G Protein-regulated Inducer of Neurite Outgrowth (GRIN) Modulates Sprouty Protein Repression of Mitogen-activated Protein Kinase (MAPK) Activation by Growth Factor Stimulation

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Background: GRIN is an effector of Go/i known to regulate neurite extension.

Results: GRIN modulates MAPK by sequestering Sprouty, an inhibitor of the MAPK pathway, and this is reversed by active Goαi binding.

Conclusion: Activation of Goαi can inhibit the MAPK pathway by releasing Sprouty from GRIN binding.

Significance: This is a novel mechanism of GPCR regulation of the MAPK pathway.

Goαi interacts directly with GRIN (G protein-regulated inducer of neurite outgrowth). Using the yeast two-hybrid system, we identified Sprouty2 as an interacting partner of GRIN. Goαi and Sprouty2 bind to overlapping regions of GRIN, thus competing for GRIN binding. Imaging experiments demonstrated that Goαi expression promoted GRIN translocation to the plasma membrane, whereas Sprouty2 expression failed to do so. Given the role of Sprouty2 in the regulation of growth factor-mediated MAPK activation, we examined the contribution of the GRIN-Sprouty2 interaction to CB1 cannabinoid receptor regulation of FGF receptor signaling. In Neuro-2A cells, a system that expresses all of the components endogenously, modulation of GRIN levels led to regulation of MAPK activation. Overexpression of GRIN potentiated FGF activation of MAPK and decreased tyrosine phosphorylation of Sprouty2. Pretreatment with Goαi-coupled CB1 receptor agonist attenuated subsequent FGF activation of MAPK. Decreased expression of GRIN both diminished FGF activation of MAPK and blocked CB1R attenuation of MAPK activation. These observations indicate that Goαi interacts with GRIN and competes GRIN from bound Sprouty. Free Sprouty then in turn inhibits growth factor signaling. Thus, here we present a novel mechanism of how Goαi-coupled receptors can inhibit growth factor signaling to MAPK.

Goαi, the most abundant G protein in the mammalian brain, belongs to the Goαi family, containing ~80% sequence homology to Goαi (1). Goαi is also expressed in adenohypophysis, heart, kidney, medulla, olfactory epithelium, and chromaffin cells (2). Despite its discovery over two decades ago, the direct signaling partners of Goαi and effectors are not fully characterized. Studies, using yeast two-hybrid screens and cDNA expression cloning, have yielded novel direct effectors of Goαi. They include the GTPase-activating protein for the small G protein Rap (RapGAP), Goαi-c-GAP (also known as RG520), the putative tyrosine phosphatase paladin, the regulator of G protein signaling 17 (RGS-17), the Purkinje cell protein-2 (Pcp2), and the G protein-regulated inducer of neurite outgrowth (GRIN) (3–5). The GRIN protein family, consisting of three gene products, GRIN1–3 (GPRIN1–3), is highly expressed in the brain and binds selectively to activated forms of Goαi, Goαs, and Goζ (5). GRIN isoforms co-localize with Goαi at the growth cone of neuronal cells and promote neurite extension in Neuro-2A cells when co-expressed with the constitutively active mutant Goαi, Q205L (6). Goαs-GRIN1 modulates Cdc42 activation and neuronal morphology (6). We have previously identified GRIN3 (originally named IP6) as a direct interacting protein of Goαi in a yeast two-hybrid study using the constitutively active form Q205L-Goαs as bait (3). This family member shows sequence homology to KIAA0514 (amino acids 205–459), which was later renamed GRIN2 (3, 5).

In the present study, we used the yeast two-hybrid system to identify potential downstream partners of the Goαs-GRIN2 pathway. GRIN2 was used as the bait to screen a human brain cDNA library, and among the candidate proteins hits was Sprouty2, a known regulator of receptor tyrosine kinase signal-
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ING. Sprouty2 has been shown to inhibit both neuronal differentiation and survival through a negative-feedback loop that down-regulates neurotrophic-driven signals (7, 8). Our results show that Sprouty2 and GRIN isoforms interact in exogenously expressed and endogenous systems. We have characterized the interaction and functional consequence of GRIN in communicating G<sub>i</sub> signals to Sprouty2. In a fully endogenous system, activation of G<sub>i1</sub>-coupled receptor can inhibit subsequent stimulation of MAPK1,2 by receptor tyrosine kinase, and thus the GRIN-Sprouty interaction can serve as a temporal bridge to connect heterotrimERIC receptor G protein pathway regulation of receptor tyrosine kinase signaling to MAPK1,2.

EXPERIMENTAL PROCEDURES

Cell Culture and Transfection—HEK-293T cells were maintained in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum and 1% penicillin-streptomycin, and Neuro-2A cells were maintained in minimum essential medium supplemented with 10% fetal bovine serum, 2 mM glutamine, 1 mM sodium pyruvate, and 1% penicillin-streptomycin. Neuro-2A cells were obtained from ATCC (CCl-131). Lipofectamine 2000 (Invitrogen) was used for introduction of exogenous DNA into HEK-293T and Neuro-2A cells according to the manufacturer’s instructions. For HEK-293T cells, 1.8 × 10<sup>6</sup> cells were plated on 60-mm dishes, and 24 h later, the cells were transfected with 5 µg of DNA, or 5.5 × 10<sup>6</sup> cells were plated on 100-mm dishes, and 24 h later, the cells were transfected with 10 µg of DNA. For imaging experiments, Neuro-2A cells (1.2 × 10<sup>6</sup>) were plated on 35-mm MatTek<sup>®</sup> plates and transfected using Lipofectamine 2000 the following day. Neuro-2A cells were serum-starved in serum-free media (0.1% BSA modified Eagle’s medium).

Treatment with Growth Factors or Agonists—Cells were treated with various growth factors or agonists in culture using serum-free media. Basic fibroblast growth factor (bFGF, Invitrogen) was dissolved in PBS to a stock concentration of 100 ng/ml and stored at 20 °C. Glial-derived neurotrophic factor (Promega) was dissolved in PBS at a stock concentration of 50 ng/ml and stored at 20 °C. EGF (Millipore) was dissolved in PBS with 0.1% BSA to a stock concentration of 100 ng/ml and stored at 20 °C. HU-210 (Sigma) was dissolved in DMSO at a stock concentration of 10 mM and stored at 20 °C. Carbachol (Tocris) was dissolved in (Sigma) monoclonal antibody at 4 °C or 4 °C with orthovanadate, 5 µg/ml leupeptin, 1 µg/ml pepstatin, 1 mM sodium orthovanadate, 5 µg/ml benzamidine, 5 mM sodium fluoride, 1 mM β-glycerophosphate, and 1 mM phenylmethylsulfonyl fluoride. One mg of whole protein was precleared with protein G-agarose (Roche Applied Science) at 4 °C for 3 h. Immunoprecipitations were performed overnight with 4 µg of M2-FLAG (Sigma) monoclonal antibody at 4 °C or 4 µg of anti-HA monoclonal antibody followed by a 4-h incubation with 30 µl of protein-G agarose. Samples were washed three times with lysis buffer containing protease inhibitors and then resolved with SDS-PAGE. After transfer to nitrocellulose, the membranes were blocked with 5% nonfat dry milk and immunoblotted with M2-FLAG, anti-G<sub>α</sub>o, or anti-HA antibodies followed by either rabbit or mouse TrueBlot (eBioscience) or routine rabbit or mouse (Pierce) HRP-conjugated secondary antibodies. Whole cell lysates were resolved on SDS-PAGE, transferred, blocked, and immunoblotted with anti-M2-FLAG, anti-G<sub>α</sub>o, or anti-HA antibodies followed by either rabbit or mouse HRP-conjugated secondary antibodies (Pierce). Bands were visualized using the ECL detection method.

RT-PCR—Neuro-2A cells were grown on 100-mm plates to 70% confluency. Total RNA was isolated using TRIzol (Invitrogen) according to the manufacturer’s protocol. One-step RT-PCR (Qiagen) was used to run PCRs for mouse GRIN2, mouse GRIN1, mouse Sprouty1, Sprouty2, Sprouty3, and Sprouty4 from Neuro-2A total RNA. The primers used were as follows: mSprouty1, 5′-TACGATTCTGTCCTAGACC-3′ (forward)
and 5'-TATTCACCACATGCTCTCC-3' (reverse); mSprouty2, 5'-GATCAGATCAAGCCATCC-3' (forward) and 5'-ACA-CAGCACAACAAAGTCC-3' (reverse); mSprouty3, 5'-GCA-TATGATTATGGAAGACG-3' (forward) and 5'-AGGCAGG-GTAAGAAAAGG-3' (reverse); mSprouty4, 5'-ATTGACCA-GATGAAGACC-3' (forward) and 5'-AGTGATAGAAGATA-CCTGTCG-3' (reverse). Each reaction contained 0.6 μM of each gene-specific primer, reaction mix, and 2 μg of total RNA. The annealing temperature was 54 °C, and the extension time was 40 s.

**Real-time RT-PCR**—Total RNA was isolated from cells using TRIzol reagent (Invitrogen), and real-time RT-PCR was performed on a LightCycler (Roche Applied Science) using the QuantiTect primer assay collection (Qiagen) according to the manufacturer’s instructions. Mouse GRIN1 (QT00262136) and tubulin (QT00251664) primer pairs were purchased from Qiagen from the QuantiTect primer assay collection. Data were analyzed using the LightCycler data analysis software. Crossing points were calculated using the second derivative maximum method. All reactions were done in triplicate, and data for GRIN were normalized against tubulin RNA levels for each sample.

**Antibodies**—Antibodies specific for GRIN were made by immunizing rabbits with a keyhole limpet hemocyanin-conjugated peptide specific for GRIN2 (Sigma Genosys). The sequence for the synthesized peptide for GRIN2 was N-DPEV-LGVAIQKHLQEMIKE-C. Antibodies were affinity-purified on peptide-conjugated columns ( Pierce).

**Imaging**—Neuro-2A cells were trypsinized and counted 1 day prior to transfection. 5.0 × 10⁴ cells were plated onto 35-mm MatTek® dishes. Cells were transfected with the appropriate GFP constructs using Lipofectamine 2000 according to the manufacturer’s instructions. Twenty-four hours after transfection, living Neuro-2A cells were imaged using a Zeiss LSM 510 META microscope in an inverted configuration with a pinhole setting of 1.0 using a UV Plan-Apochromat 63 × 1.4 NA oil objective and an optical zoom 2× in one x-y focal plane with the appropriate stage adapter configured for 35-mm MatTek® dishes. Multiple (6–8) 2–5-μm Z section slices and single images were obtained for each sample and condition. Representative images are shown.

**siRNA Knockdown Experiments**—Small interfering RNA smart pool reagents designed for mGRIN1 (catalog number M-046253-00) and siCONTROL Non-Targeting siRNA pool (catalog number D-001206-13-05) were purchased from Dharmacon. Neuro-2A cells were trypsinized and counted 1 day prior to transfection. Then, 2.5 × 10⁵ Neuro-2A cells were plated onto 35-mm dishes. Twenty-four hours later, cells were transfected with the appropriate siRNA oligonucleotide (final concentration 50 nM) using DharmaFECT 2 transfection reagent, according to the manufacturer’s instructions. After 48 h, one set of cells was trypsinized, split, and plated onto four 35-mm plates at a density of 3.5 × 10⁴ cells. RNA was harvested from a parallel set of transfected cells using the TRIzol method. Six to eight hours after plating, cells were serum-starved with 

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**TABLE 1**

Results of hGRIN2 (1–360-aa) two-hybrid screen

| hGRIN2-interacting protein | GenBank accession no. | β-Gal assay |
|---------------------------|----------------------|------------|
| E2F4                      | NM_001950            | ++         |
| Nell-2                    | NM_006159            | +++        |
| Solute carrier family 9 (antiporter, Na+/H+ exchanger) | NM_003047 | +++/+++ |
| Sprouty2                  | NM_005842            | +++        |
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RESULTS

GRIN Proteins Interact with Goαi—Our laboratory and others have found that GRIN isoforms can interact with Goαi (3, 5, 6). We characterized the nature of the interaction by GST pull-downs and co-immunoprecipitation assays. To identify the region on GRIN involved in the interaction with Goαi, GST fusion constructs were made with human GRIN2 (hGRIN2) full-length sequence and with truncated N-terminal region of hGRIN2 (containing 1–204 aa) and truncated C-terminal region of hGRIN2 (containing 205–461 aa). The various bacteria-expressed GST-tagged proteins were then incubated with HEK-293T cell lysates expressing an empty vector (pcDNA3.1) control or the constitutively active mutant Goαi Q205A. Only the GST-hGRIN2 full-length sequence and the GST-hGRIN2 205–461-aa truncation were able to pull down Goαi Q205A (Fig. 1A, left panel), suggesting that the C-terminal region of hGRIN2 is required for the interaction with Goαi. The interaction of hGRIN2 and Goαi was confirmed by co-immunoprecipitation of overexpressed FLAG-tagged hGRIN2 and Goαi Q205L (Fig. 1B). FLAG-TRAF2, a non-Goαi interacting protein, failed to immunoprecipitate Goαi (Fig. 1B). Whole cell lysates showed that FLAG-TRAF-2, FLAG-GRIN2, and Goαi were expressed accordingly (Fig. 1B, right panel). A GenBank™ search uncovered hGRIN mouse homologs mGRIN1 (long isoform) and mGRIN2 (short isoform). Although these two isoforms share only 48% homology at the C-terminal region, both FLAG-mGRIN1 and FLAG-mGRIN2 interact with Goαi, as shown by immunoprecipitation (Fig. 1, A and C).

Sprouty2 Interacts with GRIN—To identify potential interactors of GRIN, a yeast two-hybrid screen was performed using full-length GRIN2 as bait. This resulted in the induction of reporter genes in the absence of interacting proteins, suggesting that full-length GRIN2 contains a transcriptional activation domain (supplemental Fig. S1). To overcome this, we made truncations of GRIN2 and found that the truncation GRIN2 1–360 was the construct that lacked the transcriptional activation domain, and thus, we used it as bait in the yeast two-hybrid study. We screened a human brain cDNA library and found a number of potential GRIN2 interactors (Table 1). These inter-
actors were confirmed both in growth (on plates lacking leucine, tryptophan, and histidine) and in β-gal assay (β-galactosidase activity). The first protein identified was E2F4, a known transcription repressor that has been shown to function in growth suppression and differentiation (10). E2F4 represses genes during quiescence, heterodimerizes with p130 after cell cycle exit, and thereby induces differentiation in developing neurons (11). A second interactor candidate identified was neural-epidermal growth factor-like like 2 (Nell2). Nell2 is a neuron-specific thrombospondin-1-like extracellular protein containing six epidermal growth factor-like domains that is highly expressed in the hippocampus (12, 13). Nell2 was found to act within the CNS and peripheral nervous system progenitors to promote neuronal differentiation. Nell2/−/− mice have defects in spatial learning (14, 15). The Na+/H+ exchanger (NHE1) was shown to play a major role in intracellular pH homeostasis and cell volume regulation. Stimulation of NHE1 is required for cell proliferation, and its activity can be modulated by pH, tumor promoters, growth factors, and cell surface receptor signaling (16–19). Sprouty2, another direct interacting protein, belongs to a family of four mammalian proteins with a conserved C-terminal cysteine-rich domain that has been shown to regulate tyrosine kinase receptor signaling (20, 21). hSprouty2 and hGRIN2 direct interaction was confirmed by GST pulldown (Fig. 2A). Co-transfection of HA-mSprouty2 with mGRIN1 or mGRIN2 in HEK-293T cells followed by immunoprecipitation showed that both GRIN isoforms interact with Sprouty2 (Fig. 2B). Of note, Sprouty2 expression appears as a doublet in whole cell lysate gels, and Sprouty2 expression has been shown by other groups to exist as a doublet and sometimes triplet due to serine phosphorylation (22–24). Given their sequence homology, other members of the Sprouty family can potentially interact with GRIN2 proteins. We overexpressed FLAG-tagged mouse Sprouty1, Sprouty2, Sprouty3, and Sprouty4 in conjunction with control vector (pcDNA3.1) or HA-GRIN2 in 293T cells. After immunoprecipitation Sprouty proteins and immunoblotting for GRIN2, we found that Sprouty1, Sprouty2, and Sprouty4 were able to interact with GRIN2 whereas Sprouty3 could not (Fig. 2C). Whole cell lysates showed that Sprouty proteins and GRIN2 proteins were expressed in 293T cells (Fig. 2C, lower panel).

The Conserved C-terminal Domain of Sprouty2 Is Important for Interaction with GRIN2—To further characterize the Sprouty2-GRIN interaction, we sought to determine the regions of Sprouty2 responsible for GRIN2 binding. The ability of Sprouty to inhibit the MAPK signaling pathway is dependent on its C terminus (region 295–315 aa) (25). The C terminus also contains a dimerization domain (209–238 aa, Fig. 3A). This dimerization domain is one of several domains responsible for mediating interactions with other proteins (26). Furthermore, the C-terminal region of Sprouty contains an EGF-like domain which is known to function in protein-protein interactions (27). To map the regions of GRIN2 necessary for Sprouty2 and GRIN interaction, we sought to determine the regions of GRIN2 that interact with Sprouty2. We overexpressed various truncated versions of mSprouty2 tagged with hemagglutinin (HA) or DsRed monomer as follows: HA-Sprouty2 1–315 full-length, HA-Sprouty2 1–295 (Δ1), HA-Sprouty2 1–279 (Δ2), HA-Sprouty2 1–250 (Δ3), HA-Sprouty2 1–239 (Δ4), HA-Sprouty2 1–209 (Δ5), DsRed monomer N1-Sprouty2 1–73 (Δ6), and DsRed monomer N1 Sprouty2 174–315 (Δ7) (Fig. 3B). We co-expressed these truncations with FLAG-mGRIN2 in HEK-293T cells and immunoprecipitated with M2-FLAG antibody. The only mSprouty2 truncations detected after mGRIN2 immunoprecipitation were those containing region 250–279 (Fig. 3C). This suggests that the region of Sprouty2 that interacts with GRIN2 lies in the C-terminal conserved cysteine region and resides between amino acids 250–279.

Sprouty2 and Goαs Compete for GRIN2 Binding—We next set out to map the regions of GRIN2 necessary for Sprouty2 and Goαs binding. We made truncated HA-tagged mGRIN2 vari-
mGRIN2 is required for Gαo binding. HEK-293T cells were co-transfected with the indicated expression constructs, and total cell lysates were prepared as described previously. Lysates were immunoprecipitated (IP) with the anti-Gαo antibody or with the M2-FLAG antibody and immunoblotted (WB) with the anti-HA antibody (panel 1), with the anti-Gαo antibody (panel 2), and with the M2-FLAG antibody (panel 3). Panel 3 contains bands for Gαo (single band) and FLAG-Sprouty2 (double bands). Representative immunoblots from one of two independent experiments are shown. mSpr2, mouse Sprouty2; mG2, mouse GRIN2. B, Sprouty2 and Gαo compete for GRIN2 binding. HEK-293T cells were co-transfected with the indicated plasmids: Gαo Q205L (Q/L) mutant (1.5, 3.0, or 6.0 μg), FLAG-mSprouty2 (2.0, 4.0, or 6.0 μg), and HA-mGRIN2 (4 μg). Vector pcDNA3.1 was co-transfected to make the total amount of DNA transfected equal. Lysates were prepared as described previously and immunoprecipitated with anti-HA antibody. Immunoprecipitates were immunoblotted with anti-Gαo (panel 1), M2-FLAG (panel 2), or anti-HA (panel 3) specific antibodies. The triangle indicates increasing concentration of DNA transfected, and the rectangle indicates the same concentration of DNA transfected. A representative blot from three independent experiments is shown. Lower panel, whole cell lysates were immunoblotted with anti-Gαo, anti-FLAG (panel 1), M2-FLAG (panel 2), or anti-HA (panel 3) specific antibodies. Representative immunoblots from one of three independent experiments are shown. mSpr2, mouse Sprouty2; mG2, mouse GRIN2. C, corresponding densitometry for the average of 3–6 independent experiments testing the following DAN concentrations: Gαo, Q205L (Q/L) mutant (1.5, 3.0, 5.0, or 6.0 μg), FLAG-mSprouty2 (2.0, 4.0, 5.0 or 6.0 μg), and HA-mGRIN2 (4 μg). AU, arbitrary units. Error bars are S.E.

Tyrosine Phosphorylation Modulates Sprouty2-GRIN2 Interaction—Sprouty2 has been shown to be tyrosine-phosphorylated, and this phosphorylation is important for its inhibitory activity of the MAPK pathway (23, 25–27). This tyrosine phosphorylation, at Tyr-53, is induced by bFGF signaling (25). We wanted to determine whether the interaction with GRIN2 affected the tyrosine phosphorylation state of Sprouty2. To do so, we co-transfected Neuro-2A cells with FLAG-mSprouty2 with vector or mGRIN2. Transfected cells were serum-starved and then stimulated for 30 min with bFGF in the presence or absence of a 1-h pretreatment with sodium orthovanadate to inhibit protein phosphotyrosyl-phosphatases. FLAG-tagged mSprouty2 was immunoprecipitated with mGRIN2 (Fig. 5A). Immunoblotting was then performed using anti-phospho-Tyr antibody (4G10) followed by anti-GRIN and M2-FLAG antibodies. mSprouty is tyrosine-phosphorylated upon bFGF treatment (lanes 1 and 3). mGRIN2 is also tyrosine-phosphorylated.
GRIN Modulates Sprouty Repression of MAPK Activation

**A**

| Lane | mGRIN2 | FGF | Flag-mSpry2 |
|------|--------|-----|-------------|
| 1    | -      | -   | -           |
| 2    | +      | -   | +           |
| 3    | -      | +   | -           |
| 4    | +      | +   | -           |
| 5    | -      | +   | +           |
| 6    | +      | -   | +           |
| 7    | -      | +   | +           |
| 8    | +      | +   | +           |

**B**

| Lane | mGRIN2 | FGF | M2-Flag |
|------|--------|-----|---------|
| 1    | -      | -   | -       |
| 2    | +      | -   | +       |
| 3    | -      | +   | -       |
| 4    | +      | +   | -       |
| 5    | -      | +   | +       |
| 6    | +      | -   | +       |
| 7    | -      | +   | +       |

**FIGURE 5.** Expression of mGRIN2 inhibits tyrosine phosphorylation of mSprouty2. A, co-immunoprecipitation of tyrosine-phosphorylated (pY) GRIN and Sprouty. Neuro-2A cells were transfected with the indicated expression constructs. Cells were serum-starved overnight. One set of cells was treated with sodium orthovanadate (1 μM) for 1 h prior to stimulation with 40 ng/ml FGF for 30 min as indicated. Total cell lysates were immunoprecipitated (IP) with M2-FLAG antibody and immunoblotted (WB) with anti-phospho-tyrosine (G410) (top panel); anti-GRIN antibody (middle panel); and M2-FLAG antibody (bottom panel). In the whole cell lystate (WCL) panels, whole cell lysate was immunoblotted with anti-GRIN antibody (panel 1) and M2-FLAG antibody (panel 2). Of note, the M2-FLAG antibody detected some nonspecific bands (panel 2), and the third band is in fact Sprouty2. B, mSprouty2, mouse Sprouty2, Na3VO4, sodium orthovanadate. 8, whole cell lysates from A were immunoblotted with anti-phospho-p44/42 ERK1/2 antibody (panel 1), anti-total p44/42 ERK1/2 antibody (panel 2), and anti-tubulin antibody (panel 3). Representative immunoblots from one of three independent experiments are shown.

in the presence or absence of bFGF. Pretreatment with sodium orthovanadate enhanced the tyrosine phosphorylation of mGRIN2 (Fig. 5A, row 1, compare lanes 2 and 6 with lanes 4 and 8). Tyrosine-phosphorylated mGRIN2 co-immunoprecipitated with FLAG-mSprouty2 irrespective of bFGF stimulation. On the other hand, when mGRIN2 was co-expressed with mSprouty2, the amount of tyrosine-phosphorylated mSprouty2 co-immunoprecipitated decreased (lanes 3 and 4), suggesting that mGRIN2 co-expression prevents the tyrosine phosphorylation of mSprouty2. Pretreatment with sodium orthovanadate shifts up the motility of mSprouty2 band upward, suggesting further modifications (lanes 3 and 7). However, the amount of tyrosine-phosphorylated mSprouty2 co-immunoprecipitated decreased in the presence of mGRIN2 even when the cells were treated with sodium orthovanadate (lanes 7 and 8). Whole cell lysates show that FLAG-mSprouty2 and GRIN2 proteins were expressed similarly (Fig. 5A, lower panels). Cells treated with bFGF and/or sodium orthovanadate have higher phosphorylated ERK1/2 levels, indicating that the stimulation and treatment worked as expected (Fig. 5B). The fibroblast growth factor (FGF) stimulation of MAPK is damped due to overexpression of Sprouty.

**GRIN2-GFP Fusion Proteins Localize to Plasma Membrane in Gαo-expressing Cells but Not Sprouty2-expressing Cells**—To determine whether GRIN2 interacts with Gαo or Sprouty2 had any effect on GRIN2 localization, we made GRIN2-GFP fusion proteins. GRIN2 was placed in the N terminus of GFP, and co-immunoprecipitation experiments showed that this fusion protein retains the ability to interact with Gαo and Sprouty2 (data not shown). We determined the localization of the GRIN2-GFP construct when co-expressed with Gαo-Q205L, Sprouty2, or empty vector expression in Neuro-2A cells. When overexpressed along with empty vector, GRIN2-GFP localized to the cytoplasm (Fig. 6, B and E, DIFFUSE). However, upon expression of Gαo-Q205L, GRIN2-GFP primarily localized to the plasma membrane (Fig. 6, C and E, PM). Gαo Q205L expression had no effect on enhanced green fluorescent protein (EGFP) localization (Fig. 6A). This plasma membrane enrichment was less pronounced with Sprouty2 co-expression (Fig. 6, D and E, CYTO+PM). Sprouty2 localization was not affected by expression of Gαo-Q205L (supplemental Fig. S2).

**Active Gαo Signaling Attenuates FGF Activation of MAPK**—The previous experiments were performed using exogenous expression systems. We sought to find a cell line expressing all three proteins: GRIN, Sprouty2, and Gαo. Neuro-2A cells endogenously express GRIN1 and Gαo (5, 6). Sprouty2 is also endogenously present in Neuro-2A cells (data not shown). Sprouty2 has been shown to be regulate growth factor activation of ERK1/2 (25). The growth factors EGF, FGF, and glial-derived neurotrophic factor activate MAPK in Neuro-2A cells (Fig. 7A). Because the GRIN-Sprouty2 interaction can be outcompeted by Gαo signaling, we determined the functional significance of this regulation in the FGF-dependent activation of MAPK. We used HU-210, a CBI receptor agonist, to examine the effects of Gαo signaling on FGF stimulation of MAPK pathway in Neuro-2A cells. Serum-starved Neuro-2A cells were pretreated with HU-210 (500 nM) for various time periods (15, 30, and 60 min) followed by FGF stimulation for 5 min. HU-210 pretreatment for 30 or 60 min was able to decrease FGF activation of MAPK (Fig. 7, B and C), whereas a 15-min pretreatment with HU-210 had no effect. Simultaneous co-treatment with HU-210 and FGF enhanced MAPK activation (Fig. 7B, acute). This is expected as Gβγ subunits from Gαi/o can activate MAPK (28–32). We sought to test whether FGF activation of MAPK could be repressed by other Gαi/o-coupled receptors. Pretreatment by carbachol, a muscarinic agonist, also decreased FGF
activation of MAPK (Fig. 7, D and E), suggesting that this is a general mechanism not limited to CB1R signaling.

**GRIN Potentiates FGF Activation of MAPK**—Because Sprouty2 negatively regulates activation of MAPK, we determined whether exogenous mGRIN expression could modulate FGF-mediated MAPK activation. Neuro-2A cells were transfected with vector or FLAG-mGRIN1 or GRIN2. Cells were then serum-starved for 16 h and stimulated with 20 ng/ml FGF for 5 min. Expression of either mGRIN1 or mGRIN2 potentiated FGF activation of MAPK (Fig. 8, A–C). To implicate GRIN in the HU-210-dependent attenuation of MAPK activation by FGF, we abolished endogenous mGRIN1 expression using small interfering RNAs. We hypothesized that by decreasing GRIN1 levels, we would attenuate FGF activation of MAPK even further because without GRIN expression, sequestration and inhibition of Sprouty2 would decrease, and thus, Sprouty2 inhibition of the MAPK pathway would be enhanced. Neuro-2A cells were transfected with negative control pool siRNA or mGRIN1 pool siRNA. GRIN1 mRNA was decreased by 80% after 48 h of transfection with the GRIN1 pool siRNA (supplemental Fig. S3). siRNA transfected cells were serum-starved and then pretreated with DMSO or HU-210 (500 nM) for 30 min followed by FGF stimulation for 5 min. In cells exposed to mGRIN1 siRNA, pretreatment with HU-210 produced no difference from DMSO control after 5 min of FGF stimulation (Fig. 8, D and E). In addition, the levels of phospho-p44/42 for the GRIN1 siRNA pool decreased when compared with the negative control pool siRNA. This suggests that decreasing mGRIN1 expression attenuates FGF activation of ERK1,2.

**DISCUSSION**

Our laboratory and others have found GRIN to be a direct interactor of Goα, (3, 5, 33). GRIN preferentially interacts with activated Goα, over the wild-type form (Fig. 1C), suggesting that GRIN proteins may be direct downstream effectors for Goα signaling. GRIN family proteins contain conserved motifs that are important for Goα binding and for Sprouty2 binding. A Goα binding region lies within the semiconserved 200–300-amino acid region of GRIN2 where Sprouty2 binding occurs as well. Within this region, there are 15 conserved residues between GRIN1 and GRIN2, suggesting that these residues are potential contact sites for Sprouty2 and Goα binding. Mutational analysis and perhaps further truncations within the GRIN2 200–300-aa region can further define the area of interaction on GRIN2 that binds Sprouty2 and Goα. Similar to GRIN2, GRIN1 has two established C-terminal binding regions (821–851 and 902–932 aa) for Goα, (6). Goα expression induces the translocation of GRIN2 from the cytoplasm to the plasma membrane, and the C-terminal region of GRIN2 is critical for this localization (data not shown). Similarly, GRIN1 has been shown to be membrane-bound, and the C-terminal region of GRIN1 (region 772–827) is required for its localization to the plasma membrane (6). In fact, two conserved C-terminal cysteine residues, found in GRIN proteins, are required for GRIN1 membrane localization. These two cysteine residues (923–924 aa) are putative palmitoylation sites (6) and appear to be conserved in GRIN2. Palmitoylation, a reversible modification, has been shown to affect protein activity as well as subcellular localization by changing the proximity of the protein to its activators and/or targets (34–37).

The mechanism by which GRIN connects the Go signaling pathway to the receptor tyrosine kinase-activated MAPK depends on the ability of GRIN to bind Sprouty. Based on our results, we propose a modulatory role for GRIN (Fig. 9). In the absence of Go activity, GRIN binds and sequesters Sprouty (Fig. 9A). GRIN-bound Sprouty is unable to inhibit the MAPK cas-
Upon activation of G_{\alpha}\text{o}, by activation of the CB1R, the activated G_{\alpha}\text{o} subunit interacts with GRIN, thus releasing Sprouty, which can then be activated. Sprouty2 has been shown to inhibit FGF activation of MAPK through a number of loci on the FGF pathway. Sprouty contains a PXXPXR motif that competes with Sos for binding to the SH3 domain of Grb2 (24). In addition, phosphorylation of the conserved tyrosines Tyr-55 and Tyr-221 has been shown to be essential for the inhibitory activity of Sprouty (23, 25, 39). GRIN binds Sprouty within the region of 250–279 aa. By binding to that region, GRIN may inhibit Sprouty2 binding of Grb2. GRIN2 expression also appears to decrease the tyrosine phosphorylation of Sprouty2 after FGF stimulation. In this model, Sprouty upon dissociation from GRIN and activation by tyrosine phosphorylation is able to inhibit the FGF-ERK1,2 pathway (Fig. 9B). Thus, GRIN functions as a mediator of G_{\alpha}\text{o}-coupled receptor function to modulate the inhibitory effects of Sprouty2 on growth factor stimulation of MAPK activation. This model is supported by our observation that exogenous GRIN expression in Neuro-2A cells results in potentiation of MAPK activation by FGF stimulation (Fig. 9), whereas knocking down native GRIN proteins using siRNA in the same cell line attenuates FGF activation of MAPK (Fig. 9).

In examining the potential role of GRIN in neuronal processes, GRIN proteins, such as G_{\alpha}\text{o}, have been implicated in...
FIGURE 8. GRIN expression levels modulate MAPK activation by FGF. A, overexpression of GRIN2 enhances FGF stimulation of MAPK. Neuro-2A cells were transfected with either pcDNA3.1 (vector control) or pcDNA3.1 mGRIN2. Eight hours after transfection, cells were serum-starved overnight. They were then treated with FGF (20 ng/ml) for 5 min as indicated. Total cell lysate was immunoblotted (WB) with monoclonal anti-dual phospho-p44/42 ERK1/2 (top panel), polyclonal anti-dual total p44/42 ERK1/2 (second panel), anti-GRIN (third panel) antibody, and anti-tubulin antibody (bottom panel). Representative immunoblots from one of three experiments are shown. 

B, overexpression of GRIN1 enhances FGF stimulation of MAPK. Neuro-2A cells were transfected with either pcDNA3.1 FLAG (vector control) or pcDNA3.1 FLAG-mGRIN1. Eight hours after transfection, cells were serum-starved with 0.5% FBS modified Eagle’s medium for 16 h before stimulation. They were then treated with FGF (40 ng/ml) for 5 min as indicated. Cells were harvested, and 60 μg of total cell lysate was resolved by SDS-PAGE and immunoblotted with monoclonal anti-dual phospho-p44/42 ERK1/2 (top panel), polyclonal anti-dual total p44/42 ERK1/2 (second panel), M2-FLAG (third panel) antibody, and anti-tubulin antibody (bottom panel). Representative immunoblots from one of three experiments are shown. C, densitometric data for the average of three independent experiments of panel B. Error bars indicate S.E. ns, not significant. D, GRIN1 knockdown attenuates FGF stimulation of MAPK. Negative pool siRNA and GRIN1-specific siRNA (Dharmacon) were transfected into Neuro-2A cells. Cells were serum-starved overnight and pretreated with DMSO or HU-210 (500 nM) for 30 min followed by stimulation with FGF (20 ng/ml) for 5 min as indicated. For the acute stimulation, HU-210 (500 nM) and FGF (20 ng/ml) were added simultaneously for 5 min. Whole cell lysate was immunoblotted with monoclonal anti-dual phospho-p44/42 ERK1/2 (top panel), polyclonal anti-dual total p44/42 ERK1/2 (second panel), and anti-tubulin antibody (bottom panel). Representative immunoblots from one of three independent experiments are shown. CT, control. E, the corresponding densitometric data for the average of three experimental trials are shown. *, p < 0.05. Error bars indicate S.E.

FIGURE 9. Proposed mechanism of Gsα,GRIN mediated modulation of Sprouty2 inhibitory effects on MAPK activation. A, in the absence of Hu210, Gsα is inactive, and GRIN sequesters Sprouty and prevents it from inhibiting FGF-induced MAPK activation. B, upon stimulation of Gsα-coupled receptors, Gsα is activated and binds to GRIN. Sprouty is released and is able to attenuate FGF activated MAPK. Sprouty2 can potentially inhibit at the level of Grb2 and/or at c-Raf.
neurite outgrowth (5, 40). Overexpression of \( \alpha_{s} \) and GRIN1 in Neuro-2A cells resulted in neurite extension, and both proteins are expressed at the growth cone of neuronal cells (5). GRIN2, a related family member, is also expressed in the brain, particularly enriched in the cerebellum. Its expression in the neurites and role in neurite outgrowth is still under investigation. In addition, Sprouty2 has also been implicated in regulating neurite extension and neuronal development (7, 8, 25). Expression of a nonphosphorylated Sprouty2 mutant promoted FGF-induced outgrowth of neurites in PC12 cells, thus suggesting that Sprouty2 negatively regulates neurite outgrowth (9, 25). GRIN2 has been shown to inhibit tyrosine phosphorylation of Sprouty2 and thus may be a potential mechanism that promotes neurite outgrowth as well. GRIN1 can also interact with Sprouty2 and perhaps, like GRIN2, may modulate the inhibitory activity of Sprouty2.

Our laboratory has shown that activation of the \( \alpha_{i} \)-coupled cannabinoid CB1 receptors in Neuro-2A promotes the proteasomal degradation of Rap1GAPII, which subsequently activates Rap1 to induce neurite outgrowth. Whether \( \alpha_{i} \) signaling through the inhibition of the FGF pathway by GRIN is another mechanism that promotes neurite outgrowth in Neuro-2A cells will be addressed in future studies.

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