GDP-bound Goi2 regulates spinal motor neuron differentiation through interaction with GDE2

Goran Periz, Ye Yan, Zachary T. Bitzer, Shanthini Sockanathan *

The Solomon Snyder Department of Neuroscience, Johns Hopkins University School of Medicine, 725 North Wolfe Street, Baltimore, MD 21205, USA

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Goi proteins play major roles in the developing and mature nervous system, ranging from the control of cellular proliferation to modulating synaptic plasticity. Although best known for transducing signals from activated seven transmembrane G-protein coupled receptors (GPCRs) when bound to GTP, key cellular functions for Goi-GDP are beginning to emerge. Here, we show that Goi2 is expressed in motor neuron progenitors that are differentiating to form postmitotic motor neurons in the developing spinal cord. Ablation of Goi2 causes deficits in motor neuron generation but no changes in motor neuron progenitor patterning or specification, consistent with a function for Goi2 in regulating motor neuron differentiation. We show that Goi2 function is mediated in part by its interaction with GDE2, a known regulator of motor neuron differentiation, and that disruption of the GDE2/Goi2 complex in vivo causes motor neuron deficits analogous to Goi2 ablation. Goi2 preferentially associates with GDE2 when bound to GDP, invoking GPCR-independent functions for Goi2 in the control of spinal motor neuron differentiation.

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Introduction

The generation of neuronal diversity in the central nervous system is critical for the formation of functional neural circuits. This complex process involves the integration of signals that trigger neuronal differentiation with those specifying neuronal fates to regulate the timely differentiation of distinct neuronal subtypes (Jessell, 2000; Kintner, 2002). Perturbation of the regulatory networks that control the transition from cell proliferation to differentiation can have serious consequences such as the depletion of progenitor pools, imbalances in neuronal components, tumor formation and ultimately, the disruption of neural networks (Bertram, 2000; Kintner, 2002). However, the molecular mechanisms that regulate the process of neuronal differentiation are still not well understood.

The G-protein subunit, Goi plays diverse roles in the central and peripheral nervous system that include controlling key cognitive and sensory processes such as synaptic plasticity, pain, taste, and olfaction (Wetscherek et al., 2004; Malbon, 2005). These functions primarily involve their ability to mediate signals from a varied and large number of seven transmembrane G-protein coupled receptors (GPCRs) (Neer, 1995; Neves et al., 2002). Goi proteins bind quiescent GPCRs in an inactive GDP-bound form that associates in a complex with Qi and GY subunits. However, upon activation of GPCRs through chemical or physical stimulation, the Goi subunit undergoes a GDP to GTP exchange that causes the Goi/β/γ heterotrimer to release from the GPCR, and dissociate into an active Goi-GTP monomer and a Qi/β/γ dimer. The Goi-GTP subunit subsequently binds to downstream targets or effector proteins, thereby functioning as a central component in GPCR signaling pathways (Neer, 1995; Neves et al., 2002). The Goi subunit also controls the duration of the GPCR-mediated signal by its intrinsic GTPase activity. Cleavage of GTP to the GDP form inactivates Goi, and results in the disassociation of the α/β/γ heterotrimer with the GPCR. Although initially thought to facilitate Goi binding to GPCRs, Qi/β/γ heterodimers also mediate GPCR signaling, albeit to a lesser extent than Goi (Neer, 1995; Neves et al., 2002).

Interestingly, emerging studies provide evidence that Goi proteins have additional functions that are distinct from their roles in transducing GPCR signals. In C. elegans, Drosophila and vertebrates, Goi proteins function as important regulators of asymmetric cell division through their ability to orientate and position the mitotic spindle (Gotta and Ahringer 2001; Schaefer et al., 2001; Hampoolz and Knoblich, 2004; Afshar et al., 2004; Du and Macara, 2004). They mediate this function by associating with Pins (LGN in vertebrates) through the Pins GoLoco domain. In contrast to its function in GPCR signaling, the active form of Goi in spindle positioning is Goi-GDP, which binds to Pins/LGN instead of Goi-GTP (Schaefer et al., 2001; Hampoolz and Knoblich, 2004; Du and Macara, 2004). Pins/Goi complexes are also implicated in vesicle trafficking and localizing NMDA receptors to the cell membrane, a process central to synaptic plasticity (Sans et al., 2005; Knoblich, 2005). In this paradigm, Goi-GDP elevates the levels of the NMDA receptor subunit NR2B on the membrane in the presence of Pins, thereby increasing synaptic plasticity.
transmission. These two examples thus indicate that GDP-bound forms of Goα have important regulatory functions in the nervous system that are distinct from their direct roles in GPCR-mediated signaling.

Given the importance of Goα proteins in nervous system function, we considered the possibility that Goα proteins may be required for regulating neuronal diversity. One model system where the molecular pathways that regulate neuronal differentiation and subtype specification are relatively well characterized is in developing spinal motor neurons (Jessell, 2000; Price and Briscoe, 2004). Spinal progenitors located in the ventricular zone (VZ) of the spinal cord are patterned into discrete dorsal–ventral domains through integrating sonic hedgehog (shh), fibroblast growth factor (FGF), and retinoic acid signals (RA) (Diez del Corral et al., 2003; Novitch et al., 2003). Each progenitor domain expresses a unique profile of transcription factors that ultimately regulates their capacity to generate a particular neuronal subtype (Jessell, 2000; Price and Briscoe, 2004). In the case of motor neurons, RA signals induce the bHLH protein Olig2 in ventral progenitors, which acts as a key determinant of motor neuron identity by priming cells to implement motor neuron fate specification programs (Mizuguchi et al., 2001; Novitch et al., 2001; Novitch et al., 2003; Lee et al., 2005). RA subsequently initiates the differentiation of Olig2+ progenitors into postmitotic motor neurons through upregulating the expression of GDE2, a six transmembrane protein containing an extracellular glycerophosphodiester phosphodiesterase (GDPD) domain (Nogusa et al., 2004). Xiao and Sockanathan, 2004; Yanaka, 2007; Yan et al., 2009). GDE2 GDPD activity triggers Olig2 downregulation, and synchronizes Ngn2-dependent neurogenic pathways and motor neuron fate determination programs (Mizuguchi et al., 2004; Rao and Sockanathan, 2005; Yanaka, 2007; Yan et al., 2009). GDE2 GDPD activity triggers Olig2 downregulation, and synchronizes Ngn2-dependent neurogenic pathways and motor neuron fate specification networks to drive the differentiation of postmitotic motor neurons (Rao and Sockanathan, 2005; Yan et al., 2009).

Here, we investigate the expression of Goα proteins in spinal motor neurons and the function of Goα2 in motor neuron differentiation with respect to known regulators of motor neuron differentiation. We find that different members of the Goα family are expressed in differentiating and postmitotic motor neurons, suggesting sequential roles for Goα proteins in motor neuron development. Using loss of function and overexpression assays in the chick spinal cord, we show that Goα2 plays roles in regulating motor neuron differentiation, and that its function is mediated in part through its interaction with GDE2. Strikingly, Goα2 preferentially interacts with GDE2 when bound to GDP. These findings identify a role for Goα2 in spinal motor neuron development, and invoke GPCR-independent functions for Goα2 in regulating motor neuron differentiation.

Materials and methods

In situ hybridization and immunohistochemistry

Embryos were prepared for immunohistochemistry and in situ hybridization as described (Sockanathan and Jessell, 1998). Tissues were embedded in Tissue-Tek O.C.T. (Sakura Finetek) and 12-µm serial sections were obtained. Primary antibodies used are as follows: K5 (rabbit-anti-IsL1/2), 1:2500; guinea pig anti-IsL1/2, 1:10,000 (provided by T.M. Jessell); 49H anti-IsL2, 1:100; 81.5C10 anti-(His8/MNR2), 1:100 (Developmental Studies Hybridoma Bank, [DSHB]); rabbit anti-MNR2, 1:8000 (provided by B. Novitch); goat anti-βGal, 1:3000 (Arnel); rabbit anti-GPP, 1:2000 (Molecular Probes); mouse anti-PCNA, 1:2000 (SIGMA); rat anti-BrdU 1:100 (Abcam); rabbit anti-phospho-Histone H3 (Ser10), 1:200 (Millipore); mouse anti-Nkx6.1 F55A10, 1:50 (DSHB); mouse anti-Pax6 1:250 (DSHB); rabbit anti-Nkx2.2, 1:4000 (provided by T.M. Jessell); rabbit anti-Irx3, 1:8000 (provided by T.M. Jessell); mouse anti-Lim1/2 (1:1; DHSB). Images were captured using a Zeiss LSM 5 Pascal confocal microscope. In situ hybridization was performed as described (Shaeren-Wiemers and Gerfin-Moser, 1993). Quantitation of neuronal numbers was carried out using 5–10 sections/embryo from 5 embryos.

In ovo electroporation and siRNAs

All cdNAS were derived from the chick and subcloned into pCAGGS or a 250 bp fragment from the mouse H89 promoter-based vector for in ovo electroporation (MNz; Lee et al., 2004). For siRNA experiments, Goα2 siRNA duplexes were electroporated as previously described (Rao et al., 2004). Goα2 siRNA sequences (Dharmacon) are as follows: 5′- ACAUCCGACGAAGUUGAUU 3′; 3′- UCAAAUCCUCUGGAGUU-5′ Control DsRed siRNA sequences are as published in Rao et al. (2004).

Co-immunoprecipitation assays

Flag or 6xHis epitope tags were fused to the N-terminus of GDE2 or C-terminus of Goα2 and subcloned into pCAGGS or pcS2 vectors. Transiently transfected HEK293T cells were harvested and homogenized in lysis buffer using standard procedures (Yan et al., 2009). Lysates were incubated with anti-Flag M2 (Sigma) antibody and GammaBind G Sepharose beads (GE Healthcare) or anti-Flag M2 (Sigma)-bound agarose beads overnight at 4 °C under constant rotation. After centrifugation and extensive washing, the precipitated proteins were analyzed by SDS-PAGE and western blot using rabbit anti-His (Santa Cruz), anti-FLAG M2 or rabbit anti-GDE2 antibodies (Yan et al., 2009). For the GDP/AlF4− experiments, HEK293T cells were separately transfected with either Flag-GDE2 or Goα2-6xHis plasmids. Cells were lysed, using 0.1% TritonX lysis buffer (0.1% Triton-X100; 25 mM Tris–Cl, pH 7.5; 150 mM NaCl; 10 mM MgCl2; 1/200 Sigma Protease Inhibitors), spun to remove debris, and supernatants were transferred to fresh tubes. Goα2 lysates were split into aliquots, and water, GDP (10 µM final), or NaF (10 mM final) + AlCl3 (30 µM final) were added. Goα2 lysates were incubated for 30 min at RT, prior to mixing with fresh tubes containing gamma-bind slurry (pretreated with 2% BSA and washed in lysis buffer). Beads and lysates were rotated 1 h at 4 °C, and washed 4 times with lysis buffer supplemented with appropriate concentrations of GDP or NaF/AlCl3. Samples were eluted in sample buffer and run on 10% SDS/polyacrylamide gels, transferred to PVDF membranes, and bands visualized with ECL+ chemiluminescence autoradiography (Amersham).

Large scale co-immunoprecipitation experiments and LC–MS/MS analysis were performed as described in Yan et al. (2009). Rabbit anti-mGDE2 CT antibodies were used at dilutions 1:1000.

BrdU labeling

To calculate the proliferative index, electroporated embryos were exposed to BrdU for 30 min before dissection and processing as described above (Yan et al., 2009).

Results

Goαs are expressed in developing spinal motor neurons

As a first step to define the function of Goα proteins in neuronal development, we examined the expression of three members of the Goα family in the ventral spinal cord of Hamburger Hamilton stage (St) 20 chick embryos: Goα2, Goα3, and Goα1. At this stage of development, cells at different stages of differentiation are present; progenitor cells are located medially in the VZ, newly differentiating cells are present in the intermediate zone (IZ), and fully differentiated motor neurons are beginning to settle in the lateral marginal zone (MZ) of the ventral horn (Hollyday, 2001). In situ hybridization analysis shows that Goα2 is expressed medially in ventral progenitors, newly differentiating and newly born motor neurons (Fig. 1B). In contrast, Goα3 is expressed in newly differentiating and postmitotic motor neurons, while Goα1 is...
detected in postmitotic motor neuron populations (Figs. 1D, E). The medial to lateral distribution of these Gai family members in developing motor neurons suggests that these proteins may have sequential roles in the progression of motor neuron development, with Gai2 regulating early steps in motor neuron differentiation. Consistent with Gai2 function in early stages of motor neuron differentiation, Gai2 expression initiates in laterally located cells in the ventral spinal cord just prior to postmitotic motor neuron generation (arrow, Fig. 1A). Interestingly, Gai2 expression is maintained in the ventral spinal cord in medial cells and in postmitotic motor neurons after neurogenesis is complete, suggesting that Gai2 is required for later aspects of motor neuron development or function (Fig. 1C).

Gai2 ablation causes a reduction in motor neuron numbers

We focused our analysis on Gai2 as its high expression in IZ cells suggests a potential role in controlling spinal motor neuron differentiation. In order to determine the function of Gai2 in developing motor neurons, we designed 21 bp double-stranded RNA oligonucleotides designed against the Gai2 coding sequence (Gai2 siRNA) and electroporated them into embryonic chick spinal cords at St 11–13, prior to the onset of motor neuron differentiation (Rao et al., 2004). The efficacy of the Gai2 siRNAs to ablate Gai2 expression was tested in vitro and in electroporated chick spinal cords and shown to effectively ablate Gai2 protein and mRNA levels when compared with control siRNA oligonucleotides (Figs. 2A, F, and S1). Furthermore, Gai2 siRNAs were specific to Gai2, as they did not reduce Gai1 and Gai3 protein expression (Fig. S1). In our hands, the siRNAs did not cause stable knockdown of Gai2 expression; we thus focused our analyses at St 20, when motor neuron differentiation in the spinal cord is at its peak.

To investigate the consequences of Gai2 loss on motor neuron development, we first stained sections of chick spinal cords electroporated with either Gai2 or control siRNAs with antibodies against markers specific for ventral progenitors required for dorsal-ventral patterning, and for the motor neuron progenitor determinant, Olig2 (Jessell, 2000; Price and Briscoe, 2004). Patternning of ventral progenitors and the number of Olig2+ motor neuron progenitors were equivalent in control embryos and embryos silenced for Gai2 expression, suggesting that Gai2 is not required for the specification of motor neuron progenitors (Figs. 2B, C, G, H, M, N). However, embryos electroporated with Gai2 siRNAs showed an approximately 25–30% reduction of postmitotic motor neurons marked by Islet1, Islet2 and HB9 expression (Figs. 2D, E, I, J, M, N). Interestingly, a number of electroporated embryos also showed a broadening of the zone of cells expressing NeuroM, a marker for newly differentiating neurons, suggesting that loss of Gai2 may lead to a lengthening of the transition from dividing progenitors to differentiated postmitotic neurons (Fig. 2K; Roztocil et al., 1997). In support of this, all of the NeuroM+ cells in the ventral domain of the spinal cord in many cases continue to express Olig2 when Gai2 is ablated, in contrast to the contralateral non-electroporated side where laterally located NeuroM expressing cells downregulate Olig2 (arrows, Fig. 2L). Gai2 is expressed in the dorsal spinal cord, leading us to ask if Gai2 is required for dorsal spinal interneuron differentiation. Embryos electroporated with Gai2 siRNAs showed a reduction in Lim1/2 expression, suggesting a requirement for Gai2 in the differentiation of d12, d14, d16 and V1 spinal interneuron classes (Fig. S1; Helms and Johnson, 2003). Taken together, these observations are consistent with the model that Gai2 can control neuronal differentiation in the spinal cord in multiple cellular contexts, but that it is not required for the specification of progenitor identities or for regulating progenitor number.

Gai2 loss does not affect progenitor proliferation

One alternative explanation for the loss of motor neurons when levels of Gai2 are reduced is that the rate of progenitor proliferation is decreased. To test this possibility, we analyzed embryos electroporated with control and Gai2 siRNAs for the ability of cells to progress through S-phase and M-phase of the mitotic cell-cycle. The frequency of cells to exit the cell-cycle could not be tested as nucleotide analogues have extremely long half lives in the egg due to the closed environment and the lack of clearing by maternal metabolic pathways (Bannigan, 1981). To analyze the number of cells in S-phase, the nucleotide analogue bromodeoxyuridine (BrdU) was added to electroporated chick embryos in ovo for 30 min prior to dissection in order to label S-phase cells. Sectioned embryos were then stained for PCNA, which marks all cycling cells, and the labeling index, which equals the proportion of all PCNA+ cells that had incorporated BrdU, was calculated (Chenn and Walsh, 2002). The
mitotic index was computed by staining adjacent sections with the mitotic marker PH3, and obtaining the ratio of PH3+ cells to the total number of proliferating cells (Chenn and Walsh, 2002). Using these measurements of cell-cycle progression, we found that embryos electroporated with $G_\alpha_{i2}$ siRNAs showed similar S-phase and M-phase profiles as control embryos (Figs. 3A–D). These observations suggest that $G_\alpha_{i2}$ does not regulate progenitor cell proliferation, supporting our model that $G_\alpha_{i2}$ regulates the differentiation of spinal motor neurons.

$\alpha_{i2}$ binds GDE2, a regulator of motor neuron differentiation

In order to investigate the mechanism by which $G_\alpha_{i2}$ might regulate motor neuron differentiation, we took a candidate approach to identify proteins known to regulate motor neuron differentiation that may interact with $G_\alpha_{i2}$. While GPCRs are likely candidates for this purpose, no GPCRs have been implicated in the regulation of motor neuron differentiation to date. Moreover, the large number and diversity of GPCRs decreases the feasibility of screens using the candidate approach. Known mediators of motor neuron differentiation include transcription factors such as Neurogenin 2; but, these factors are unlikely to be relevant contenders, as $G_\alpha_{i2}$ localizes to the cell membrane and its presence in the nucleus has not been reported (Scardigli et al., 2001; Lee and Pfaff, 2003; Lee et al., 2005). However, one plausible candidate is the six transmembrane protein GDE2, which controls spinal neuronal differentiation through extracellular GDPD activity (Nogusa et al., 2004; Rao and Sockanathan, 2005; Yanaka, 2007; Yan et al., 2009). In addition to being membrane bound, loss of GDE2 results in a similar phenotype to $G_\alpha_{i2}$ ablation in that

![Fig. 2. $G_\alpha_{i2}$ ablation results in reduced numbers of motor neurons.](image)

(A–L) Transverse sections of St 20 embryonic chick spinal cords electroporated on the left side. (A, F) In situ hybridizations of $G_\alpha_{i2}$ mRNA expression. (B–E, G–L) Immunohistochemical analysis of markers of progenitors, newly differentiating and postmitotic motor neurons. Bars in K denote extent of zone of cells expressing NeuroM. Arrows in L show downregulation of Olig2 expression in control NeuroM+ cells; however, Olig2 expression is maintained in NeuroM+ cells when $G_\alpha_{i2}$ is ablated. (M, N) Graphs quantifying Olig2+ progenitors and postmitotic Isl1/2+ motor neurons. EP/NEP = ratio of neurons of electroporated versus non-electroporated sides of the spinal cord. mean ± s.e.m.; Student's t-test, *p = 0.003, n = 5.

![Fig. 3. $G_\alpha_{i2}$ ablation does not alter progenitor proliferation.](image)

(A, C) Representative images of transverse sections of St 20 chick spinal cords electroporated on the left. Dashed line marks the dorsal margin of the ventral spinal cord (V); V regions were counted to compute the indices shown in (B, D). Proliferation index = PCNA+Brdu+/total PCNA+ cells; mitotic index PCNA+PH3+/total PCNA+ cells. Graphs quantifying indices comparing non-electroporated side (Ctrl) with contralateral side electroporated with $G_\alpha_{i2}$ siRNAs ($G_\alpha_{i2}$KD) mean ± s.e.m.; Student's t-test, p > 0.05, n = 5.
progenitor specification is not perturbed, but there is a major loss of motor neurons, and specific classes of ventral and dorsal interneurons (Rao and Sockanathan, 2005; Yan et al., 2009).

To explore if Goxi2 might exert its effects on neuronal differentiation through GDE2, we examined if Goxi2 and GDE2 are able to form a complex using co-immunoprecipitation (IP) assays. Plasmids expressing FLAG-tagged GDE2 (FLAG-GDE2) and His-tagged Goxi2 (chGoxi2-His) were transfected into HEK293T cells, and GDE2 containing complexes were IP-ed using anti-FLAG antibodies. Western blots of the IP-ed complexes using anti-His antibodies detected a band of the appropriate molecular weight for chGoxi2-His, only when GDE2 and Goxi2 were coexpressed, consistent with the ability of GDE2 and Goxi2 to interact (Fig. 4A). In addition, we performed unbiased proteomic screens where we carried out large scale IPs of GDE2 containing complexes in transfected HEK293T cells followed by Liquid Chromatography–Mass Spectrometry/Mass Spectrometry (LC–MS/MS). Analysis of a protein band of approximately 40 kDa that corresponds to the molecular weight of Gα proteins and that was detected in GDE2 containing complexes but not controls, identified three separate tryptic peptides corresponding to endogenous human Goxi2 (Fig. 4B). Taken together, these data are consistent with the model that Goxi2 and GDE2 are capable of interacting to form a complex in vivo.

Goxi2 interacts with GDE2 through its intracellular C-terminal domain

GDE2 contains a 43 amino acid intracellular N-terminal region, six transmembrane domains, an intracellular C-terminal domain of 82 residues, and two 13 amino acid intracellular connecting loops between the transmembrane domains. As a first step to determine which of these regions binds to Goxi2, we generated versions of GDE2 that lack the first 38 amino acids of the N-terminal domain (GDE2ΔN38). IP analysis using extracts from HEK293T cells transfected with chGoxi2-His and FLAG-GDE2ΔN38 show that Goxi2 is capable of interacting with GDE2ΔN38, suggesting that the N-terminal region of GDE2 is not required for Goxi2 binding (Fig. 4A). We next constructed variants of GDE2 that lack the C-terminal intracellular domain (GDE2ΔC); however, deletion of the C-terminal domain rendered GDE2 unstable and prone to degradation (data not shown). To circumvent this problem, we expressed three different forms of the C-terminal (CT) region of GDE2 in HEK293T cells. Western blots of extracts containing the GDE2 CT domain alone (CT-cyt), a myristoylated/palmitoylated form of the GDE2 CT region that targets it to the membrane (CT-lyn) and the CT region attached to the last transmembrane domain of GDE2 (CT-tm6), showed that all three forms of GDE2 were expressed in HEK293T cells. This observation is consistent with the increased stability of the CT domain compared with GDE2ΔC, although CT-cyt was consistently expressed at lower levels compared with CT-lyn and CT-tm6. To determine if the CT of GDE2 was sufficient to interact with Goxi2, we performed IP assays using extracts from HEK293T cells transfected with chGoxi2-His and either CT-cyt, CT-lyn or CT-tm6. All three forms of the GDE2 CT domain were found to complex with Goxi2, suggesting that sequences located within this 82 amino acid domain are sufficient for Goxi2 and GDE2 to interact (Fig. 4C).

To obtain further evidence that the CT domain is the site of GDE2/Goxi2 interaction, we tested the ability of the GDE2 CT domain to interfere with GDE2/Goxi2 complex formation in the context of the full-length GDE2 protein. We repeated the IP assay using extracts from HEK293T cells transfected with FLAG-GDE2, chGoxi2-His, and plasmids expressing HA-tagged CT-cyt. Western blots of the complexes IP-ed with anti-FLAG antibodies showed that GDE2 and Goxi2 could interact in the absence of CT-cyt, but that GDE2/Goxi2 complex formation was abolished in the presence of the GDE2 CT domain (Fig. 4D). Notably, addition of the CT domain did not interfere with interactions between GDE2 and its known activator Prdx1 (Fig. S2). Taken together, these data suggest that Goxi2 interacts with the intracellular C-terminal domain of GDE2 and that the CT region of GDE2 can effectively interfere with GDE2/Goxi2 complex formation.
**GDE2 CT expression phenocopies Goα2 ablation in the spinal cord**

If Goα2 mediates its effects on spinal motor neuron differentiation through its interactions with GDE2, then disruption of endogenous GDE2/Goα2 complexes should result in motor neuron phenotypes that are comparable to when Goα2 is ablated. To test this hypothesis, we wanted to express GDE2 CT-cyt in cells that express GDE2 and Goα2 in the spinal cord, namely, within cells that are located in the IZ that directly abut cycling VZ progenitors (Fig. 1B). We specifically did not want to perturb Goα2 function in VZ cells given the established roles of GPCRs in regulating progenitor proliferation in the spinal cord (Megason and McMahon, 2002). Two fragments derived from the mouse HB9 promoter have been consistently used to express heterologous genes in developing spinal motor neurons; a 250 bp element (MN_e) in combination with a minimal promoter, and a larger 3.0 kb fragment (3.0HB9) (Lee and Pfaff, 2003; Lee et al., 2005; Lee et al., 2004). To compare the onset of gene expression driven by these promoters we electroporated MN_e-GFP and HB9-3.0GFP into chick spinal cords to monitor when GFP expression initiates in relation to VZ progenitors. The lateral margin of the VZ is marked by cells that are in S-phase, while cells in the IZ lie immediately lateral to this boundary (Hollyday, 2001). Examination of electroporated spinal cords pulsed with BrdU showed that GFP expression driven by HB9-3.0 GFP initiates in cells in the final cell-cycle, while the MN_e element drives GFP expression in IZ cells immediately adjacent to the VZ (Figs. 5A, B). Thus, in order to express GDE2 CT-cyt in IZ cells where endogenous GDE2 is normally expressed, we subcloned GDE2 CT-cyt under the control of the MN_e promoter element (MN_e–GDE2 CT).

We electroporated MN_e-GDE2 CT into chick spinal cords at St 11–13 prior to motor neuron differentiation and assayed the effects of GDE2 CT expression on motor neuron development at St 20. Expression of GDE2 CT in IZ cells resulted in the loss of postmitotic motor neurons assessed by the expression of Islet1, Islet2 and HB9, with no changes in dorsal–ventral patterning or the number of Olig2+ progenitors (Figs. 5C, D, F, H). In addition, we observed a similar expansion of cells expressing NeuroM in some cases, consistent with cells exhibiting delays in their transition to a differentiated state (Fig. 5E). This phenotype is remarkably similar to the changes observed when Goα2 expression is reduced using siRNAs (Fig. 2) and suggests that the ability of Goα2 to regulate spinal motor neuron differentiation occurs in part, through its interaction with GDE2.

**GDP-bound forms of Goα2 preferentially interact with GDE2**

A major function of Goα2 is to transduce signals from activated GPCRs to downstream effector molecules. GDP-bound Goα2 interacts with Gβ and Gγ to form an inactive heterotrimer that binds to seven transmembrane GPCRs. Upon stimulation of relevant GPCRs, GDP to GTP exchange occurs on Goα2, and G-protein heterotrimers disassociate to GTP bound Goα2 and Gβ/Gγ dimers, both of which can regulate downstream effector proteins (Neer, 1995; Neves et al., 2002). Our finding that Goα2 is involved in regulating spinal motor neuron differentiation through its interaction with GDE2 raises the possibility that GDE2 is a target of GPCR signaling pathways. If so, then one prediction is that GTP and not GDP-bound forms of Goα2 will preferentially bind GDE2. To investigate the GDP/GTP status of Goα2 when bound to GDE2, we took advantage of two point mutants of Goα2 that are known to alter the equilibrium of GDP/GTP exchange on Goα2. Alteration of the glutamine (Q) residue at amino acid position 205 of the
Gαi2 protein to leucine (L), limits the intrinsic GTPase activity of the protein and results in a constitutively active form of Gαi2 (Lowndes et al., 1991). Thus, Q205L Gαi2 mutants maintain a GTP bound state for significantly longer periods than WT forms of the Gαi2 protein. In contrast, Q205L Gαi2 mutants that contain a second mutation at amino acid position 273, where aspartic acid (D) is changed to asparagine (N), are unable to bind GTP or GDP and essentially exist in an “empty” state that is more analogous to the inactive GDP-bound form of Gαi2 (Goel et al., 2004). We performed IP assays using extracts from HEK293T cells transfected with FLAG-GDE2 and His-tagged WT Gαi2 or Q205L/D273N Gαi2 mutants. Surprisingly, the Q205L Gαi2 mutant showed decreased binding to GDE2 when compared with equivalent amounts of WT Gαi2 or the Q205L/D273N Gαi2 mutant protein (Fig. 6A). This result suggests that GDP-bound forms of Gαi2 are more likely to interact with GDE2 than GTP-Gαi2.

To address this possibility more directly, we assessed the binding of Gαi2 with GDE2 under conditions where we increased the availability of either GDP, or a stable analogue of GTP formed by the reaction of GDP and AlF4− (Chen et al., 1997). Specifically, extracts from HEK293T cells transfected with chGαi2-His and FLAG-GDE2 were preincubated with GDP, or GDP and AlF4− in the binding reaction prior to the addition of anti-FLAG antibodies. Consistent with our previous results, addition of AlF4− resulted in decreased binding of Gαi2 and GDE2, whereas inclusion of GDP alone did not perturb Gαi2/GDE2 interactions (Fig. 6B). Taken together, our results indicate that Gαi2 interacts preferentially with GDE2 when bound to GDP, suggesting that GDE2 is not a downstream effector of Gαi2 mediated GPCR signaling. Instead, our observations raise the possibility that Gαi2/GDE2 interactions may utilize non-canonical functions of Gαi2 in the control of motor neuron differentiation.

To assess the consequences of Gαi2 on motor neuron differentiation, we overexpressed Gαi2 under the MNc promoter (MNc–Gαi2), thus targeting Gαi2 expression to cells in the IZ. Chick embryos were electroporated at St 11–13 prior to motor neuron differentiation and analyzed at St 19/20, when motor neuron differentiation is at its peak. Expression of Gαi2 caused a small decrease in the number of Islet1/2 motor neurons without affecting Olig2+ motor neuron progenitor generation (Figs. 6C, D). One possible explanation for this subtle phenotype is that higher cellular levels of GTP compared with GDP, combined with endogenous opposing GEF and GAP protein activities, result in a modest increase in GTP bound Gαi2, thus decreasing GDE2-dependent motor neuron differentiation (Neer, 1995; Malbon, 2005).

To test this possibility, we electroporated plasmids expressing the Q205L Gαi2 mutant that prolongs the GTP bound state of Gαi2 into chick spinal cords and evaluated the production of postmitotic motor neurons (MNc–Q205L Gαi2). Expression of Q205L Gαi2 consistently caused a marked decrease in Islet1/2+ motor neurons, compared with the contralateral non-electroporated side of the spinal cord (Figs. 6E, F). Taken together, these observations suggest that elevated levels of GTP bound forms of Gαi2 interfere with motor neuron generation, and are thus consistent with the model that GDP-bound Gαi2 regulates neuronal differentiation in the spinal cord.

**Discussion**

Our study describes the expression of Gαi2 in the spinal cord, and identifies a function for Gαi2 in the control of spinal motor neuron differentiation. We show that GDP-bound forms of Gαi2 preferentially interact with GDE2, a known regulator of motor neuron differentiation and that this interaction is necessary for generating the normal complement of spinal motor neurons. Our data suggest that Gαi2 interactions are unlikely to be mediated by upstream GPCR signals and invoke instead, a non-canonical function for Gαi2 in regulating motor neuron differentiation.

The progression of motor neuron differentiation can be monitored by the cell–body position of prospective motor neurons within the spinal cord. Cycling progenitors are located medially within the VZ, while cells in transition to a differentiated state are situated in the IZ and terminally differentiated motor neurons are located laterally in the MZ (Jessell, 2000; Hollyday, 2001; Price and Briscoe, 2004). We show that different members of the Gαi family of proteins are expressed in overlapping patterns along the medial–lateral axis of the spinal cord. This expression pattern suggests that different Gαi proteins might function at different stages of motor neuron development, where medially expressed Gαi proteins such as Gαi2, play roles in motor neuron differentiation while more laterally expressed subunits might function in specifying postmitotic motor neuron fate or in regulating their function. Consistent with this prediction, knockdown of Gαi2 by siRNAs results in a reduction of postmitotic motor neurons, but no changes in the patterning of motor neuron progenitors, the number of Olig2+ progenitors or the rate of...
progenitor proliferation. We find that Gαi3 expression overlaps with Gαi2 in IZ cells of the spinal cord, suggesting potential redundant roles for Gαi3 in regulating motor neuron differentiation. Indeed, reports indicate that there is functional redundancy between Gαi2 and Gαi3, for instance, single Gαi2 and Gαi3 null mutants are viable but Gαi2/Gαi3 double knockouts are lethal (Wettschurek et al., 2004). In support of this possibility, we find that Gαi3 is capable of binding GDE2. Moreover, Gαi3 interacts with GDE2 when bound to GDP rather than GTP, suggestive of shared mechanisms between Gαi2 and Gαi3 in regulating GDE2-dependent motor neuron differentiation (G.P. and S.S., unpublished observations).

Our results suggest that Gαi2 mediates motor neuron differentiation in part through its interaction with GDE2. Interestingly, Gαi2 binds preferentially to GDE2 when bound to GDP rather than GTP. We infer from this observation that GDE2 is not an effector of Gαi2 mediated signaling from upstream GPCRs. If so, how might Gαi2 interactions with GDE2 operate to regulate motor neuron differentiation? One possibility is that GDE2 regulates downstream pathways through mechanisms utilizing Gαi2 in a manner similar to the mode of GPCR function. Some studies have described GDE2 as adopting a seven transmembrane structure, but these conclusions were based on predictions generated from computer based structural algorithms (Zheng et al., 2000; Nogusa et al., 2004; Yanaka, 2007). In contrast to these predictions, epitope tagging experiments indicate that the N and C-termini of GDE2 are intracellular while the GDP domain is extracellular, thus providing strong biochemical evidence that GDE2 is a six transmembrane protein (Rao and Sockanathan, 2005). Since GDE2 does not conform to the typical seven transmembrane GPCR structure, it is highly unlikely that it functions as a classical GPCR. However, it is possible that the GDE family of proteins utilizes similar components of the GPCR signal transduction machinery to perform its functions. In support of this idea, the related two pass transmembrane GDPD protein GDE1 is known to bind RGS16, a protein that regulates G-protein signaling through its GTPase activity (Zheng et al., 2000).

Alternative possibilities for how Gαi2–GDE2 might function in concert with GDE2 to regulate motor neuron differentiation arises from known GPCR-independent roles for Gαi2–GDE2 in asymmetric cell division and in receptor trafficking (Hampopelz and Knoblich, 2004; Sans et al., 2005). While the possibility that Gαi2–GDE2 interactions with GDE2 are involved in asymmetric cell division, which is a known prerequisite for triggering neuronal differentiation, is exciting, this function is unlikely, as GDE2 and Gαi2 expression overlap in non-dividing cells of the ventral spinal cord. In terms of receptor trafficking, it remains possible that Gαi2–GDE2 is required for optimal transport of GDE2 to or from the cell surface. By analogy with the NR2B subunit of the NMDA receptor, Gαi2–GDE2 would increase levels of GDE2 to the membrane, thereby acting as a positive regulator of GDE2 signaling and promoting motor neuron differentiation (Sans et al., 2005). This model is consistent with our results where ablation of Gαi2 causes reductions in postmitotic motor neuron numbers and a broadening of the domain of NeuroM expression in the ventral spinal cord. The sustained expression of NeuroM in the ventral spinal cord therefore suggests the possibility that NeuroM is required for optimal transport of GDE2 to or from the cell surface. By analogy with the NR2B subunit of the NMDA receptor, Gαi2–GDE2 would increase levels of GDE2 to the membrane, thereby acting as a positive regulator of GDE2 signaling and promoting motor neuron differentiation (Sans et al., 2005).

In conclusion, our study identifies a role for GDP-bound forms of Gαi2 in regulating neuronal differentiation that is likely to be distinct from its roles in mediating GPCR signaling. These observations raise the possibility that GDP forms of Gαi proteins may have new cellular functions in regulating multiple aspects of neuronal development and function.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ydbio.2010.02.032.

References


