Neural Expression of G Protein-coupled Receptors GPR3, GPR6, and GPR12 Upregulates cyclic AMP Levels and Promotes Neurite Outgrowth*

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Running Title: GPR-mediated Regulation of Intraneuronal cAMP

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Cyclic AMP regulates multiple neuronal functions, including neurite outgrowth and axonal regeneration. GPR3, GPR6, and GPR12 make up a family of constitutively active G protein-coupled receptors (GPCRs) that share greater than 50% identity and 65% similarity at the amino acid level. They are highly expressed in the central nervous system, and their expression in various cell lines results in constitutive stimulation of cAMP production. When the constitutively active GPCRs were overexpressed in rat cerebellar granule neurons in culture, the transfected neurons exhibited significantly enhanced neurite outgrowth and overcame growth inhibition caused by myelin-associated glycoprotein (MAG). GPR12-mediated neurite outgrowth was the most prominent and was shown to depend on Gs, and cAMP-dependent protein kinase (PKA).

Moreover, the GPR12-mediated rescue from MAG inhibition was attributable to PKA-mediated inhibition of the small GTPase, RhoA. Among the 3 receptors, GPR3 was revealed to be enriched in the developing rat cerebellar granule neurons. When the endogenous GPR3 was knocked down, significant reduction of neurite growth was observed, which was reversed by expression of either GPR3 or GPR12. Taken together, our results indicate that expression of the constitutively active GPCRs upregulates cAMP production in neurons, stimulates neurite outgrowth, and counteracts myelin inhibition. Further characterization of the GPCRs in developing and injured mammalian neurons should provide insights into how basal cAMP levels are regulated in neurons and could establish a firm scientific foundation for applying receptor biology to treatment of various neurological disorders.

Neurons in the adult mammalian central nervous system, unlike those in the embryonic or peripheral nervous system, cannot regenerate their axons after injury. The failure of regeneration is attributable to the non-permissive environment for axonal growth, such as the presence of myelin-associated inhibitory molecules (1), formation of glial scar (2), and a deficiency in growth-promoting substrates and factors (3), as well as the intrinsic inability of adult neurons to survive and regrow their axons after injury. Intracellular level of cAMP was identified as one of such intrinsic factors that could influence axonal regeneration (4). Indeed, recent reports demonstrate that activating cAMP signaling by treating neurons with dibutyryl-cAMP (db-cAMP; a cell-permeable non-hydrolysable analogue of cAMP) or a variety of neurotrophins, such as brain-derived neurotrophic factor (BDNF) and neurotrophin-3 (NT-3), overcomes inhibition caused by myelin-associated glycoprotein (MAG) and myelin in vitro (5-7). Exposure to neurotrophins activates their specific receptor tyrosine kinases (Trk A, Trk B, and Trk C), which are expressed in various types of neurons and form a complex with p75 neurotrophin receptor. The activated receptor tyrosine kinases then transiently activate extracellular signal-regulated kinases (ERKs), which, in turn, cause phosphorylation and...
inhibition of phosphodiesterases (PDEs), the enzymes that degrade cAMP. As a result, the intracellular concentration of cAMP is upregulated in the neurotrophin-treated neurons (5).

The activity of adenylate cyclases, a class of enzymes that synthesize cAMP from ATP, is regulated through a subset of G protein-coupled receptors (GPCRs). However, to the best of our knowledge, the involvement of GPCRs in regulating intraneuronal cAMP levels and axonal regeneration has not been well documented. We previously cloned 2 highly related, orphan GPCR genes, GPCR01 and GPCR21, which are expressed predominantly in the mouse central nervous system (8). Subsequently, human counterparts of these genes, GPR12 and GPR3, as well as a third member of the family, GPR6, were cloned and reported by other investigators (9-12). Interestingly, later studies demonstrated that these GPCRs, when expressed in a variety of cell lines, stimulate adenylate cyclases to levels similar to those obtained with other Gs-coupled receptors that are fully stimulated by their ligands (13,14).

Because these 3 constitutively active GPCRs are highly expressed in neurons in the central nervous system (8,11,14,15), their developmentally regulated expression may account in part for the molecular mechanisms regulating the cAMP levels of these neurons. In this study, we chose rat cerebellar granule neurons as a model system and showed for the first time that the expression of the constitutively active GPCRs (GPR3, GPR6, and GPR12) upregulates intraneuronal cAMP, enhances neurite outgrowth, and blocks myelin inhibition in rat primary neurons.

**EXPERIMENTAL PROCEDURES**

**Plasmid Construction** - The cDNA clone for the monomeric red fluorescent protein (mRFP1; AF506027) (16) was kindly provided by R. Y. Tsien (University of California, San Diego, CA). The cDNA clones for the dominant negative mutant of rat G, α subunit (GsDN) and control G, α subunit (Gsc), originally named αs(a3β5/G226A/A366S) and αs(R280K) (17), were kindly provided by C. H. Berlot (Geisinger Medical Center, Danville, PA). The full-length cDNA library clones for mouse GPR3 and GPR12 genes were originally named GPCR21 (D21062) and GPCR01 (D21061), respectively (8). The full-length GPR6 coding sequence was amplified from mouse genomic DNA with Pfu Turbo® DNA polymerase (Stratagene, La Jolla, CA) using a pair of primers, 5'-CAGGGAGCAACATCTAGCGCGATGAA-3' and 5'-TGTTGGGTGTTGTTTGTGAGGGA-3', and cloned into a pCR4-TOPO® -blunt cloning vector (Invitrogen, Carlsbad, CA), which was designated as pCR4-GPR6. The 1.2-kb KpnI-XbaI fragment of GPR3 cDNA, 1.2-kb Pmel-NotI fragment of the pCR4-GPR6, and 1.4-kb HindIII-MscI fragment of GPR12 cDNA were cloned into the pHGCX expression plasmid (18) between the unique KpnI and XbaI, EcoRV and NotI, and HindIII and EcoRV sites, respectively. The resulting expression vectors were designated as pHGC-GPR3, -GPR6, and -GPR12, respectively. A control vector that expresses mouse adrenomedullin receptor (ADMR; D17292) (19), pHGC-ADMR, was also constructed. Each of these plasmid vectors expresses receptor protein and enhanced green fluorescent protein (EGFP; Clontech Laboratories Inc., Mountain View, CA) under the control of the human cytomegalovirus (CMV) immediate-early (IE) promoter and herpes simplex virus (HSV) IE 4/5 promoter, respectively. A second set of expression vectors, pHGC-GPR3-RHA, -GPR6-RHA, and -GPR12-RHA, were also constructed. These vectors express each receptor fused with mRFP1 followed by influenza virus hemagglutinin (HA) epitope (RHA) at the COOH terminus. Finally, we constructed 4 expression vectors, pHGC-GPR12-RHA-GsDN, -GPR12-RHA-Gsc, -RHA-GsDN, and -RHA-GsC, which contain a third expression cassette: GsDN or Gsc under the control of the CMV promoter. The construction details and sequences of these vectors are available upon request.

**Cell lines and cell culture** - Mouse neuroblastoma Neuro2a cells (American Tissue Culture Collection, Manassas, VA) and human embryonic kidney (HEK)-293 cells (Invitrogen) were maintained and propagated in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% fetal bovine serum (FBS), penicillin (100 U/mL), and streptomycin.
(100 μg/mL). All cells were cultured at 37°C in an atmosphere containing 5% carbon dioxide.

**Immunoblotting** - HEK-293 cells were transfected with plasmid DNA using LipofectAMINE™ (Invitrogen) following manufacturer’s recommendations. Twenty-four hours after transfection, the cells were harvested and lysed by incubating in lysis buffer containing 50 mM Tris (pH 8.0), 150 mM NaCl, 1% Triton X-100, 100 mM iodoacetamide (Sigma-Aldrich, St. Louis, MO), and Complete™ protease inhibitor cocktail (Roche Diagnostics, Indianapolis, IN) on ice for 30 min with gentle agitation. Cell lysates were cleared by brief centrifugation, and supernatants were collected. Twenty micrograms of protein from each sample were supplemented with 20 mM dithiothreitol, boiled at 98°C for 5 min, and separated by Tris-glycine SDS-polyacrylamide gel electrophoresis (10% gel) and transferred onto a polyvinylidene difluoride membrane (Millipore, Bedford, MA). The membrane was blocked by incubating in 0.1% Tween 20 in phosphate-buffered saline (PBS) containing 3% skim milk (Bio-Rad Laboratories, Inc., Hercules, CA) for 1 hour at room temperature. The blot was then incubated with anti-HA rat monoclonal antibody (1:3000; Roche Diagnostics) overnight at 4°C, washed 3 times in 0.1% Tween 20 in PBS, and incubated with anti-rat IgG conjugated to horseradish peroxidase (1:10000; Amersham Biosciences, Pittsburgh, PA) for 1 hour. Immunoreactive protein bands were then detected using the ECL Plus™ Western Blotting Detection System (Amersham) and Kodak BioMax XAR films (Eastman Kodak, Rochester NY). The same blot was incubated with Restore™ stripping buffer (Pierce Chemical, Rockford, IL) and reused for the detection of β-actin (1:3000; mouse monoclonal antibody; Sigma) as a loading control.

**Chemicals, proteins, and siRNAs** - Protein kinase inhibitors, KT5720, KT5823, Gö6976, and Rp-cAMP, as well as forskolin and db-cAMP were purchased from Calbiochem (La Jolla, CA). Recombinant human BDNF and rat MAG-human IgG Fc chimera (MAG-Fc) proteins were purchased from Sigma-Aldrich. Lysoosphatidic acid (LPA) was purchased from Biomol International LP (Plymouth Meeting, PA). G Silencer® siRNA against rat GPR3 (5’-CCUACUCAGAGACAAcU/ 5’ GUUGUCUCAGAGUGUAGGtg) and negative control #1 siRNA were purchased from Ambion, Inc. (Austin, TX).

**Isolation of rat cerebellar granule neurons and DNA electroporation** - Cerebellar granule neurons were prepared from Sprague Dawley rats (Harlan, Indianapolis, IN) according to an established procedure (20) with modifications. Briefly, pups were sacrificed at postnatal day 7 (P7), and the whole cerebellum was removed. The meninges were carefully stripped off, and whole tissue was then washed in calcium- and magnesium-free PBS and dissociated into single cells using the Worthington Papain Dissociation System (Worthington Biochemical Corporation, Lakewood, NJ). The dissociated cerebellar cells were applied immediately to a two-step gradient of Percoll (35%/60% in PBS; Sigma-Aldrich). After centrifugation at 2,000 × g for 10 min at 4°C, a fraction enriched with granule neurons was collected from between the 35% and 60% Percoll layers. The isolated neurons were washed once with PBS and subjected to electroporation using the Nucleofector™ system (Amaxa Inc., Gaithersburg, MD). Briefly, 5 × 10⁶ dissociated neurons were spun down at 138 × g for 5 min at 4°C, resuspended in 100 µL of Rat Neuron Nucleofector™ solution kept at room temperature, combined with 3 µg of plasmid DNA, and transferred into a cuvette and electroporated using program G-13 of the Amaxa system. For RNA interference experiments, neurons were cotransfected with 1.5 µg of siRNA and 2 µg of GFP-expressing plasmid DNA using the Amaxa system. The electroporated neurons were immediately resuspended in 600 µL of DMEM supplemented with 10% fetal bovine serum and plated onto 24-well culture plates (BD Falcon, BD Biosciences, Franklin Lakes, New Jersey) or chamber glass slides (Nalge Nunc International, Rochester, NY) coated with 100 µg/mL of poly-D-lysine (PDL; Sigma-Aldrich) and 2 µg/mL of laminin (BD Biosciences, San Jose, CA). Four to 6 hours after electroporation, the culture medium was replaced with B27-supplemented neurobasal-A medium (Invitrogen) with 200 µM L-glutamine. Chemicals, such as protein kinase inhibitors, cAMP analogues, BDNF, or LPA, were added 4
4 hours after plating neurons and kept in the culture for 18 to 48 hours. Transfected GFP-positive neurons were imaged using a Zeiss LSM510 META confocal microscope (Carl Zeiss Microimaging, Inc., Thornwood, NY). Images were taken using a 10 × objective lens, GFP-labeled neurites were traced using the LSM 5 Image Browser software (Zeiss) built in to the microscope, and the length of the longest neurite process for each neuron was measured (at least 150 neurons per each condition).

**RNA isolation, cDNA synthesis, and real-time polymerase chain reaction (PCR)** - Rat cerebellar granule neurons were prepared from 4 different developmental stages of Sprague Dawley rats, P1, P4, P7, and P14, according to the above described protocol. Total RNA was isolated from the prepared neurons using TRIzol reagent (Invitrogen). The RNA samples were treated with the TURBO DNA-free™ kit (Ambion) and then reverse transcribed into first-strand cDNA by Superscript II™ reverse transcriptase (Invitrogen) using oligo-dT primers according to the manufacturer’s protocols. In addition to the prepared rat cDNA samples, a Mouse Multiple Tissue cDNA Panel (BD Biosciences Clontech, Mountain View, CA) was used for quantitative PCR analyses. The sequences of PCR primers and VIC-labeled TaqMan probes specific for rat GPR3, GPR6, and GPR12 genes were selected using Primer Express® software (Applied Biosystems, Foster City, CA): 5′-CGCCAACCTCTCTCTCTCTAC-3′, 5′-CGGGTTGATCATGGAGTTGTAA-3′, and 5′-VIC-CCTACCTACCTTACCCTGC-TAMRA-3′ for rat GPR3; 5′-AGATCCACGTCCTTTGTTG-3′, 5′-GGAGCGGAAGGGCATTCT-3′, and 5′-VIC-GCTTGTTCTGCTGTTCTCCA-TAMRA-3′ for rat GPR6; 5′-GGGCAGCCGGTGACTTC-3′, 5′-ATCGTTGCTTCTGCCTGTGA-3′, and 5′-VIC-CTCTCTACATTCCCTGCTGC-TAMRA-3′ for rat GPR12; 5′-CTGACCGCGTGGCTCTAGA-3′, 5′-CACTTGGGCTGTGAGACATTTC-3′, and 5′-VIC-TGTTCCAGATGGTCAGGGTCCCATC-3′ for mouse GPR6; 5′-GGTGCTCTAAGGATGCTTCTGCTT-3′, and 5′-VIC-CTTCCTTTAAATTCTTTGCACTGGA-TCTCACA-TAMRA-3′ for mouse GPR12. Ready-made FAM-labeled TaqMan probes and primers specific for rat β-actin (Rn00667869_m1) or mouse β-actin (Mm00607939_s1, Applied Biosystems) were used as internal controls. Real-time PCR analyses were carried out in 96-well optical reaction plates using an ABI PRISM® 7900 HT sequence detection system according to the manufacturer's protocol. After 2-min incubation at 50°C, the samples were denatured by a 10-min incubation at 95°C and subjected to 40 cycles of amplification (95°C for 15 s, 60°C for 1 min). The fluorescence signal from each well was normalized using an internal passive reference. The cycle threshold (C_T) values obtained with the GPR3, GPR6, and GPR12-specific probes and primers were compared with those of β-actin-specific probe and primers using the comparative C_T method as described in the user manual (User Bulletin #2 for ABI Prism® 7700).

**Intracellular cAMP measurement** - In a 24-well culture plate (BD Falcon), 2 × 10^5 HEK-293 cells were transfected with plasmid vector DNA using LipofectAMINE™. Four hours after transfection, the cells were washed twice and fed with DMEM supplemented with charcoal-stripped FBS (Gemini Bio-Product, West Sacramento, CA). Alternatively, 5 × 10^6 cerebellar neurons were electroporated with plasmid vector DNA using the Amaxa Nucleofector™ system as described above and plated into each well of a 48-well culture plate coated with PDL/laminin. The transfected 293 cells and neurons were lysed and harvested 24 hours after transfection. Levels of intracellular cAMP were measured using the cAMP Biotrak enzymeimmunoassay (EIA) kit (Amersham) according to the non-acetylation EIA procedure.

**MAG-Fc inhibition assay and Rho-GTP assays** - Five × 10^6 cerebellar neurons were electroporated with vector DNA using the Amaxa Nucleofector™ system and plated onto a 10-cm culture dish (BD Falcon) coated with PDL/laminin. Four hours after electroporation,
MAG-Fc pre-conjugated with human anti-Fc (Jackson ImmunoResearch, West Grove, PA) was added to the culture medium at a final concentration of 25 μg/mL. Twenty-four hours after transfection, the length of the longest neurite process for each neuron was measured as described above. Amounts of Rho-GTP in the transfected cerebellar neurons were quantitated using the G-LISA™ RhoA activation assay kit (Cytoskeleton, Inc., Denver, CO) according to manufacturer's recommendations. The chemiluminescence signal of each sample was detected and analyzed using a FLUOstar OPTIMA microplate reader (BMG LABTECH Inc., Durham, NC).

**Statistical analyses** - Statistical analysis was performed by one-way ANOVA followed by Fisher’s PLSD test. \( P < 0.05 \) was considered statistically significant.

**RESULTS**

**GPR3, GPR6, and GPR12 are highly expressed in the central nervous system** - We originally reported molecular cloning of the mouse GPCR21 (GPR3) and GPCR01 (GPR12) genes and characterized their predominant expression in the central nervous system (8). To verify the expression levels of the 3 closely related G protein-coupled receptor (GPCR) genes, GPR3, GPR6, and GPR12, in mammalian tissues, quantitative real-time PCR analysis was performed using a panel of mouse tissue cDNA as a template. As shown in Figure 1, all 3 receptor genes are transcribed abundantly in adult brain. GPR12 showed the highest level of expression in the brain, and a significant amount of its transcript was detected in liver. The expression of GPR6 was mostly restricted to the brain, with only a trace amount detected in the testis. GPR3, on the other hand, was mainly expressed in the brain and testis. We and others have reported that GPR3 is also highly expressed in mouse oocytes and is responsible for the maintenance of meiotic arrest in mammalian oocytes (21-24).

**Functional assessment of GPR3, GPR6, and GPR12 in HEK-293 and Neuro2A cell lines** - To investigate the functions of GPR3, GPR6, and GPR12, we constructed 2 versions of plasmid vectors expressing each of the 3 receptors, either as the receptors themselves or as RHA (mRFP1 and HA tag) fusion proteins. All the vectors contain an additional GFP expression cassette that allows easy visualization of the transfected cells (Figure 2A). To verify expression of these receptor proteins, we first transfected HEK-293 cells with the RHA fusion constructs and performed Western blot analysis using an anti-HA rat monoclonal antibody. Single major bands of 60 to 65 kD were detected in the lanes for GPR3-, GPR6-, and GPR12-RHA (Figure 2B), which is consistent with the expected molecular weights of GPR3-, GPR6-, and GPR12-RHA proteins (62.1, 64.8, and 63.3 kD, respectively).

We then examined levels of cAMP in the transfected cells. HEK-293 cells transfected with pHGC-GPR3, -GPR6, or -GPR12 showed significantly increased levels of cAMP (Figure 2C). Similar results were also obtained with cells transfected with pHGC-GPR3-RHA, -GPR6-RHA, and -GPR12-RHA (data not shown). Notably, GPR6- or GPR12-transfected cells showed higher levels of cAMP than did GPR3-transfected cells. The samples transfected with an empty vector (pHGCX) or another control vector expressing adrenomedullin receptor (ADMR), a GPCR that can couple to \( G_s \) in the presence of its peptide ligand (19), (pHGC-ADMR) showed no cAMP elevation.

We next evaluated the functional activity of these receptors in Neuro2a mouse neuroblastoma cells. Interestingly, the GFP-positive, transfected Neuro2a cells were often transformed into neuron-like cells extending their neurites when transfected with the vectors expressing GPR3, GPR6, and GPR12, but not with the control vectors (data not shown). Similar findings were also obtained with the vectors expressing the RHA-fused receptors, indicating that addition of the RHA tag at the COOH terminus does not affect their functions. Moreover, the RFP-labeled receptor proteins were highly expressed and localized along the cytoplasmic membrane as well as in a perinuclear compartment of the transfected Neuro2a cells (Figure 2D).

**Expression of GPR3, GPR6, and GPR12 in rat primary neurons facilitates neurite outgrowth** - To determine whether expression of GPR3, GPR6, or GPR12 increases intracellular cAMP and promotes neurite extension in primary neurons.
mammalian neurons, we repeated similar experiments using primary cerebellar granule neurons prepared from P7 rat pups using Percoll density gradient centrifugation. More than 90% of the cells were confirmed positive for neuron-specific markers, such as microtubule-associated protein 2 (MAP2) and growth associated protein 43 (GAP43) (data not shown), which was consistent with a previous report (25).

Five × 10⁶ rat cerebellar neurons were electroporated with each expression vector using the Amaxa system, plated onto PDL/laminin-coated plates, and fixed with 4% paraformaldehyde 24 hours after transfection, and neurite lengths of the transfected neurons were evaluated. As shown in Figure 3A, we found the transfection efficiency to be approximately 50% and confirmed that the majority of the GFP-positive cells were MAP2-positive neurons. Neurons transfected with pHGC-GPR3, -GPR6, or -GPR12 demonstrated prominent neurite outgrowth, whereas neurite growth of the neurons transfected with control vectors was very limited (Figure 3B and 3C). There was also a trend showing more enhanced neurite outgrowth with GPR6 and GPR12 than with GPR3.

We then measured cellular cAMP level using an EIA method 24 hours after electroporation. As shown in Figure 3D, cAMP level was elevated 2- to 3-fold when neurons were transfected with pHGC-GPR6 or -GPR12, whereas no significant increase was observed in the pHGC-GPR3-transfected neurons. However, assays using the more sensitive acetylation protocol revealed approximately 40% higher intracellular cAMP levels in the pHGC-GPR3-transfected neurons than in the control pHGCX-transfected neurons. These results indicated that all of these 3 constitutively active GPCRs upregulate intracellular cAMP and enhance neurite outgrowth in rat primary neurons. Among them, GPR12 possesses the most potent neurite outgrowth activity, and cAMP levels correlate with the degrees of neurite extension.

We next evaluated the time-course of neurite outgrowth mediated by GPR12 overexpression using a Zeiss LSM510 confocal microscope. Time-lapse images of pHGCX-transfected (Control) and pHGC-GPR12-transfected (GPCR12) cerebellar granule neurons at 20, 22, 24, 26, and 28 hours after transfection are shown in Figure 4 (Time-lapse movies from 20 to 32 hours after transfection are supplied as supplemental data.). GPR12-expressing neurons showed extensive neurite growth at rates ranging from 20 to 40 µm/hour, whereas control neurons showed limited neurite growth (5 to 15 µm/hour). Notably, cell bodies of the GPCR12-expressing neurons tend to stay at the same locations throughout the period, whereas those of the control neurons occasionally move as the neurites retract. We further compared the GPR12-mediated effects on neurite outgrowth with those mediated by treatment with 1 mM db-cAMP or 200 ng/mL BDNF. Neurons treated with db-cAMP showed the most enhanced growth of their neurites at any given time point, and GPR12-expressing neurons demonstrated the second most potent activity of neurite elongation (Figure 5).

**GPR3 is predominantly expressed in developing rat cerebellar granule neurons, and knocking down GPR3 inhibits neurite growth in P7 neurons** - We next investigated if any of these 3 receptors are expressed in rat cerebellar granule neurons. We thus isolated granule neurons from rat cerebella at 4 different developmental stages (P1, P4, P7, and P14), extracted total RNA, and performed quantitative real-time RT-PCR analysis. GPR3 was abundantly transcribed in granule neurons at all stages, whereas the levels of GPR6 and GPR12 transcription were low throughout these stages (Figure 6A). We then asked if the endogenous GPR3 expression contributes to the intrinsic ability of P7 granule neurons to extend their neurites. GPR3 expression was knocked down by siRNA with the transcriptional level being reduced down to ~ 60% of control (Figure 6B). Neurites of neurons cotransfected with pHGCX and control siRNA showed no growth inhibition, whereas significant inhibition of neurite growth was observed (P < 0.0001) with neurons transfected with GPR3 siRNA (Figure 6C and 6D). These results suggest that GPR3 is necessary for neurite outgrowth, most likely by increasing intracellular cAMP levels in cerebellar granule neurons. The inhibitory phenotype of neurons transfected with GPR3 siRNA was reversed by cotransfection of a vector expressing GPR3 or GPR12 (Figure 6C and 6D), indicating that GPR3 and GPR12 act in
a redundant manner at least in regard to promoting neurite outgrowth in culture.

**Stimulatory G protein Gs and activation of PKA are essential for GPR12-mediated neurite outgrowth** - To investigate signal pathways and molecular targets of GPR12, we first asked whether Gs is involved. For this purpose, we constructed plasmid vectors that express GPR12-RHA together with a dominant negative mutant of Gs (GsDN) or a control Gs subunit (GsC). Additional control vectors that express RHA and either GsDN or GsC were also constructed. Co-expression of GsDN completely inhibited neurite growth mediated by GPR12 (Figure 7A). The intracellular cAMP level of the neurons expressing both GPR12 and GsDN was no higher than that of control (Figure 7B). Taken together, these results indicated that the GPR12-mediated effects, intracellular cAMP increase and neurite elongation, are Gs dependent in rat neurons.

We next examined if activation of PKA is involved in the GPR12-mediated neurite outgrowth. Cerebellar granule neurons derived from P7 rat pups were treated with inhibitors against various protein kinases after transfection with pHGC-GPR12. PKA inhibitors (KT5720 or Rp-cAMP) completely abrogated the GPR12-mediated neurite extension, whereas PKG inhibitor (KT5823) or PKC inhibitor (Gö6976) had no effect (Figures 7C). These results indicated that the GPR12 facilitates neurite outgrowth through the cAMP-PKA pathway.

**GPR12-expressing neurons overcome MAG inhibition through PKA-dependent Rho inactivation** - We then asked if postnatal mammalian neurons in the central nervous system could be rescued from myelin-mediated neurite inhibition by overexpressing GPR12. P7 rat cerebellar granule neurons were electroporated with the GPR12-expressing vector or control vectors, plated on PDL/laminin-coated dishes, and treated with either rat MAG-human IgG Fc chimera (MAG-Fc) or control human IgG Fc proteins. Twenty-four hours after transfection, the length of the longest neurite was measured for each transfected neuron. As anticipated, MAG-Fc treatment inhibited neurite extension of control neurons, whereas it showed no inhibitory effects on the GPR12-expressing neurons (Figure 8A). Similar effects were also observed when GPR3 or GPR6 was expressed (data not shown). When GPR12-transfected neurons were treated with MAG-Fc, co-treatment of the neurons with PKA inhibitors (KT5720 or Rp-cAMP) significantly inhibited growth of their neurites, whereas co-treatment with PKG inhibitor (KT5823) had no effect (Figure 8B).

The Rho-family GTPases regulate the actin cytoskeleton. RhoA is involved in regulating axonal growth and growth cone behavior (26-28). Studies using non-neuronal cells have indicated that the cAMP-PKA signaling regulates the activity of RhoA (29,30). PKA-mediated phosphorylation of RhoA decreases the ability of GTP-bound RhoA to interact with Rho kinase, resulting in neurite extension (30). To investigate the molecular mechanisms of GPR12-mediated rescue of cerebellar granule neurons from MAG inhibition, we examined the activity of RhoA using an enzyme-linked immunosorbent assay (ELISA) for GTP-bound RhoA. MAG treatment increased RhoA activity in control neurons (empty vector-transfected), and the activation of RhoA by MAG treatment was less in the GPR12-transfected neurons. Treating the neurons with PKA inhibitors KT5720 or Rp-cAMP diminished the GPR12-mediated inhibitory effects on RhoA activation (Figure 9).

**DISCUSSION**

In neurons, cAMP-PKA signaling plays an important role in diverse functions, including survival (31-33), neuronal differentiation (34), axon guidance (35-37), neurite outgrowth (32,33), and axonal regeneration (4,5,38,39). Cyclic AMP-dependent pathways can be modulated by many signals, including various neurotransmitters, neuromodulators, adhesion molecules, and Ca2+. However, it is not well known how the levels of intracellular cAMP are regulated in developing or injured neurons.

In this study, we demonstrated that neural expression of the constitutively active GPCRs (GPR3, GPR6, and GPR12) regulates intracellular cAMP levels, promotes neurite outgrowth, and blocks myelin inhibition in mammalian neurons. Transient expression of the GPR3, GPR6, or GPR12 in HEK-293 cells...
resulted in a significant increase in intracellular cAMP levels, which is consistent with previous reports (13,14,22,40). When the receptors were expressed in Neuro2a neuroblastoma cells, striking morphological changes, transformation into neuron-like cells and extension of their neurites, were observed. These findings prompted us to investigate whether overexpression of the constitutively active GPCRs in primary neurons results in activation of cAMP signaling, neurite outgrowth, and rescue from myelin inhibition. To this end, we chose to use cerebellar granule neurons from early postnatal rats because of their abundance and well characterized development (41,42). Use of a dominant negative mutant of the G$_s$ α subunit revealed that GPR12 does stimulate cAMP production and neurite outgrowth in a G$_s$-dependent manner. In addition, we confirmed that the GPR12-mediated activities of neurite outgrowth and rescue of MAG inhibition require cAMP-dependent activation of PKA. Quantitative real-time PCR analysis revealed that rat cerebellar granule neurons express GPR3 transcripts abundantly throughout all of the developmental stages examined, whereas the transcriptional levels of GPR6 and GPR12 are maintained at very low levels. When endogenous expression of GPR3 was knocked down with siRNA, neurite growth of the transfected P7 granule neurons was significantly inhibited. This finding implicates GPR3’s critical role in maintaining appropriate level of intracellular cAMP in developing cerebellar granule neurons. Furthermore, the intracellular cAMP level regulated by GPR3 is likely to play important roles in the terminal differentiation, migration, neurite projection, and survival of developing cerebellar granule neuron, which needs further investigation using GPR3 knockout mice. Recent studies by Ledent and colleagues implicated that the GPR3 knockout mice show no apparent neurological phenotype (23). More rigorous histological and behavioral assessments may be required to reveal some neurological phenotypes in the knockout mice. Alternatively, the redundant expression of GPR6 and GPR12 in the central nervous system may fully complement the lack of GPR3. If the latter is the case, double or triple knockout mice may need to be generated to assess the neuronal functions of these constitutively active receptors.

Cyclic AMP has been reported as a key regulator that affects axonal growth of developing neurons towards various neurotrophic factors and axon guidance molecules (35-37). The developmental decrease in cAMP levels in mammalian neurons has been proposed to underlie the developmental loss of axonal regeneration in the presence of myelin-associated inhibitors (4). Upregulating cAMP signaling in damaged central nervous system neurons by injection of membrane-permeant cAMP analogues was shown to promote axonal regeneration of damaged dorsal root ganglia neurons (43,44). Our current study thus provides an additional line of evidence supporting the conclusion that cAMP-PKA is a major signaling pathway that facilitates neurite outgrowth and axonal regeneration in mammalian neurons. It is interesting to hypothesize that differential expression of the constitutively active G$_s$-linked receptors is responsible for regulating cAMP levels in developing and/or damaged neurons. If this turns out to be the case, GPR3, GPR6, and GPR12 can be significant molecular targets for treating a variety of neurological disorders, including brain and spinal cord injuries, stroke, and neurodegenerative disorders.

PKA-mediated phosphorylation of synapsins was reported to be involved in the cAMP-dependent neurite outgrowth of *Xenopus* embryonic neurons (45). It would be interesting to investigate, in the future, the significance of synapsin phosphorylation in GPR12-mediated activities in mammalian neurons. In the current study, the activity of the small GTPase RhoA, which regulates focal adhesion and formation of actin stress fibers, was confirmed to be upregulated by MAG in P7 cerebellar granule neurons. Moreover, GPR12-mediated PKA activation was shown to inhibit RhoA activation in the MAG-treated neurons, suggesting that upregulated expression of GPR12, and probably also that of GPR3 and GPR6, could facilitate axonal regeneration in the adult central nervous system, at least in part by inhibiting Rho activation.

In addition to coupling with G$_s$, GPR3, GPR6, and GPR12 may also interact with G$_i$ (13,15,46). Uhlenbrook and colleagues reported that sphingosine 1-phosphate (S1P) is a potential...
ligand for all of these 3 receptors (13). Ignatov and colleagues, on the other hand, reported that GPR6 is a high-affinity receptor for S1P (15) and GPR12 is a high-affinity receptor for sphingosylphosphorylcholine (SPC) (46). Further investigation is required to clarify how these potential ligands modulate the activity of the constitutively active GPCRs in different types of neurons.

Although the detailed molecular mechanisms for the cAMP-dependent axonal growth are still elusive, potential regulations of cAMP levels in mammalian neurons by cell type- and developmental stage-specific expression of the constitutively active GPCRs may open up a new research area in the fields of developmental neurobiology as well as translational research for neurorregeneration. Moreover, based on the present findings, upregulating expression of the constitutively active GPCRs in damaged neurons may hold potential as a therapeutic strategy to treat various neurological disorders, including spinal cord injuries and stroke. More investigation is needed to elucidate expression of these 3 receptors during normal development and in various disease conditions.

REFERENCES


FOOTNOTES

*This work was supported by NIH grant R21 NS44514 to YS and by the Dardinger Center Fund for Neuro-oncology Research at the Arthur G. James Cancer Hospital, The Ohio State University Medical Center. The authors thank Drs. Roger Y. Tsien and Catherine H. Berlot for plasmids. The authors also acknowledge Drs. Laurinda A. Jaffe and E. Antonio Chiocca for their critical advice and Ms. Rosalyn Uhrig for her technical editing of the manuscript.

The abbreviations used are: GPCR, G protein-coupled receptor; MAG, myelin-associated glycoprotein; PKA, cyclic AMP-dependent protein kinase; db-cAMP, dibutyryl-cyclic AMP; BDNF, brain-derived neurotrophic factor; NT-3, neurotrophin-3; ERK, extracellular signal-regulated kinase; PDE, phosphodiesterase; ADMR, adrenomedullin receptor; EGFP, enhanced green fluorescent protein; CMV, cytomegalovirus; IE, immediate-early; HA, hemagglutinin; HEK-293, human embryonic kidney-293; DMEM, Dulbecco’s modified Eagle’s medium; FBS, fetal bovine serum; PBS, phosphate-buffered saline; LPA, lysophosphatidic acid; PDL, poly-D-lysine; PCR, polymerase chain reaction; Ct, cycle threshold; EIA, enzymeimmunoassay; MAP2, microtubule-associated protein 2; GAP43, growth...
associated protein 43; ELISA, enzyme-linked immunosorbent assay; S1P, sphingosine 1-phosphate; SPC, sphingosylphosphorylcholine; MCS, multiple cloning sites; and SEM, standard error of measurement.

**FIGURE LEGENDS**

**Figure 1.** Expression profiles of mRNA for GPR3, GPR6, and GPR12 in different mouse tissues. Mouse Multiple Tissue cDNA Panel (BD Biosciences Clontech) was used as a template for quantitative PCR analyses. All data were normalized using the levels of mouse β-actin mRNA as an internal control.

**Figure 2.** Construction and characterization of plasmid vectors that express GPR3, GPR6, GPR12, or their RFP-HA (RHA) fusion proteins. (A) Schematic maps of the vector constructs used in this study. All vectors are derived from the pHGCX plasmid expressing GFP under the control of the HSV IE4/5 promoter, allowing easy visualization of transfected cells. The cDNA sequences for murine adrenomedullin receptor (ADMR), GPR3, GPR6, GPR12 as well as the RHA-tagged GPR3, GPR6, and GPR12 were inserted into the multiple cloning sites (MCS) downstream of the CMV promoter. (B) Immunoblotting of RHA-tagged GPR3, GPR6, and GPR12 expressed in HEK-293 cells. Anti-HA rat monoclonal antibody was used for their detection. Cells transfected with pHGCX were used as a negative control, whereas those transfected with pCHA-Luc, which expresses an HA-tagged firefly luciferase (HA-Luc), were used as a positive control. β-actin was used as a loading control. (C) Elevation of the intracellular concentration of cAMP in HEK-293 by overexpressing GPR3, GPR6, and GPR12. Cells were transfected with one of the expression vectors using LipofectAMINE™. Untransfected, pHGCX- or pHGC-ADMR-transfected cells were used as negative controls, whereas 50 µM forskolin-treated cells (incubated for 15 min prior to assay) were used as positive controls. Twenty-four hours after transfection, cells were harvested for cAMP EIA. The results were presented as mean + standard error of measurement (SEM) (n = 3). * represents P < 0.0001 compared to the pHGCX-transfected samples. (D) Intracellular localization of RHA-tagged GPR3, GPR6, and GPR12 proteins expressed in Neuro2a cells. Cells were transfected using LipofectAMINE™ with one of the vectors expressing RHA-tagged GPR3, GPR6, or GPR12. Twenty-four hours later, the transfected cells were analyzed and imaged using a Zeiss LSM510 confocal microscope. RFP-labeled receptor proteins were detected along the cytoplasmic membrane as well as in the perinuclear region. The scale bar represents 20 µm.

**Figure 3.** Evaluation of neurite extension and intracellular cAMP elevation in primary neurons transfected with GPR3-, GPR6-, and GPR12-expressing vectors. pHGCX and pHGC-ADMR were used as control vectors. Electroporated P7 rat cerebellar granule neurons were plated onto PDL/laminin-coated chamber slides. Twenty-four hours after transfection, neurons were imaged and analyzed using a Zeiss LSM510 META confocal microscope. (A) Cells transfected with pHGC-GPR12 were subjected to immunofluorescence staining using an antibody against neuron-specific MAP2 (red; left panel). The transfected cells were visualized by their GFP expression (green; middle panel). The merged image (right panel) confirmed that the transfected cells were all neurons. (B) Cerebellar granule neurons transfected with pHGC-GPR3, -GPR6, and -GPR12 showed enhanced neurite outgrowth compared to neurons transfected with the control vectors. The scale bar represents 50 µm. (C) P7 cerebellar granule neurons transfected with pHGC-GPR3, -GPR6, and -GPR12 demonstrated a 1.5- to 3-fold increase in their neurite lengths compared to those transfected with control pHGCX or pHGC-ADMR. The length of the longest neurite for each GFP-positive transfected neuron was measured (> 150 neurons per each condition). The mean and SEM were calculated for each condition and presented as percentage of length relative to pHGCX-transfected neurons. * and ** represent P < 0.05 and P < 0.0001 compared to pHGCX, respectively. (D) Intracellular cAMP levels of transfected P7 cerebellar granule neurons were measured by EIA 24 hours after transfection. The results are presented as mean + SEM (n = 3). * represents P < 0.0001.
compared to pHGCX.

**Figure 4.** Time-lapse images of pHGCX-transfected (Control) and pHGC-GPR12-transfected (GPR12) cerebellar granule neurons in culture. The images were taken using a Zeiss LSM 510 Meta confocal microscope at 20, 22, 24, 26, and 28 hours after transfection. The scale bar represents 50 µm.

**Figure 5.** Time course of neurite outgrowth of P7 rat cerebellar granule neurons transfected with pHGC-GPR12. Neurons were electroporated with either pHGC-GPR12 or control pHGCX. Subsets of pHGCX-transfected neurons were treated with either 1mM db-cAMP or 200 ng/mL BDNF 4 hours after electroporation. (Upper panel) Representative images of cerebellar granule neurons at 24 and 48 hours after electroporation. (Lower panel) At 24, 48, and 96 hours after electroporation, the length of the longest neurite for each GFP-positive transfected neuron was measured; the results are presented as mean + SEM; n > 150).

**Figure 6.** Expression profiles of GPR3, GPR6, and GPR12 in developing rat cerebellar granule neurons (A) and RNA interference experiments using P7 rat cerebellar granule neurons (B-D). (A) Total RNA was extracted from cerebellar granule neurons prepared from rats at 4 different developmental stages (P1, P4, P7, and P14). The RNA samples were subjected to quantitative RT-PCR analysis using probes and primers specific to rat GPR3, GPR6, and GPR12. All quantitative data were adjusted using the levels of β-actin mRNA as internal controls. (B-D) P7 rat neurons were electroporated with a combination of siRNA and a GFP-expressing vector: (a) control siRNA and pHGCX; (b) GPR3 siRNA and pHGCX; (c) GPR3 siRNA and pHGC-GPR3; and (d) GPR3 siRNA and pHGC-GPR12. The transfected neurons were plated on PDL/laminin-coated plates and cultured for 24 hours. (B) At 24 hours post transfection, total RNA was prepared from the neurons and subjected to quantitative RT-PCR analysis using primers and probe specific to rat GPR3. (D) After fixation, the length of the longest neurite for each GFP-positive neuron was measured, and the results were presented as mean + SEM; n > 150) relative to the mean neurite length of neurons transfected with control siRNA and pHGCX. Neurite extension was significantly (P < 0.0001) inhibited when siRNA specific to rat GPR3 was electroporated, and this was reversed by expression of mouse GPR3 or GPR12.

**Figure 7.** The stimulatory G protein α subunit Gs-α and PKA activation are essential for GPR12-mediated neurite elongation in neurons. (A, B) Rat cerebellar neurons were electroporated with 4 different vectors expressing dominant negative Gs (GsDN) or control Gs (GsC) with or without GPR12. The transfected neurons were plated on PDL/laminin-coated plates and cultured for 24 hours. (A) After fixation, the length of the longest neurite for each GFP-positive neuron was measured; the results are presented as mean + SEM; n > 150) relative to the mean neurite length of neurons transfected with pHGC-RHA-GsC. (B) Levels of intracellular cAMP were also measured 24 hours after transfection. The results are presented as mean + SEM (n = 3) relative to the mean concentration of cAMP in pHGC-RHA-GsC transfected neurons. (C) Rat cerebellar neurons were electroporated with either pHGCX or pHGC-GPR12 vectors. The transfected neurons were plated on PDL/laminin-coated plates and cultured with or without various protein kinase inhibitors. Twenty-four hours after transfection, the length of the longest neurite for each GFP-positive neuron was measured; the results are presented as mean + SEM (n > 150) relative to the mean neurite length of neurons transfected with pHGC-GPR12. PKA inhibitors KT5823 and Rp-cAMP inhibited neurite elongation mediated by GPR12 overexpression.

**Figure 8.** Overexpression of GPR12 causes P7 rat cerebellar neurons to overcome myelin-associated glycoprotein (MAG)-mediated neurite retraction. (A) Neurons were electroporated with either pHGCX, pHGC-ADMR, or pHGC-GPR12 and plated on PDL/laminin-coated plates. Four hours later, the transfected neurons were treated with or without 25 μg/mL MAG-human IgG Fc chimera (MAG-Fc). Twenty-four hours after transfection, the neurons were fixed, and the length of the longest neurite for
each GFP-positive neuron was measured. The results are presented as mean ± SEM; n > 150) relative to the mean neurite length of neurons transfected with pHGCX (*P < 0.05). (B) P7 rat cerebellar neurons were electroporated with pHGCX or pHGC-GPR12. Four hours later, the transfected neurons were treated with MAG-Fc and/or protein kinase inhibitors. Twenty-four hours after transfection, the length of the longest neurite for each GFP-positive neuron was measured. The results are presented as mean ± SEM (n > 150) relative to the mean neurite length of neurons transfected with pHGC-GPR12 in the absence of MAG-Fc or inhibitors. * represents P < 0.0001 compared to pHGC-GPR12 plus MAG-Fc.

Figure 9. RhoA activity assay of transfected cerebellar neurons. P7 rat cerebellar neurons were electroporated with either pHGCX or pHGC-GPR12 and plated on PDL/laminin-coated plates. Twenty-four hours after transfection, the neurons were incubated for 15 min with MAG-Fc and/or protein kinase inhibitors. Three hundred µg/mL lysocephatidic acid (LPA) was also used as a positive control. The GTP-bound active form of RhoA was detected and quantitated using the G-LISA™ RhoA activation assay kit (Cytoskeleton). The results are presented as mean relative light units (RLU) ± SEM; n = 4). * and ** represent P < 0.001 and P < 0.05, respectively.

Supplemental data. Time-lapse movies of pHGCX-transfected (Control) and pHGC-GPR12-transfected (GPR12) cerebellar granule neurons in culture taken using a Zeiss LSM 510 Meta confocal microscope 20 through 32 hours after transfection. The scale bar represents 50 µm. The time counter in the upper left corner represents minutes after initiation of recording.
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J. Biol. Chem. published online February 6, 2007

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