline I_{Ca} using the equation I_{drug}/I_{baseline} × 100, where I_{drug} and I_{baseline} are I_{Ca} amplitudes during and before drug application, respectively. Mean ± S.E.M. drug responses are represented as lines on graph. The n values for each group are indicated on graph in parentheses. Means values between uninjected and untagged GPR18-injected neurons were not significantly different (unpaired t test, P > 0.05).
HVA-I_{Ca} was also not significantly different between Ca_{3.1} alone and Ca_{3.1} with GPR18-expressing neurons (Fig. 4D; unpaired t test, \( P > 0.05 \)).

To ensure the external recording solution was not interfering with drug activity at the receptor, a Tris-based external IC_{Ca} solution was also tested. No significant difference in NAGly responses was observed between uninjected and GPR18-expressing SCG neurons (for controls, 122.4% ± 3.2% baseline IC_{Ca}, \( n = 9 \); for GPR18-expressing neurons, 115.5% ± 2.2% baseline IC_{Ca}, \( n = 10 \); unpaired t test, \( P > 0.05 \) or NE responses (for controls, 44.1% ± 4.2%, \( n = 9 \); for GPR18-expressing neurons, 40.8% ± 2.7%, \( n = 10 \), unpaired t test, \( P > 0.05 \)).

Although a positive effect from GPR18 has been observed from mouse-derived cell lines endogenously expressing GPR18 (McHugh et al., 2010; Burstein et al., 2011; Takenouchi et al., 2012), most GPR18 studies have used human GPR18 (Gantz et al., 1997; Kohno et al., 2006; Qin et al., 2011; McHugh et al., 2012). To assess the variability of GPR18 across species, protein sequences from different species were analyzed by protein alignment. On the basis of GPR18 protein sequence alignment (Supplemental Fig. 1C), mouse GPR18 is 95.2% identical, 97% similar to rat GPR18 and 85.8% identical, 92.1% similar to human GPR18. The greatest divergence in GPR18 protein appears in the N terminus. Because the sequences are highly similar in the transmembrane domains and important signaling regions of GPCRs, we are confident our mouse GPR18 clone is similar to studies of human GPR18. Also, because mouse and rat GPR18 protein sequences are highly similar, we do not anticipate difficulty heterologously expressing the mouse GPR18 clone in rat neurons.

Thus, we observed direct effects of NAGly only on LVA-I_{Ca} and HVA-I_{Ca} that are not G protein mediated. Furthermore, we did not observe NAGly-mediated inhibition of HVA-I_{Ca} in GPR18-injected neurons.

**Other Potential Agonists of GPR18 Do Not Inhibit I_{Ca}**

Other proposed agonists of GPR18 were tested on SCG neurons expressing GPR18, and inhibition of IC_{Ca} was used as a measure of G protein activation (Fig. 5). N-arachidonoyl-L-serine (10 \( \mu \)M), another lipoamino acid, potentiated IC_{Ca} in all groups tested: uninjected, GPR18-expressing, and CB_{1}R-expressing SCG neurons. No significant difference between groups was observed (one-way ANOVA, \( P > 0.05 \)). AEA (10 \( \mu \)M), the endocannabinoid neurotransmitter, did not inhibit IC_{Ca} in uninjected or GPR18-injected cells. AEA inhibited IC_{Ca} in CB_{1}R-expressing neurons, which was significantly different from all groups (one-way ANOVA, \( P < 0.05 \)). Synthetic cannabinoids, abnormal cannabidiol and O-1602, had no effect on baseline IC_{Ca} in uninjected or GPR18-expressing neurons. NE was applied to all cells as a positive control for G protein modulation and was found to inhibit IC_{Ca}. NE responses from CB_{1}R-injected neurons are significantly less than the other groups tested (one-way ANOVA, \( P < 0.001 \)), possibly because of the overexpression of exogenous receptor and sequestration of available G proteins from other GPCRs.

**Mutations in GPCRs that Induce Tonic Receptor Activity Do Not Activate GPR18.** To bypass the need for

![Fig. 4. NAGly-mediated inhibition of LVA-I_{Ca} heterologously expressed in rat SCG neurons. (A) Sample IC_{Ca} trace from an uninjected SCG neuron, elicited by the low-voltage IC_{Ca} protocol illustrated as inset. Two 25-millisecond test pulses, the first to \(-40\) mV and the second to 10 mV, separated by a 60-millisecond pulse to \(-60\) mV to inactivate LVA-I_{Ca}. (B) Sample superimposed IC_{Ca} traces from a Ca_{3.1}-injected SCG neuron, elicited by the low-voltage IC_{Ca} protocol. For all sample traces displayed in the figure, solid black trace is the baseline IC_{Ca}; solid gray trace is the IC_{Ca} during application of 10 \( \mu \)M NAGly, y-axis scale bar is 0.5 nA, and x-axis scale bar is 10 milliseconds. Note the inhibition of LVA-I_{Ca}, elicited during the first test pulse and the potentiation of HVA-I_{Ca}, elicited during the second test pulse by application of NAGly. (C) Sample superimposed IC_{Ca} traces from an SCG neuron co-injected with Ca_{3.1} and GPR18, elicited by the low-voltage IC_{Ca} protocol. In this set of experiments, an untagged version of GPR18 was used. (D) Changes in IC_{Ca} amplitude, both LVA- and HVA-I_{Ca} produced by NAGly (10 \( \mu \)M) from each cell are represented as individual points in the dot plot graph. NAGly responses were normalized to baseline IC_{Ca}. Mean ± S.E.M. NAGly responses are represented as lines on graph. The n values for each group are indicated on graph in parentheses. Mean values between Ca_{3.1} alone and Ca_{3.1} with GPR18-injected groups were not significantly different (unpaired t test, \( P > 0.05 \)).
agonists to activate GPR18, mutations that induce constitutive activity of receptors were introduced. These mutations were based on mutagenesis studies of the α₁B-adrernergic receptor (Cotecchia et al., 1990; Kjelsberg et al., 1992; Scheer et al., 1996, 1997), as illustrated in Supplemental Fig. 2 with snake plot diagrams (Supplemental Fig. 2A) and sequence alignments (Supplemental Fig. 2B). Site-directed mutagenesis and primers listed in Table 1 were used to generate mutants of GPR18 and a Gαi/o protein–coupled receptor, ADRA2A.

The properties of ICₐ in inhibition induced by constitutively active Gαi/o-coupled receptors are analogous to the inhibition induced by agonist application; a high FR and kinetic slowing during prolonged voltage depolarization. Other GPCR responses may also be induced by expression of constitutively active receptors because excess free-Gβγ is loaded onto downstream effectors, thereby effectively reducing the dynamic range of Gβγ-mediated responses. Constitutively active GPCRs that inhibit ICₐ through voltage-independent mechanisms are more difficult to assess because inhibited currents display similar kinetics as uninhibited currents and the mechanisms responsible for ICₐ inhibition can be quite diverse. The overall reduction in ICₐ amplitude, or ICₐ density, may indicate tonic receptor activity for such mechanisms of ICₐ inhibition. Thus, basal FR, NE-mediated ICₐ inhibition, and ICₐ density were used as measures of tonic receptor activity.

The D/ERY motif is common in all GPCRs, and mutations in the first residue of the motif can induce constitutive activity of receptors (Scheer et al., 1996, 1997). Expression of a GPR18 version of this mutation, GPR18 D118T-EGFP, was localized primarily in a membrane network inside the cell, reminiscent of the endoplasmic reticulum, and not in the plasma membrane (Fig. 6A, left). A substitution mutation of the first residue of the motif can induce constitutive activity of GPR18 (A108) and constitutively activate the ADRA2A receptor produced significantly larger basal FR than from SCG neurons expressing ADRA2A D130T (Table 2). On the other hand, mutations designed to constitutively activate the ADRA2A receptor produced significantly larger basal FR than did similar mutations in GPR18 (Table 2; unpaired t test, P < 0.017) and the ADRA2A N51A mutant significantly reduced the effectiveness of NE-induced inhibition of ICₐ.

Mutations in the highly conserved asparagine residue within the first transmembrane domain of GPCRs (Supplemental Fig. 2) can induce constitutive activity (Scheer et al., 1996). In addition, mutations in the C-terminal end of the third intracellular loop have been described for ADRA1B (Cotecchia et al., 1990; Kjelsberg et al., 1992) and ADRA2A (Ren et al., 1993) to induce constitutive activity. Analogous mutations were generated in GPR18 and ADRA2A, based on sequence alignment to ADRA1B (Supplemental Fig. 2B) and topographic location (Supplemental Fig. 2A), and constitutive activity was assessed. Mutations introduced into GPR18 failed to tonically activate receptor, as measured by basal FR, NE response, and ICₐ density (Table 2). On the other hand, mutations designed to constitutively activate the ADRA2A receptor produced significantly larger basal FR than did similar mutations in GPR18 (Table 2; unpaired t test with multiple comparison correction, P < 0.017) and the ADRA2A N51A mutant significantly reduced the effectiveness of NE-induced inhibition of ICₐ.

Thus, mutations predicted to induce constitutive receptor activity did not activate GPR18 but were effective when introduced into ADRA2A receptors, which tonically activate Gαi/o signaling pathways.

Proposed GPR18 Agonists Do Not Inhibit ICₐ in SCG Neurons Expressing a Nonconstitutively Active Mutant of GPR18. A recent study found endogenous GPR18 is constitutively active and differentially expressed in melanoma metastases (Qin et al., 2011). However, we found no indication GPR18 expressed in SCG neurons was constitutively active (basal FR, 1.3 ± 0.04, n = 25; NE-mediated ICₐ inhibition, 47.4% ± 3.6% baseline ICₐ, n = 17; ICₐ density, 2.2 ± 2.4 picoamperes per picofarad, pA/pF, n = 25). This paper also describes a single amino acid residue responsible for conferring constitutive activity of GPR18 (A108) and mutating the residue to an asparagine restored NAGly-induced G protein signaling. We tested this constitutively active null mutant by monitoring ICₐ in SCG neurons during agonist application.

![Fig. 5](https://example.com/figure5.png)
Fig. 6. Mutant class A GPCRs designed to induce or alter tonic activity of receptors. (A) Left: Confocal image of GPR18 D118T-EGFP construct expressed in HeLa cells. Note the fluorescence primarily inside of the cell in the internal membrane network, reminiscent of the endoplasmic reticulum. Scale bar is 20 μm. Right: Sample superimposed I_Ca traces from an untagged GPR18 D118T-injected SCG neuron, elicited by the double-pulse I_Ca protocol. For all sample traces displayed in the figure, solid black trace is the baseline I_Ca, dashed black trace is the I_Ca during application of 10 μM NE, solid gray trace is the I_Ca during application of 10 μM NAGly, y-axis scale bar is 0.5 nA, and x-axis scale bar is 10 milliseconds. Horizontal dashed gray line from the peak of the postpulse I_Ca highlights the relief of tonic Ca²⁺ channel inhibition by the conditioning pulse. (B) Sample superimposed I_Ca traces from an ADRA2A D130T-injected SCG neuron, elicited by the double-pulse I_Ca protocol. Note the kinetic slowing and inhibition of prepulse I_Ca, and the large relief of tonic Ca²⁺ channel inhibition by the conditioning pulse in the baseline I_Ca trace. (C) Responses of a proposed nonconstitutively active mutant of GPR18, GPR18 A108N (Qin et al., 2011), to NAGly and other agonists of GPR18. Left: Confocal image of GPR18 A108N-EGFP construct expressed in HeLa cells. Note the “rim-like” fluorescence in transfected cells. Scale bar is 20 μm. Right: Sample superimposed I_Ca traces from an untagged GPR18 A108N-injected SCG neuron, elicited by the double-pulse I_Ca protocol. Below: Time course of I_Ca amplitude in an untagged GPR18 A108N-expressing neuron during exposure to 10 μM NAGly, 10 μM AEA, and 10 μM Abn-Cbd. ○ represents the prepulse I_Ca. Note the potentiation of pre- and postpulse I_Ca after application of NAGly and lack of I_Ca response to AEA and Abn-Cbd application. (D) Changes in I_Ca amplitude, normalized to baseline I_Ca, from each cell, are represented as individual points in the dot plot graph. Mean ± S.E.M. drug responses are represented as lines on graph. The n values for each group are indicated on graph in parentheses. To compare groups, a one-way ANOVA followed by Newman-Keuls post-test was used. *P < 0.05, ***P < 0.001.

HeLa cells transfected with GPR18 A108N-EGFP displayed a “rim-like” fluorescence pattern (Fig. 6C, left). In SCG neurons expressing GPR18 A108N, NAGly potentiated pre- and postpulse I_Ca elicited with the double-pulse voltage protocol (Fig. 6C, right). Other proposed agonists of GPR18, AEA and abnormal cannabidiol, were also tested in GPR18

### TABLE 2

Summary of constitutively active mutants

<table>
<thead>
<tr>
<th>Construct</th>
<th>Basal FR</th>
<th>NE Response (% Baseline I_Ca)</th>
<th>I_Ca Density (pA/pF)</th>
</tr>
</thead>
<tbody>
<tr>
<td>GPR18 D118T</td>
<td>1.3 ± 0.04 (13)</td>
<td>36.7 ± 3.1 (13)</td>
<td>-28.5 ± 3.5 (13)</td>
</tr>
<tr>
<td>ADRA2A D130T</td>
<td>2.2 ± 0.23 (18)*</td>
<td>42.9 ± 5.0 (16)</td>
<td>-22.8 ± 3.1 (18)</td>
</tr>
<tr>
<td>GPR18 D118A</td>
<td>1.2 ± 0.03 (23)</td>
<td>46.2 ± 3.2 (22)</td>
<td>-23.6 ± 2.6 (23)</td>
</tr>
<tr>
<td>ADRA2A D130A</td>
<td>1.8 ± 0.19 (12)*</td>
<td>35.3 ± 4.5 (12)</td>
<td>-25.1 ± 3.4 (12)</td>
</tr>
<tr>
<td>GPR18 N40A</td>
<td>1.3 ± 0.04 (14)</td>
<td>43.6 ± 2.3 (13)</td>
<td>-29.4 ± 3.3 (14)</td>
</tr>
<tr>
<td>ADRA2A N51A</td>
<td>2.6 ± 0.21 (11)*</td>
<td>74.6 ± 11.1 (6)*</td>
<td>-23.9 ± 4.8 (11)</td>
</tr>
<tr>
<td>GPR18 E231E</td>
<td>1.2 ± 0.04 (5)</td>
<td>40.8 ± 6.8 (5)</td>
<td>-17.7 ± 3.8 (5)</td>
</tr>
<tr>
<td>ADRA2A T373E</td>
<td>2.1 ± 0.3 (7)*</td>
<td>32.7 ± 3.5 (6)</td>
<td>-22.1 ± 3.3 (7)</td>
</tr>
<tr>
<td>Un injected</td>
<td>1.3 ± 0.03 (14)</td>
<td>39.9 ± 2.8 (13)</td>
<td>-34.1 ± 4.1 (14)</td>
</tr>
<tr>
<td>ADRA2A</td>
<td>1.6 ± 0.2 (14)</td>
<td>35.6 ± 5.5 (14)</td>
<td>-20.8 ± 2.8 (14)</td>
</tr>
</tbody>
</table>

* P < 0.017.
A108N-injected cells, but neither induced ICa inhibition (Fig. 6C, lower panel). Only the positive control, NE, produced a significant reduction in ICa in GPR18 A108N-expressing neurons (Fig. 6D, one-way ANOVA, \( P < 0.001 \)).

**No Evidence of GPR18 Coupling to Gz.** The lack of GPR18 activation by NAGly prompted us to explore possible coupling of GPR18 to other Gz proteins not endogenously expressed in SCG neurons. We have previously expressed Gz in SCG neurons (Jeong and Ikeda, 1998) and found coupling of Gz to endogenous GPCRs (Fig. 7A). In Gz-expressing cells, NE-induced ICa inhibition by a Gzγ-mediated mechanism: there were kinetic slowing of ICa during NE application in the first test pulse (Fig. 7Al), substantial relief of prepulse ICa inhibition by the conditioning pulse, and thus increase in FR during NE application (Fig. 7Aii). The kinetics of NE activation and deactivation in Gz-injected cells (Fig. 7Aii) were slower than endogenous Gz10 protein coupling (Fig. 7Aiii), which is consistent with the slower intrinsic GTPase activity of Gz compared with Gz or Gz. Together with the persistence of NE-mediated inhibition of ICa in Gz-expressing neurons after overnight PTX treatment (Fig. 7C) suggests coupling of endogenous \( \alpha \)-adrenergic receptors to Gz.

NAGly slightly potentiated ICa in Gz-injected SCG neurons (Fig. 7Al), increasing both pre- and postpulse ICa (Fig. 7Aii) resulting in no change of the FR (Fig. 7Aiii). In SCG neurons injected with Gx and GPR18, NE, but not NAGly, induced inhibition of ICa (Fig. 7Bl) and increased the FR during NE application (Fig. 7Bii). NAGly responses were not significantly different between uninjected, Gx alone, GPR18 coexpressing Gx and GPR18 A108N coexpressing Gx groups (Fig. 7C, one-way ANOVA, \( P > 0.05 \)). NE responses in PTX-treated neurons were significantly lower in Gx alone, GPR18 coexpressing Gx, and GPR18 A108N coexpressing Gx groups compared with uninjected controls (Fig. 7C; one-way ANOVA, \( P < 0.001 \)).

Mutants of GPR18 designed to induce constitutive activity were also coexpressed with Gz to test possible coupling. Basal FR was significantly reduced in SCG neurons expressing Gz coexpressed with uninjected controls (one-way ANOVA, \( P < 0.001 \)), but no significant difference between basal FR was found between the various GPR18 mutants when coexpressed with Gz (Fig. 7Dl). NE-induced inhibition of ICa was also unchanged across all groups tested (Fig. 7Dii).

Therefore, we found no evidence of GPR18 coupling to Gz.

**No Evidence of GPR18 Coupling to Gz15.** Potential coupling of GPR18 to Gz15 was also tested. Gz15 is highly expressed in hematopoietic cells (Giannone et al., 2010), similar to GPR18 expression. Although Gz15 is considered a promiscuous G protein, capable of coupling various classes of GPCRs, reconstitution of Gz15 signaling and functional coupling of this G protein to endogenous GPCRs in SCG neurons has yet to be demonstrated.

To demonstrate functional coupling of a GPCR to Gz15 in SCG neurons, we used ICa inhibition as an assay for G protein activity. Expression of Gz15 alone suppressed endogenous NE-mediated signaling (Supplemental Fig. 3A). This artifact of Gz protein overexpression, which sequesters available Gzγ protein, could be alleviated by coinjecting \( \beta \)z and \( \gamma \)z with Gz15 to restore the stoichiometric balance of heterotrimeric G protein signaling (Supplemental Fig. 3B). The basal FR of Gz15 was also restored to uninjected control levels after coexpressing \( \beta \gamma \)z (for Gz15 alone, basal FR, 1.0 ± 0.01, \( n = 16 \); for Gz15β1γ2, basal FR, 1.3 ± 0.02, \( n = 36 \)). Endogenous \( \alpha \)-adrenoceptors do not couple to PTX-insensitive Gz15 because there was no NE-mediated inhibition of ICa in Gz15β1γ2-expressing cells after overnight PTX treatment (Supplemental Fig. 3C). Because mGluR2 has been shown to couple to Gz15 in vitro (Gomeza et al., 1996), mGluR2 and Gz15β1γ2 were coexpressed in SCG neurons and glutamate-induced inhibition of ICa after PTX treatment was measured (Fig. 8A). The properties of ICa inhibition by mGluR2 coupled to Gz15 were distinct from mGluR2 coupled to Gz10 (Supplemental Fig. 3, D and E): There was no kinetic slowing during the first test pulse of the double-pulse protocol, almost equal inhibition of the pre- and postpulse ICa, and therefore a smaller change in the FR, and insensitivity to PTX. Gz10–mediated pathways still dominated mGluR2 signaling because glutamate induced a change in FR in cells coexpressing Gz10β1γ2 and mGluR2 (Supplemental Fig. 3F).

In PTX-treated SCG neurons injected with Gz15β1γ2 and GPR18, NAGly potentiated pre- and postpulse ICa evoked by the double-pulse voltage protocol (Fig. 8, Bi and Bii), with no change in the FR (Fig. 8Biii). No NAGly-induced inhibition of ICa was observed in PTX-treated SCG neurons coexpressing Gz15β1γ2 and GPR18 or GPR18 A108N (Fig. 8C), and NAGly responses were not significantly different between all groups tested (one-way ANOVA, \( P > 0.05 \)). No NE-induced inhibition of ICa was observed in any PTX-treated group tested (Fig. 8C).

Mutants of GPR18 designed to induce constitutive activity were also coexpressed with Gz15β1γ2 to test possible coupling. ICa density was significantly reduced in Gz15β1γ2 and mGluR2-injected neurons during glutamate application (one-way ANOVA, \( P < 0.05 \)) but mutants of GPR18 coexpressed with Gz15β1γ2 did not change ICa density compared with uninjected controls (Fig. 8D).

Therefore, we found no evidence of GPR18 coupling to Gz15.

**Potentiation of I GIRK by NAGly but No GPR18-mediated Potentiation of I GIRK.** Previous work has suggested GPR18 couples to Gz10 protein signaling pathways (Kohn et al., 2006; McHugh et al., 2010, 2012; Takeuchi et al., 2012), but we have not observed any GPR18-mediated inhibition of ICa which is a primary downstream effector of Gz10. We tested other effectors of Gz10 proteins, including G protein–coupled inwardly-rectifying K+ currents (I GIRK), to further investigate GPR18 signaling. GIRK channels expressed in SCG neurons open upon G protein activation by a Gβγ-mediated mechanism.

SCG neurons do not endogenously express GIRK channels, so we injected cDNA encoding homomeric GIRK channel subunits, GIRK4 S143T (Vivaudou et al., 1997). I GIRK was evoked at 0.1 Hz using a voltage ramp (Fig. 9Ai, inset) in solutions designed to isolate I GIRK. In SCG neurons expressing GIRK4 S143T, peak I GIRK increased during NAGly treatment (Fig. 9, Ai and Aii). As a positive control for Gz10 protein activation, NE was applied and peak I GIRK also increased (Fig. 9, Ai and Aii). In SCG neurons expressing GIRK4 S143T and GPR18, both NAGly and NE potentiated I GIRK (Fig. 9, Bi and Bii). NE significantly increased I GIRK in SCG neurons expressing GIRK4 S143T with or without GPR18 (Fig. 9C; one-way ANOVA, \( P < 0.001 \)). But the modulation of I GIRK by NAGly was modest and only reached significance in SCG neurons expressing GIRK4 S143T alone with and without PTX treatment (Fig. 9C), suggesting that the effect of NAGly on I GIRK is independent of GPR18 expression. Furthermore,
NAGly’s effect on $I_{\text{GHRK}}$ is not $G_{\alpha_{i/o}}$ protein mediated because overnight PTX treatment did not block NAGly responses on $I_{\text{GHRK}}$ but did block NE responses (Fig. 9C).

Thus, NAGly activation of GPR18 was not observed with G protein modulation of $I_{\text{GHRK}}$ currents as an assay.

**No NAGly-induced Change in cAMP Levels in GPR18-Expressing HEK Cells.** Another downstream effector of G proteins is adenylyl cyclase, which converts ATP to cAMP. Live-cell cAMP levels were monitored in HEK cells transfected with the BRET-based cAMP sensor, CAMYEL (Jiang et al., 2007), loaded onto a multiwell luminescence plate reader. Net BRET was calculated from measurements of light intensity from the acceptor and donor channels after application of enzyme substrate, h-coelanterizene. High net BRET values indicate high intracellular cAMP levels, and low net BRET values indicate low intracellular cAMP levels.

The ability of $G_{\alpha_{i/o}}$ protein–coupled receptors to inhibit adenylyl cyclase and reduce intracellular cAMP levels after forskolin stimulation was used as a measure of $G_{\alpha_{i/o}}$ protein activation. Application of forskolin (1 μM) produced a decrease in net BRET, and NAGly application failed to change net BRET levels in empty vector- or GPR18-expressing HEK cells (Fig. 10Aii). A comparison of net BRET values 4 minutes after NAGly application shows no significant difference from the ratio of postpulse to prepulse ICa of the same sample cell as in Bii. (C) Changes in ICa and ICa amplitude produced by NAGly (10 μM) and NE (10 μM) from each cell are represented as individual points in the dot plot graph. Mean ± S.E.M. drug response are represented as lines on graph. NE-mediated responses were elicited from SCG neurons after overnight PTX treatment. The $s$ values for each group are indicated on graph in parentheses. To compare groups, a one-way ANOVA was performed, followed by Newman-Keuls post-test was performed. *** $P < 0.001$. (D) Testing possible coupling of constitutively active GPR18 with $G_{\alpha_{i/o}}$ in SCG neurons. (Di) The basal FR, a sensitive indicator of tonic receptor activity, was determined from the ratio of postpulse to prepulse ICa of the first recording obtained from each cell. Basal FR values from each cell are represented as individual points in the dot plot graph. Mean ± S.E.M. basal FR are represented as lines on graph. To compare groups, a one-way ANOVA followed by Newman-Keuls post-test was performed. *** $P < 0.001$. (Dii) Changes in ICa amplitude in response to NE compared with uninjected SCG neurons. (Di) The basal FR, a sensitive indicator of tonic receptor activity, was determined from the ratio of postpulse to prepulse ICa of the first recording obtained from each cell. Basal FR values from each cell are represented as individual points in the dot plot graph. Mean ± S.E.M. NE response are represented as lines on graph. No significant difference was observed between groups, as determined by one-way ANOVA followed by Newman-Keuls post-test ($P > 0.05$).
after forskolin stimulation, which was blocked by overnight PTX treatment (Fig. 10Bi). The increase in net BRET values 4 minutes after glutamate application was significant for the mGluR2-transfected group (Fig. 10Bii; one-way ANOVA, $P < 0.05$).

$\text{G}_{\alpha_\gamma}$-coupled pathways stimulate adenylate cyclase and the ability of GPCRs to increase intracellular cAMP levels was used as a measure of $\text{G}_{\alpha_\gamma}$-protein activation. NAGly did not significantly change net BRET levels in empty vector- or GPR18-expressing HEK cells (Fig. 10C; unpaired $t$-test, $P > 0.05$). But dopamine applied to D1R-transfected HEK cells was able to decrease net BRET values, which was blocked by overnight CTX treatment (Fig. 10Di). Only the D1R-transfected group significantly reduced net BRET values 4 minutes after dopamine application (Fig. 10Dii; one-way ANOVA, $P < 0.001$).
With the BRET-based cAMP sensor, we found no evidence of NAGly-mediated GPR18 coupling to Go12 or Go5 signaling pathways.

Discussion

In the present study, we could not activate GPR18 that was heterologously expressed in a native neuronal system. NAGly is the proposed endogenous ligand for GPR18 (Kohno et al., 2006) and mediates microglia migration through a PTX-sensitive pathway (McHugh et al., 2010); however, we did not observe activation of Go12 protein signaling after NAGly application on heterologously expressed GPR18 in SCG neurons (Fig. 3). Moreover, other putative agonists, including AEA, abnormal cannabidiol and O-1602 did not induce ICa in GPR18-injected SCG neurons (Fig. 5). Likewise, a high-throughput screen of 43 lipid ligands, which included AEA and NAGly, also failed to activate GPR18 (Yin et al., 2009). This was surprising because most, if not all, endogenous or heterologously expressed GPR18 protein–coupled receptors in SCG neurons negatively couple to N-type Ca2+ channels via a PTX-sensitive pathway. Instead, NAGly produced a consistent increase in ICa (Fig. 3), similar to what has previously been reported by our laboratory (Guo et al., 2008a). As positive controls, NAGly potently inhibited heterologously expressed T-type Ca2+ channels confirming agonist activity (Fig. 4), and full-length GPR18 protein was expressed and trafficked to the plasma membrane, as demonstrated with the “rim-like” fluorescence pattern of a GFP-tagged version of the receptor (Fig. 1) and positive staining of external HA-tagged GPR18 (Fig. 2). However, in the absence of an actual receptor response, expression of functional GPR18 receptors in this study remains to be determined. A protein sequence alignment of GPR18 (Supplemental Fig. 1C) suggests the mouse GPR18 clone used in our study is similar to functional GPR18 in other studies (Kohno et al., 2006; Qin et al., 2011; McHugh et al., 2012) and capable of proper protein expression. Other downstream effectors of Go12 were examined, but NAGly failed to activate GPR18. ICa was potentiated by NAGly (Fig. 9), but this effect was independent of GPR18 expression and Go12 protein coupling because 1) NAGly-induced potentiation of current was present in GIRK4 S143T alone injected SCG neurons and 2) augmentation of ICa by NAGly persisted after overnight PTX treatment. In GPR18-expressing cells, cAMP levels were not altered by NAGly after forskolin-induced cAMP production (Fig. 10A), suggesting no inhibition of adenylate cyclase by GPR18. Furthermore, GPR18 failed to couple to other Go proteins, Go4 (Fig. 7) and Go15 (Fig. 8), heterologously expressed in SCG neurons. We have previously demonstrated GPCR coupling to Go4 using agonist-mediated ICa inhibition (Jeong and Ikeda, 1998), but this is the first demonstration of negative coupling of activated Go15 to HVA-Ca2+ channels. Thus, modulation of N-type Ca2+ channels is a versatile assay of G protein activity because it is a common downstream effector of multiple G protein families.

Mutants of GPR18 that are predicted to confer constitutive activity, failed to tonically activate (i.e., in the absence of overt agonist) the receptor according to the measures of tonic G protein activity used in this study (Fig. 6; Table 2). On the other hand, analogous mutations in ADRA2A (of the same class A of GPCRs as GPR18) were tonically active when
Keuls post-test, ***p < 0.001. (A and B) Test of GPR18-mediated inhibition of forskolin stimulated cAMP production. (Ai) Time course of net BRET readings from empty vector and untagged GPR18-expressing HEK cells. F, forskolin (1 μM); NG, NAGly (10 μM). (Aii) Net BRET values 4 minutes after injection of NAGly from each sample well are represented as individual points in the dot plot graph. Mean ± S.E.M. net BRET values are represented as lines on graph. A significant increase in mean net BRET value after glutamate application was observed in the mGluR2-transfected group (one-way ANOVA followed by Newman-Keuls post-test, **p < 0.01). (B and C) Test of GeGlu-mediated stimulation of cAMP production. (Bi) Time course of net BRET readings from empty vector and mGluR2-expressing HEK cells. F, forskolin (1 μM); G, glutamate (100 μM). PTX was applied to HEK cells during overnight incubation. (Bii) Net BRET values 4 minutes after injection of glutamate from each sample well are represented as individual points in the dot plot graph. Mean ± S.E.M. net BRET values are represented as lines on graph. A significant increase in mean net BRET value after glutamate application was observed in the mGluR2-transfected group (one-way ANOVA followed by Newman-Keuls post-test, **p < 0.001). (C and D) Test of GeGlu-mediated stimulation of cAMP production. (Ci) Time course of net BRET readings from empty vector and untagged GPR18-expressing HEK cells. F, forskolin (10 μM). (Cii) Net BRET values 4 minutes after injection of NAGly from each sample well are represented as individual points in the dot plot graph. Mean ± S.E.M. net BRET values are represented as lines on graph. No significant difference was observed between empty vector and GPR18-transfected cells (unpaired t test, P > 0.05). (D) Time course of net BRET readings from empty vector and D1R-expressing HEK cells. D, dopamine hydrochloride (10 μM); F, forskolin (10 μM). CTX was applied to HEK cells during overnight incubation. (Di) Net BRET values 4 minutes after injection of dopamine from each sample well are represented as individual points in the dot plot graph. Mean ± S.E.M. net BRET values are represented as lines on graph. A significant decrease in mean net BRET value after dopamine application was observed in the D1R-transfected group (one-way ANOVA followed by Newman-Keuls post-test, ***p < 0.001).

Fig. 10. Monitoring modulation of adenylate cyclase using the BRET-based cAMP sensor CAMYEL. Data are obtained from live HEK cells loaded in a microplate luminometer. HEK cells were transfected with empty vector or selected G protein–coupled receptor cDNA, CAMYEL cDNA, and PEI. Approximately 16 hours after transfection, cells were transferred to a black 96-well microplate and loaded into a luminometer for recording. Net BRET was calculated as A/D – d, where A is the light intensity measured from the acceptor channel (542/27 nm) for 1 second, D is the light intensity measured from the donor channel (460/60 nm) for 1 second, and d is the background or spectral overlap, calculated previously as the A/D value for Blue8 alone. Net BRET values are inversely related to cAMP levels (high net BRET = low intracellular cAMP levels, low net BRET = high intracellular cAMP levels). (A and B) Test of GeGlu-mediated inhibition of forskolin stimulated cAMP production. (Ai) Time course of net BRET readings from empty vector and untagged GPR18-expressing HEK cells. F, forskolin (1 μM); NG, NAGly (10 μM). (Aii) Net BRET values 4 minutes after injection of NAGly from each sample well are represented as individual points in the dot plot graph. Mean ± S.E.M. net BRET values are represented as lines on graph. No significant difference was observed between empty vector and GPR18-transfected cells (unpaired t test, P > 0.05). (B) Test of GeGlu-mediated stimulation of cAMP production. (Bi) Time course of net BRET readings from empty vector and mGluR2-expressing HEK cells. F, forskolin (1 μM); G, glutamate (100 μM). PTX was applied to HEK cells during overnight incubation. (Bii) Net BRET values 4 minutes after injection of glutamate from each sample well are represented as individual points in the dot plot graph. Mean ± S.E.M. net BRET values are represented as lines on graph. A significant increase in mean net BRET value after glutamate application was observed in the mGluR2-transfected group (one-way ANOVA followed by Newman-Keuls post-test, **p < 0.01). (C and D) Test of GeGlu-mediated stimulation of cAMP production. (Ci) Time course of net BRET readings from empty vector and untagged GPR18-expressing HEK cells. F, forskolin (10 μM). (Cii) Net BRET values 4 minutes after injection of NAGly from each sample well are represented as individual points in the dot plot graph. Mean ± S.E.M. net BRET values are represented as lines on graph. No significant difference was observed between empty vector and GPR18-transfected cells (unpaired t test, P > 0.05). (D) Time course of net BRET readings from empty vector and D1R-expressing HEK cells. D, dopamine hydrochloride (10 μM); F, forskolin (10 μM). CTX was applied to HEK cells during overnight incubation. (Di) Net BRET values 4 minutes after injection of dopamine from each sample well are represented as individual points in the dot plot graph. Mean ± S.E.M. net BRET values are represented as lines on graph. A significant decrease in mean net BRET value after dopamine application was observed in the D1R-transfected group (one-way ANOVA followed by Newman-Keuls post-test, ***p < 0.001).

expressed in rat sympathetic neurons (Fig. 6B; Table 2). After expression of ADRA2A mutants, the basal FR, a sensitive indicator of tonic G protein activation (Ikeda, 1991), was significantly elevated (Table 2). This elevation was abolished after PTX treatment implicating tonic activation of endogenous Gi alpha proteins. It should be noted that some mutations designed to confer tonic activity of GPCRs also increase agonist potency (Cotecchia et al., 1990), suggesting tonic G protein activity may actually represent increased sensitivity to agonist levels. This possibility cannot be excluded before testing receptor antagonists. Other possible consequences of tonic GPCR activity, such as activation of β-arrestin–mediated internalization, are not quantifiable with the electrophysiological techniques used in this study but may nevertheless occur. Redistribution of mutant GPR18 receptors into the endoplasmic reticulum of cells (Fig. 6A) may reflect this mechanism, or mutations in the receptor may affect receptor trafficking. The lack of receptor expression at the plasma membrane may confound measurements of G protein activity. However, we have described other heterologously expressed GPCRs where GFP-fusion constructs are located primarily inside the cell but receptors maintain functional G protein signaling (Guo et al., 2008b). Thus, we infer that a small amount of GPCR expressed in the plasma membrane, which may not be noticeable with GFP-fusion constructs, is sufficient to carry out G protein activity. Assuming the mutations introduced into GPR18 were successful in producing a tonically active receptor, the inability to measure changes in basal G protein activity may indicate the existence of a mediator that couples GPR18 to G proteins, which is missing in SCG neurons or lost by cellular dialysis during whole-cell recordings. Heterodimerization of GPR18 with another GPCR may facilitate G protein signaling of GPR18 by heterodimer directed signaling (for reviews see

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Hudson et al., 2010; Marshall and Foord, 2010) and studies in endogenously expressing GPR18 cells may be necessary to find its GPCR binding partner and to recapitulate G protein signaling of GPR18.

The only positive responses of NAGly observed in this study were its direct effects on voltage-gated Ca^{2+} channels (Figs. 3 and 4). Similar effects of lipooamino acids and arachidonic acid, a product of fatty acid amide hydrolase (FAAH) hydrolysis of NAGly (Grazia Cascio et al., 2004), on ion channel function have been documented (Chemín et al., 2007; Guo et al., 2008a; Barbara et al., 2009). However, it is unlikely that the effects observed in this study are a result of NAGly breakdown to arachidonic acid and glycine because arachidonic acid reduces N-type Ca^{2+} channel amplitude (Liu and Rittenhouse, 2000) and the effect of NAGly on T-type Ca^{2+} channels is independent of FAAH activity (Barbara et al., 2009). NAGly has many reported actions: G_{i/o}-mediated activation of high-conductance Ca^{2+}-sensitive K^{-} (BK) channels (Begg et al., 2003; Parmar and Ho, 2010), partial agonism of GPR92 (Oh et al., 2008), and blockade of glycine uptake via the glycine transporter, GLYT2 (Wiles et al., 2006), to name a few. Of note, NAGly is also a potent competitive substrate with AEA for FAAH (Huang et al., 2001) and inhibiting FAAH can increase endogenous AEA levels (Burstein et al., 2002). It is unclear which target of NAGly is responsible for the NAGly-induced cell migration or apoptosis observed in other recombinant systems (McHugh et al., 2010, 2012; Takenouchi et al., 2012) or whether GPR18 signaling is cell-type dependent. For instance, if NAGly-induced signaling is intimately related to resting endocannabinoid levels, the lack of endogenous endocannabinoid production in SCG neurons (Won et al., 2009) may be responsible for this study’s inability to reconstitute GPR18 signaling pathways.

GPR18-mediated signaling directly in neurons has not been demonstrated. GPR18 activity has been implicated in neuronal function by virtue of its regulation of microglial function (McHugh, 2012), but no detectable levels of GPR18 transcript can increase endogenous FAAH levels (Burstein et al., 2002). It is possible that GPR18 signaling in microglia is responsible for this study’s inability to reconstitute NAGly signaling. Perhaps studies examining NAGly-mediated responses from endogenously expressing GPR18 cells will shed some light on this signaling pathway.

**Authorship Contributions**

**Participated in research design:** Lu, Puhl, Ikeda

**Conducted experiments:** Lu

**Contributed new reagents or analytic tools:** Puhl

**Performed data analysis:** Lu

**Wrote or contributed to writing of the manuscript:** Lu, Puhl, Ikeda

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