GRIN1 Regulates μ-Opioid Receptor Activities by Tethering the Receptor and G Protein in the Lipid Raft*8

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Xin Ge1, Yu Qiu, Horace H. Loh2, and Ping-Yee Law3

From the Department of Pharmacology, University of Minnesota Medical School, Minneapolis, Minnesota 55455

The lipid raft location of μ-opioid receptor (MOR) determines the receptor activities. However, the manner in which MOR is anchored within the lipid rafts is undetermined. Using the targeted proteomic approach and mass spectrometry analyses, we have identified GRIN1 (G protein-regulated inducer of neurite outgrowth 1) can tether MOR with the G protein α-subunit and subsequently regulate the receptor distribution within the lipid rafts. Glutathione S-transferase fusion pulldown and receptor mutational analyses indicate that GRIN1–MOR interaction involves a receptor sequence G267-G5SEK-G271 within the MOR third intracellular loop that is not involved in Ge interaction. The GRIN1 domains involved in MOR interaction are also distinct from those involved in Ge interaction. Pertussis toxin pretreatment reduced the amount of GRIN1 co-immunoprecipitated with MOR but not the amount with Ge. Furthermore, overexpression of GRIN1 significantly enhanced the amount of MOR in lipid raft and the receptor signaling magnitude as measured by Src kinase activation. Such increase in MOR signaling was demonstrated further by determining the GRIN1-dependent pertussis toxin-sensitive neurite outgrowth. In contrast to minimal neurite outgrowth induced by etorphine in control neuroblastoma N2A cells, overexpression of GRIN1 resulted in the increase in etorphine- and non-morphine-induced neurite outgrowth in these cells. Knocking down endogenous GRIN1 by small interfering RNA attenuated the agonist-induced neurite outgrowth. Disrupting lipid raft by methyl-β-cyclodextrin also blocked neurite outgrowth. Hence, by tethering Ge with MOR, GRIN1 stabilizes the receptor within the lipid rafts and potentiates the receptor signaling in the neurite outgrowth processes.

G protein-coupled receptors (GPCRs)4 use heterotrimERIC guanine nucleotide-binding proteins (G proteins) as signaling intermediates between activated receptors and their intracellular effectors. GPCR signaling complex consists minimally of three protein components as follows: a receptor, a G protein, and a signaling effector. During the course of receptor signaling, these components at one time must physically interact with each other for signals to propagate. Lipid rafts, microdomains located within the plasma membrane, cluster receptors and components of receptor signaling cascades. Selective partitioning of proteins into lipid rafts often influences lipid transport, membrane trafficking, and signal transduction. In this manner, lipid rafts can serve as signaling platforms for receptors and effectors (1). Proteins known to interact with lipid rafts and to have activities influenced by such interaction, include GPCRs (e.g. the β2-adrenergic receptor), tyrosine kinase receptors (e.g. epidermal growth factor receptors and nerve growth factor receptors), GTPases (e.g. H-Ras and α-subunits of heterotrimeric G proteins), and nonreceptor tyrosine kinases (e.g. Src and Src family kinases), as well as mitogen-activated protein kinase (MAPK)/ERK kinase and ERK (2–8). The function of lipid rafts in organizing and mediating signal transduction of GPCRs was reported for many receptor classes and subtypes (9, 10). Physical interaction between opioid receptor and G protein in lipid raft represents the high affinity state of agonist binding (11, 12), suggesting that the localization of receptor and G protein in lipid rafts may facilitate their coupling and subsequently influence agonist binding to receptors. It is highlighted in recent studies that the influence of lipid rafts on signaling proteins is dynamic in both directions (13–15). This means that proteins are able to mediate their signaling environment (e.g. post-translational modification such as palmitoylation/des-palmitoylation or myristoylation/demyristoylation) and can affect the length of stay in the lipid rafts of signaling proteins, and the different affinities of signaling proteins for lipid rafts can affect themselves and their associated proteins to be held in or expelled out of lipid rafts, thereby modifying the properties and organization of signaling proteins in lipid rafts (16, 17). Finally, signaling amplitude can be amplified or diminished in these signaling microdomains. Collectively, the varieties of interactions between lipid rafts and proteins reflect the complexity of their relationship and the multitude of possible regulatory processes involved in GPCR-associated signal transduction (13, 18).

Opioid receptors, being members of the rhodopsin subfamily among the GPCR superfamily, have been shown to locate within the lipid raft domains both in the in vitro cell models and in vivo (19, 20). In addition, raft domains differentially affect agonist binding of different opioid receptors and the receptor coupling to the G proteins, which determine the distinct signal pathways selected for receptor signaling. For example, human κ-opioid receptors expressed in Chinese hamster ovary...
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cells are located in lipid rafts, and cholesterol depletion of these cells has no effect on the association between \( \kappa \)-opioid receptors and G protein (19), indicating receptor and G protein occupy different membrane microdomains. By contrast, the receptor location within the lipid raft regulates agonist-selected signal transduction of \( \mu \)-opioid receptors (MOR), and which raft location reflects the receptor-G protein coupling in lipid raft (20).

However, the mechanism in which the G protein can cluster MOR within the lipid raft and subsequently regulate the agonist-selective signaling remains undetermined. To address these issues, yeast two-hybrid system and GST fusion protein pulldown assay were used to identify some components of the scaffolding complex of GPCRs (21, 22). But as to these methods, only one or two domains of the receptor were used to construct a fusion protein for identifying scaffolding proteins. They cannot reflect agonist/antagonist-mediated conformational change responsible for protein recruitment. To identify the binding partners of MOR, we purified MOR complexes from neuroblastoma Neuro2A (N2A) cells stably expressing His\(_6\)-tagged MOR with Ni\(^{2+}\) affinity chromatography, separated the MOR-associated protein complex by SDS-PAGE, and determined individual protein identities with mass spectrometry analyses. Among the identified proteins, GRIN1 (G protein-regulated inducer of neurite outgrowth 1) has been found to be directly associated with MOR. GRIN1 was initially found specifically expressed in brain and interacted selectively with activated \( \alpha\)-subunits of the \( G_i \) subfamily (\( G_{i,\alpha} \), \( G_{s,\alpha} \), and \( G_{o,\alpha} \)). GRIN1 co-localizes with \( G_{s,\alpha} \) at the growth cone of neuronal cells and promotes neurite extension in N2A cells (23). It was reported that the interaction between GRIN1 and activated \( G_{s,\alpha} \) induced activation of Cdc42, which led to morphological changes in neuronal cells (24). In this study, we demonstrate that GRIN1 serves as a tether for MOR and \( G_o \) that results in the clustering of MOR within the lipid rafts. Such immobilization of MOR within lipid rafts induces neurite outgrowth in the presence of etorphine. Our data suggested that GRIN1 had a distinct function besides its reported interaction with activated \( G_{o,\alpha} \).

**EXPERIMENTAL PROCEDURES**

**Culturing of N2A Cells Stably Expressing His\(_6\)-MOR and Transient Transfection of cDNA**—The rat MOR-tagged with the His\(_6\) epitope at the N terminus was subcloned in pCDNAmp vector. N2A cells were cultured in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 100 units/ml penicillin, 100 \( \mu \)g/ml streptomycin, and 10% fetal calf serum (DMEM growth medium) in a 10% CO\(_2\) incubator. These N2A cells were transfected with 10 \( \mu \)g of the His\(_6\)-MOR plasmids. The colonies surviving 1 mg/ml antibiotic of geneticin were isolated. The cell clone that expressed MOR of 0.8 pmol/mg protein was used in the receptor complex purification.

As for transient transfection of cDNA, N2A cells were plated on 100-mm dishes (immunoprecipitation studies) at a density of 250,000 cells/ml and maintained in DMEM growth medium to 80% confluency. Transfections were performed using the Superfect transfection reagent (Qiagen, Valencia, CA). Transfection medium was replaced with medium containing fresh serum 12–18 h after transfection, and cells were harvested 24–48 h later.

**Purification of Receptor Complex and Mass Spectrometry**—The methods have been described in a previous paper (25). Briefly, 60 150-mm dishes of N2A cells stably expressing His\(_6\)-MOR, together with 60 150-mm dishes of N2A cells as control were lysed with 1% Triton X-100 at 4 °C for 2 h. Lysate was collected and centrifuged at 10,000 \( \times \) \( g \) for 15 min at 4 °C. Supernatant was collected and purified by Ni\(^{2+}\) resin columns (Invitrogen). Then columns were washed 10 times with wash buffer and eluted with elution buffer provided by the kit (Invitrogen). Eluates were concentrated with Amicon concentration cells (Amicon, Beverly, MA). Proteins were separated by SDS-PAGE and silver-stained, and the presence of MOR was identified by Western blot analysis.

As for mass spectrometry analyses and identification of the proteins, silver-stained gel bands were excised, dried, and destained by incubating in 15 mM K\(_2\)Fe(CN)\(_6\) and 50 mM NaN\(_2\)SO\(_4\) at 24 °C for 15 min, followed by washing with 100 mM NH\(_4\)HCO\(_3\). Destained gels were dried and rehydrated in 50 mM NH\(_4\)HCO\(_3\) and 5 mM CaCl\(_2\) solution with 0.01 mg/ml sequence-grade modified porcine trypsin (Promega, Madison, WI) and then incubated at 37 °C overnight. Trypsinized fragments were collected by sonicating the gel pieces in 50 \( \mu \)l of 25 mM NH\(_4\)HCO\(_3\) together with 50 \( \mu \)l of 50% acetonitrile. The supernatant was collected and sonicated repeatedly in 50 \( \mu \)l of 5% formic acid and added with 50 \( \mu \)l of 50% acetonitrile again. The supernatant was pooled together. DTT was added to a final concentration of 1 mM. Prior to MALDI-TOF analysis, the sample was desalted using Millipore C18 ZipTips, using the protocol described by Millipore, with the following modification: the elution buffer was 60:40, acetonitrile/water, 0.1% trifluoroacetic acid. The instrument used for the collection of MALDI-TOF was a Bruker Biflex III, equipped with an N2 laser (337 nm, 3-ns pulse length) and a microchannel plate detector. The data were collected in the reflectron mode, positive polarity, with an accelerating potential of 19 kV. Each spectrum is the accumulation of \(~200\) laser shots. External calibration was performed using human angiotensin II (monoisotopic mass [MH\(^+\)] 1046.5; Sigma) and adrenocorticotropin hormone fragment 18–39 (monoisotopic mass [MH\(^+\)] 2465.2; Sigma). The matrix used for samples and standards was \( \alpha\)-cyano-4-hydroxycinnamic acid (Hewlett-Packard, sold in solution, in methanol) diluted 1:1 with 50:50 acetonitrile/nanopure water, 0.1% trifluoroacetic acid. High pressure liquid chromatography-grade acetonitrile was purchased from Fisher, and +99% spectrophotometric grade trifluoroacetic acid was purchased by Sigma.

**Immunoprecipitation and Western Blot Analysis**—Confluent cells were washed in phosphate-buffered saline (PBS) at 4 °C and lysed for 30 min in solubilization buffer (0.5% polyoxyethylene (10) lauryl ether (C\(_{12}\)E\(_{9}\), 100 mM NaCl, 50 mM sodium phosphate, 1 mM DTT, pH 7.2) containing mammalian protease inhibitor mixture (Sigma) at 4 °C. Lysate was centrifuged for 30 min at 10,000 \( \times \) \( g \), and the supernatant was collected and assayed for protein by the BCA method (Pierce). A total of 500 \( \mu \)g of protein preclidean with protein G-Sepharose beads was incubated with 1 \( \mu \)g of mouse anti-FLAG antibodies for 2–3 h followed by incubation with 30 \( \mu \)l of protein G-Sepharose.
(Sigma) for 3 h at 4 °C. Sepharose beads were pelleted by brief centrifugation at 10,000 × g at 4 °C and washed three times with solubilization buffer. Proteins were eluted by resuspending the beads in 2 volumes of 2 X SDS sample buffer (10 mM Tris, 15 mM SDS, 20 mM DTT, 20% glycerol, 0.02% bromphenol blue, pH 6.8) followed by incubation at 65 °C for 30 min. Proteins were resolved by SDS-PAGE, transferred to Immobilon-P membranes (Millipore, Bedford, MA), followed by immunoblotting with anti-GRIN1 primary antibodies (T116), and detected with anti-rabbit alkaline phosphatase-linked secondary antibodies (Bio-Rad) diluted in Tris-buffered saline containing 5% powdered milk and 0.1% Tween 20 unless indicated otherwise.

In Vitro Translation—The constructs are translated in vitro using the TnT Coupled Transcription/Translation System (Promega) according to the company’s protocol. In brief, the TnT buffer, rabbit reticulocyte lysate, RNA polymerase, amino acid mixture, and RNase inhibitor were added together in a 0.5-ml microcentrifuge tube placed on ice. 1 μg of plasmid DNA was added last to the tube and to the reaction mixture. Components were spun to the bottom of tube and mixed by centrifuging at 13,000 rpm for 15 s. After that, the reaction was incubated at 30 °C for 90 min and then terminated by keeping in store at –80 °C.

Pulldown Assays with the GST-MOR Fusion Proteins and in Vitro Translation Product of GRIN1—The GST fusion proteins of MOR second intracellular loop (MORIL2), MOR third intracellular loop (MORIL3), and MOR C-tail (MORCT) were constructed by cloning the PCR products of these receptor domains into the BamHI site of the pGXT2 vector. These PCR products were generated using the rMOR cDNA cloned in pRcR-cmv and the following primers: MORIL2 sense, GGATCCCTCTGCAACTATGAGCTGGAGCAC; and antisense, GGATCCCTCAGCAGACGTTGACG-MORIL3 sense, sense 3, GATCCCGTTTGCTGGAACATGGCATGTCCGGATATCAAAGAAGCATTTGGAGCAGTACAGATTTTTTCCAAA, and antisense 4, AGCTTTTGGAAAAAATCTGACGCTCCAAATGCTTCTTTGATATCCGACCATGCCATGTTCCAGCAAACGG. The plasmids were transformed into Escherichia coli BL21-CodonPlus (DE3)-RP (Stratagene, La Jolla, CA), and the production of fusion proteins was induced with addition of 2 mM isopropyl-1-thio-β-D-galactopyranoside. GST, MORIL2, MORIL3, or MORCT proteins were adsorbed onto glutathione-agarose beads (Sigma) and washed three times with ice-cold GST buffer (50 mM Tris-HCl, 150 mM NaCl, 0.05% Tween 20, pH 7.4) and immunoblotted with mouse anti-HA primary antibodies (Covance). The direct interaction of the in vitro translated product was detected with mouse anti-alanine phosphatase-linked secondary antibodies (Bio-Rad) diluted in Tris-buffered saline containing 5% powdered milk and 0.1% Tween 20.

Construction of GRIN1 siRNA—Constructs of GRIN1 siRNA were cloned into GFP-tagged pRNAT H1.1 vector with siRNA sequence for GRIN1 (designed by GenScript siRNA design center siRNA Target Finder and siRNA Construct Builder) as follows: sense 1, GATCCGGTTTCTCGAGTCTGATGACCTTGTATCTCAGCTACTGAGGTCATGGGATCTCATGACCGACATTGATATCCGACATGCCATGTTCCAGCAAACGG; sense 2, GATCCGGTTTCTGAGGTCATGAGATCCCAAGAAGGTATGAAAATTTGCTGGAACATGGCATGTCCGGATATCAAAGAAGCATTTGGAGCAGTACAGATTTTTTCCAAA, and antisense 1, AGCTTTTTGAAAAAATTTTTCTGAGGCTAGATCAGCCGGAAGATTCTTCTGTGGATCTCATGACCGACATTGATATCCGACATGCCATGTTCCAGCAAACGG; sense 3, GATCCGGTTTCTCGAGTCTGATCAGCCGGAAGATTCTTCTGTGGATCTCATGACCGACATTGATATCCGACATGCCATGTTCCAGCAAACGG; sense 4, GATCCGGTTTCTCGAGTCTGATCAGCCGGAAGATTCTTCTGTGGATCTCATGACCGACATTGATATCCGACATGCCATGTTCCAGCAAACGG.

Terminal dUTP Nick End-Labeling Assay—Adherent cultured N2A cells transfected with GFP-GRIN1 siRNA with or without GRIN1 overexpression rescue and positive control treated by actinomycin D (1 μM) were fixed in 1% paraformaldehyde in PBS, pH 7.4, on the coverslips in a 6-well plate at room temperature. After excess liquid was removed, the cover-
slips were washed with two changes of PBS, 5 min for each wash. The coverslips were post-fixed in precooled ethanol/acetone acid 2:1 for 5 min at −20 °C, followed by washing with two changes of PBS, 5 min for each wash. Afterward, excess liquid was aspirated carefully, and 75 μL/5 cm² of equilibration buffer (Millipore) was added directly on the specimen for 10 s at room temperature. The coverslips were incubated with 100 μL/5 cm² working strength terminal deoxynucleotidyltransferase enzyme in a humidified chamber at 37 °C for 1 h. The cells were placed in working strength stop/wash buffer (Millipore), then agitated for 15 s, and incubated in the same buffer for 10 min. The cells were washed in two changes of PBS for 1 min per wash. 100 μL/5 cm² anti-digoxigenin conjugate of rhodamine (Millipore) was added directly on the specimen for 10 s at room temperature. The coverslips were incubated with 100 μL of PBS for 2 min per wash. The coverslips were washed with two changes of PBS, 5 min for each wash. Afterward, excess liquid was aspirated carefully, and 75 μL/5 cm² of equilibration buffer (Millipore) was added directly on the specimen for 10 s at room temperature. The coverslips were incubated with 100 μL of PBS for 2 min per wash. The cells were washed in two changes of PBS for 1 min per wash. 100 μL/5 cm² anti-digoxigenin conjugate of rhodamine (Millipore) was added to the slides. The slides were incubated in a humidified chamber for 30 min at room temperature while avoiding exposure to light. Afterward, the cells were washed in four changes of PBS for 2 min per wash. 4’ 6-Diamidino-2-phenylindole (1:100, 500 μL) was added to the cells for 1 h at room temperature, and the cells were washed in four changes of PBS for 2 min per wash. The cells were mounted with Fluoromount-G (Southern Biotech). For statistical analysis, images from more than 20 fields in each sample were calculated. The percentages of cells inducing apoptosis versus total cells were analyzed with Prism 4, version 4.0, by GraphPad Software, Inc.

**Live Cell Imaging by Confocal Microscopy**—Cells transfected with GFP-GRIN1 were grown in 10-mm culture plates with a glass coverslip at the bottom to 30–40% confluency and treated with GFP-tagged GRIN1s were visualized by immunofluorescence using a CARY II™ Confocal Imager (BD Biosciences) and a Leica DMIRE2 fluorescence microscope. Fluorescence images of the same cells were taken at time “0” after medium was changed and at different time points during agonist incubation as indicated. For statistical analysis, images from more than 20 fields in each sample were measured and calculated. The percentages of cells possessing neurite outgrowth versus total cells were analyzed with Prism 4, version 4.0, by GraphPad Software, Inc.

**Immunoprecipitation of GRIN1 with Adenovirus-mediated Expression of G Protein α-Subunit**—The construction of PTX-resistant adeno-Gio2-Leu, adeno-Gio3-3-Leu, and adeno-Goa-Leu was described previously (26). N2A cells stably expressing MOR with HA-tagged at the N terminus in 100-mm dishes were infected with PTX-resistant adeno-Gio2-2-Leu, adeno-Gio3-Leu, or adeno-Goa-Leu viruses using Superfect transfection reagent (QIAGEN) as described previously (26). 24 h later, cells were transfected with HA-GRIN1 using Superfect transfection reagent according to the manufacturer’s instructions. The next day, 100 ng/mL PTX was added, and the cells were incubated overnight. Then the cells were treated with 100 μM etorphine or with 100 nM etorphine as indicated. The cells were washed with two changes of PBS, 5 min for each wash. Afterward, excess liquid was aspirated carefully, and 75 μL/5 cm² of equilibration buffer (Millipore) was added directly on the specimen for 10 s at room temperature. The coverslips were incubated with 100 μL/5 cm² working strength terminal deoxynucleotidyltransferase enzyme in a humidified chamber at 37 °C for 1 h. The cells were placed in working strength stop/wash buffer (Millipore), then agitated for 15 s, and incubated in the same buffer for 10 min. The cells were washed in two changes of PBS for 1 min per wash. 100 μL/5 cm² anti-digoxigenin conjugate of rhodamine (Millipore) was added directly on the specimen for 10 s at room temperature. The coverslips were incubated with 100 μL of PBS for 2 min per wash. The coverslips were washed with two changes of PBS, 5 min for each wash. Afterward, excess liquid was aspirated carefully, and 75 μL/5 cm² of equilibration buffer (Millipore) was added directly on the specimen for 10 s at room temperature. The coverslips were incubated with 100 μL of PBS for 2 min per wash. The cells were washed in two changes of PBS for 1 min per wash. 100 μL/5 cm² anti-digoxigenin conjugate of rhodamine (Millipore) was added directly on the specimen for 10 s at room temperature. The coverslips were incubated with 100 μL of PBS for 2 min per wash. The cells were washed in two changes of PBS for 1 min per wash. 100 μL/5 cm² anti-digoxigenin conjugate of rhodamine (Millipore) was added to the slides. The slides were incubated in a humidified chamber for 30 min at room temperature while avoiding exposure to light. Afterward, the cells were washed in four changes of PBS for 2 min per wash. 4’ 6-Diamidino-2-phenylindole (1:100, 500 μL) was added to the cells for 1 h at room temperature, and the cells were washed in four changes of PBS for 2 min per wash. The cells were mounted with Fluoromount-G (Southern Biotech). For statistical analysis, images from more than 20 fields in each sample were calculated. The percentages of cells inducing apoptosis versus total cells were analyzed with Prism 4, version 4.0, by GraphPad Software, Inc.

**Live Cell Imaging by Confocal Microscopy**—Cells transfected with GFP-GRIN1 were grown in 10-mm culture plates with a glass coverslip at the bottom to 30–40% confluency and treated with 100 nM etorphine or with 100 nM etorphine as indicated. After that, cells were washed with fresh medium three times and incubated in fresh medium without fetal bovine serum at 37 °C for 48 h. GFP-tagged GRIN1s were visualized by immunofluorescence using a CARY II™ Confocal Imager (BD Biosciences) and a Leica DMIRE2 fluorescence microscope. Fluorescence images of the same cells were taken at time “0” after medium was changed and at different time points during agonist incubation as indicated. For statistical analysis, images from more than 20 fields in each sample were measured and calculated. The percentages of cells possessing neurite outgrowth versus total cells were analyzed with Prism 4, version 4.0, by GraphPad Software, Inc.
2 μg/ml leupeptin, 2 μg/ml pepstatin, and 50 mM NaF). The homogenates were centrifuged at 1000 × g to remove nuclei and large debris (P1). Supernatant was collected (S1). Pellet with crude membranes (P1) were rehomogenized and centrifuged again at 10,000 × g. Supernatant was collected as S2 and pooled together with S1, and 1.5 ml of them were taken for lipid raft fractionation as described under “Separation of Lipid Raft.”

Separation of Lipid Raft—N2A Cells stably expressing HA-MOR in 150-mm plates were grown and transfected with vector, GRIN1, and GRIN1 siRNA. To prepare rafts (29), 1.5 ml of raft buffer (500 mM sodium carbonate, pH 7.5, at 4 °C) was added after the medium was aspirated, and cells were scraped from the plates. An equal volume (1.5 ml) of modified Barth’s solution (MBS) buffer, pH 6.8, containing 80% sucrose was added, and the combined supernatants were placed in the bottom of a centrifuge tube. A 3-ml step gradient of 5 and 35% sucrose in raft buffer was layered on top of the lysate. Gradients were centrifuged for 18 h at 20,000 rpm using an SW 41 rotor in a Beckman ultracentrifuge. After centrifugation, 1-ml fractions of the gradients were collected. Total proteins in each fraction were precipitated by 5% trichloroacetic acid and washed three times with acetone at 4 °C, then dissolved in Laemmli buffer, and separated on a 10% SDS-polyacrylamide gel. Afterward, the separated proteins were transferred to Immobilon-P membrane (Millipore, Bedford, MA), and the membrane was blocked in a blocking solution of 10% dry milk and 1% Tween 20 in Tris-buffered saline. Western analyses were carried with mouse anti-HA (1:2000), mouse monoclonal anti-transferrin (1:1000), rabbit anti-Giα2 (1:1000), or rabbit anti-T116 (1:100). Primary antibody was probed with alkaline phosphatase-conjugated secondary antibodies (1:5000). Proteins bands were detected by the addition of the ECF substrate, and fluorescence of the bands was determined with Storm 860 (GE Healthcare). Band intensities were quantified and analyzed using ImageQuant (GE Healthcare). To normalize the Western analyses from separate runs, the relative percentage of the proteins in each fraction was obtained by dividing each individual gradient fraction versus the sum of the pixels from all fractions.

RESULTS

Identification of GRIN1 Binding to MOR—To identify components of MOR signaling complexes, His6-MORs stably expressed in N2A cells were partially purified with a Ni2+ resin column as described previously (25). Eluted samples containing MOR complexes were denatured by SDS-PAGE sample buffer at 65 °C for 30 min. Silver staining of the gels revealed multiple protein bands, including His6-MOR, which migrated as a diffusive band with a molecular mass between 65 and 70 kDa. The consistency in protein isolation can be demonstrated in the silver-staining results from three different experiments (25). These silver-stained bands were demonstrated to be proteins associated with His6-MOR by their absence in Ni2+ resin eluates of N2A wild type cells. The identity of MOR was further confirmed by parallel Western blot analysis, using anti-MOR carboxyl tail polyclonal antibody (25, 30). The protein band around 110 kDa was identified as GRIN1 by MALDI-TOF spectrometry analysis, indicating that GRIN1 was in the purified MOR complex. Equivalent protein candidates reported were gi|5901688 and gi|5901688. They all refer to the sequence of GRIN1.

The specific interaction between MOR and GRIN1 can be demonstrated with the co-immunoprecipitation experiments. To eliminate protein interaction after disruption of cellular boundaries during extraction, cells expressing either FLAG-MOR or HA-GRIN1 were mixed together immediately prior to detergent addition. Contrary to cells co-transfected with both FLAG-MOR and HA-GRIN1 (Fig. 1A, lane 4), mixed cells transfected with either MOR or GRIN1 alone did not co-immunoprecipitate together (Fig. 1A, lane 3). Furthermore, GRIN1 interaction with MOR appears to be affected by agonist treatment. When the N2A cells transiently transfected with HA-GRIN1 and FLAG-MOR were treated with 100 nM etorphine for 10 min, a reduction in the amount of GRIN1 co-immunoprecipitated by MOR was observed (Fig. 1B). Quantification of the band indicated around 60 ± 10.8% reduction in GRIN1-MOR co-immunoprecipitated (Fig. 1C). Western analyses of HA-GRIN1 in the total lysate and amount of FLAG-MOR immunoprecipitated indicated the observed reduction was not due to the differences in these two protein levels in the control and etorphine-treated cells.

Because the observed GRIN1-MOR interaction was demonstrated with the heterologous expression system N2A cells and to demonstrate whether GRIN1 interacts with MOR endogenously, mouse hippocampi were used subsequently. MOR from mouse hippocampus tissue was immunoprecipitated with the anti-MOR C-tail polyclonal antibodies (30). Mouse cerebellum was used as the negative control to determine the specificity of the antibodies used. As shown in Fig. 1D, MOR C-tail antibodies could immunoprecipitate the endogenous GRIN1 from hippocampus even under PTX pretreatment, whereas proteins with molecular weights similar to GRIN1 were not observed with the MOR C-tail antibody immunoprecipitates of the cerebellum extracts, which indicated GRIN1 endogenously bound to MOR regardless of G protein (Fig. 1D).

Because GRIN1 was shown to interact with Gα (23), the co-immunoprecipitation of GRIN1 with the receptor antibodies or receptor epitope antibodies could be the result of receptor-G protein interaction. To further determine whether GRIN1 directly interacts with MOR or not, gel overlay experiments were performed. In vitro translation products of GRIN1 were shown as in Fig. 1E, panel a. The total lysate of N2A cells expressing FLAG-MOR was first separated with SDS-PAGE and transferred to Immobilon-P membrane. The membrane was then incubated with the in vitro translation products of the GRIN1 construct, and the molecular mass was around 85 kDa, probably due to the absence of post-translational modification. After excess in vitro translated product was removed by repeated washings, a GRIN1-positive band with a mass of ~68 kDa was observed in the lane containing lysate from MOR-expressed N2A cells but not in lane from wild type N2A cells (Fig. 1E, panel b). The location of the in vitro translated HA-GRIN1 product coincided with the location of FLAG-MOR, as determined by a parallel experiment of Western blot using anti-MOR C-tail antibody (Fig. 1E, panel c). These data indicated that GRIN1 directly interacted with MOR. Another GRIN1-positive band was also observed in both wide type N2A cells and
MOR-expressed N2A cells, which was confirmed to be endogenous G\textsubscript{i}/H\textsubscript{9251} subunit by Western analysis using anti-G\textsubscript{i}/H\textsubscript{9251} antibody (data not shown).

**GRIN1 Directly Interacted with the MOR Third Intracellular Loop**—Next, we examined the MOR sequence involved in the binding to GRIN1 by using GST fusion protein pulldown studies. Because of the intracellular location of GRIN1, only the putative intracellular domains of MOR were used in the pulldown experiments. GST or GST-MOR second, GST-MOR third intracellular loop, and GST-MOR C-tail fusion proteins (see Table 1 for specified amino acid sequences) were expressed in *E. coli* and purified. To avoid the influence of G protein interaction, the *in vitro* translation GRIN1 product was used instead of total cell lysates in the GST fusion protein pulldown experiments. As shown in Fig. 2A, the GST-MOR third intracellular loop bound GRIN1 *in vitro* translation product to a much greater extent than GST-MOR second intracellular loop and GST-MOR C-tail fusion proteins, which showed similar bind-

![Figure 1](image-url)

**TABLE 1**

| Amino acid sequence of GST fusion protein | Amino acid sequence |
|------------------------------------------|--------------------|
| GST-MOR second intracellular loop        | GST-DRY1AVCHPVKALDFRTPR |
| GST-MOR third intracellular loop         | GST-LMILRLKSVRMLSGKDEQRNLRRITR |
| GST-MOR C-tail                           | GST-DENFKRCFREPFICTSSTIEQIQNSSVRQRNTERHPSTANTVDRTNHQLLENLEAETAPLP |

**Figure 1.** GRIN1 directly interacts with MOR. **A,** co-immunoprecipitation of FLAG-MOR and HA-GRIN1 in cells only express HA-GRIN1 (lane 1); in cells only express FLAG-MOR (lane 2); in mixed cells that individually express FLAG-MOR and HA-GRIN1 (lane 3); and in cells that express both FLAG-MOR and HA-GRIN1 (lane 4). **B,** effect of 100 nM etorphine on the co-immunoprecipitation of GRIN1 with MOR in cells overexpressed with both HA-GRIN1 and FLAG-MOR. **C,** quantification analysis of immunoprecipitated GRIN1 with MOR with or without etorphine treatment. The values represent the averages from *n* = 3 blots. **,** *p* denotes < 0.01. **D,** co-immunoprecipitation of GRIN1 with MOR in mouse brain region with no endogenous MOR expression (cerebellum) or with high endogenous MOR expression (hippocampus). GRIN1 was detected by anti-GRIN1 (T116) antibody (panel a), and endogenous MOR was determined by rabbit anti-MOR C-tail antibody (panel b). **E,** gel overlay of HA-GRIN1 with N2A-MOR. Panel a, *in vitro* translation products of HA-GRIN1, as detected by anti-HA antibody; panel b, gel overlay of HA-GRIN1 on membranes containing SDS-PAGE separated N2A extract from cells expressing or not expressing MOR; panel c, same blot was stripped and MOR expression was detected using anti-MOR C-tail antibody. IB, immunoblot.
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FIGURE 2. MOR third intracellular loop is involved in direct interaction with GRIN1. A, GST-MOR third intracellular loop bound GRIN1. Panel a, in vitro translation product of GRIN1 binding to GST, GST-MOR second intracellular loop (2nd IL), GST-MOR third intracellular loop (3rd IL), or GST-MOR C-tail fusion protein; panel b, expression level of respective fusion proteins as determined with Coomassie Blue staining. B, amino acid sequences of MOR third intracellular loop, and the corresponding sequences of MORi3-1, MORi3-2, MORi3-3, MORi3-4, and MORi3-5 mutants. WT, wild type. C, co-immunoprecipitation of GRIN1 with different MOR third intracellular loop mutant. Panel a, GRIN1 was detected with anti-HA in the co-immunoprecipitates; panel b, the expression level of HA-GRIN1 in 1:20 of the total lysates used in IP experiments; panel c, the amount of MOR in the immunoprecipitates detected with anti-MOR C-tail antibodies after the blots were stripped. D, quantification and statistical analysis of the relative percentage of immunoprecipitated GRIN1 versus MOR or each MOR third intracellular loop mutants. The values represent the averages from \( n = 3 \) blots. E, gel overlay of HA-GRIN1 or premixing of HA-GRIN1 with GST-IL-3 or HA-GRIN1 with GST-IL-3 with N2A-MOR. Panel a, expression level of GST-IL-3 or GST-IL-3 fusion proteins as determined by Coomassie Blue staining; panel b, gel overlay of HA-GRIN1 or premixing of HA-GRIN1 with GST-IL-3 or HA-GRIN1 with GST-IL-3 on membranes containing N2A extract from cells expressing MOR separated by SDSPAGE; panel c, quantification and statistical analysis of the relative percentage of MOR and G protein. Quantification results expressed as percentage of GRIN1-overlaid control values. Ctl, control, which represents the intensities of the bands that HA-GRIN1 overlays N2A-MOR, represented as mean ± S.E. from \( n = 3 \), **, \( p \) denotes < 0.05.

Among these mutants, domain i3-5 was determined to be pivotal for MOR-G protein-interacting domain. Because GST-IL-3 pulled down GRIN1 in vitro translation product (Fig. 2A), it indicated that GST-tagged MOR-binding motif still possessed the characteristics for directly binding to GRIN1 in vitro translation product. To further confirm the deleted sequence \( ^{267}\text{GSKEK}^{271} \) is pivotal for directly binding to GRIN1, gel overlay experiments were performed by premixing the in vitro translation product of GRIN1 and GST-MORi33 or GST-MORi35 as described under “Experimental Procedures.” As shown in Fig. 2E, there was no difference between gel overlay with in vitro translation product of GRIN1 and with the premixed in vitro translation product of GRIN1 and GST-MORi33, which indicates GST-MORi33 could not compete with MOR for the binding of GRIN1. In contrast, premixing with GST-MORi35 blocked GRIN1 direct interaction with MOR, which demonstrated that GST-MORi35 could interact with GRIN1 in the premixing procedure and led to attenuation of the direct interaction between in vitro translation product of GRIN1 and MOR. These overlay studies further establish that the motif \( ^{267}\text{GSKEK}^{271} \) in MOR participates in the receptor interaction with GRIN1.

GRIN1 Serves as a Tether for MOR and \( \text{G}_i\alpha \)—It has been reported that GRIN1 can bind to activated \( \text{G}_i\alpha \), \( \text{G}_o\alpha \), and \( \text{G}_z\alpha \) mutants. Five amino acids in the third intracellular loop were systematically deleted to generate the third loop deletion mutants as described previously (31). The sequences that were deleted in the mutants are summarized in Fig. 2B. Interestingly, GRIN1 remained bound to the deletion mutants of i3-1, i3-2, i3-4, and i3-5 (Fig. 2, C and D). Deletion of the sequence, \( ^{267}\text{GSKEK}^{271} \) (i3-3), within the third intracellular loop almost abolished the GRIN1 and MOR interaction. Among these mutants, domain i3-5 was determined to be pivotal for MOR-G protein interaction (31). Although GRIN1 has been reported to interact with \( \text{G}_x\omega \), directly (23), these data also suggest that GRIN1 directly binds to the MOR domain distinct from the receptor G protein-interacting domain. Because GST-IL-3 pulled down GRIN1 in vitro translation product (Fig. 2A), it indicated that GST-tagged MOR-binding motif still possessed the characteristics for directly binding to GRIN1 in vitro translation product. To further confirm the deleted sequence \( ^{267}\text{GSKEK}^{271} \) is pivotal for directly binding to GRIN1, gel overlay experiments were performed by premixing the in vitro translation product of GRIN1 and GST-MORi33 or GST-MORi35 as described under “Experimental Procedures.” As shown in Fig. 2E, there was no difference between gel overlay with in vitro translation product of GRIN1 and with the premixed in vitro translation product of GRIN1 and GST-MORi33, which indicates GST-MORi33 could not compete with MOR for the binding of GRIN1. In contrast, premixing with GST-MORi35 blocked GRIN1 direct interaction with MOR, which demonstrated that GST-MORi35 could interact with GRIN1 in the premixing procedure and led to attenuation of the direct interaction between in vitro translation product of GRIN1 and MOR. These overlay studies further establish that the motif \( ^{267}\text{GSKEK}^{271} \) in MOR participates in the receptor interaction with GRIN1.
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FIGURE 3. Activation of MOR promoted the interaction of GRIN1 with G\textsubscript{i2}, but not G\textsubscript{i3}, or G\textsubscript{o3}. A, N2A cells stably expressing MOR were infected with PTX-insensitive adenoviruses, transfected with HA-GRIN1, and treated with PTX to inactivate endogenous G\textsubscript{i/o} proteins. After cells were treated with or without 100 nM etorphine for 10 min, G\textsubscript{i2} (upper two panels), G\textsubscript{i3} (middle two panels), or G\textsubscript{o3} (lower two panels) proteins were immunoprecipitated (IP) by their corresponding antibodies, and the immunoprecipitate was then blotted (IB) with anti-HA for HA-GRIN1 and with G\textsubscript{i2}, G\textsubscript{i3}, G\textsubscript{o3} antibodies. The blots are representative of similar blots from three independent experiments. Ctrl, control, where the cells were not infected with the adenoviruses but were transfected with HA-GRIN1; -, untreated control; +E, treated with 100 nM etorphine for 10 min. B, quantification analysis of immunoprecipitated GRIN1 with MOR with or without PTX pretreatment. The values represent the averages from n = 3 blots. *, p < 0.05 versus the basal.

FIGURE 4. GRIN1 acts as a scaffolding protein between MOR and G\textsubscript{i2}. A, co-IP of GRIN1-(1–717) mutant with G\textsubscript{i2}. B, co-IP of GRIN1-(1–717) mutant with MOR with or without PTX pretreatment. D, quantification analysis of immunoprecipitated GRIN1 with MOR with or without PTX pretreatment. **, p < 0.01. The values represent the averages from n = 3 blots. C, co-IP of GRIN1 with MOR with or without PTX pretreatment. *, p < 0.05 versus the basal. The values represent the averages from n = 3 blots. D, quantification analysis of immunoprecipitated GRIN1 with G\textsubscript{i2} with or without PTX pretreatment. The values represent the averages from n = 3 blots. IB, immunoblot.

(23). To illustrate which subtype of Ga is associated with GRIN1 when MOR is activated, adenovirus was used to over-express PTX-insensitive Ga subtypes in N2A cells (G\textsubscript{i2}, G\textsubscript{i3}, and G\textsubscript{o3}). PTX pretreatment was used to suppress the receptor-mediated activation of the endogenous G\textsubscript{i/o} proteins, and then each Ga was pulled down under the conditions with or without etorphine as described under “Experimental Procedures.” G\textsubscript{i2} was found to bind GRIN1 when MOR was activated by etorphine (Fig. 3). There was 54.5 ± 8.9% increase in the amount of HA-GRIN1 immunoprecipitated by the G\textsubscript{i2} antibodies in the presence of etorphine. The amount of HA-GRIN1 immunoprecipitated by the G\textsubscript{o3} antibodies remained the same in the absence or presence of etorphine.

The association of GRIN1 with the MOR signaling complex could still be due to the protein interaction with Ga subunits within the complex. To eliminate such possibility, a GRIN1-(1–717) mutant that lacked G protein-binding motif (24) was constructed. As expected, the full-length GRIN1 could be co-immunoprecipitated with G\textsubscript{i2} (Fig. 4A, 4th lane), whereas the truncated mutant GRIN1-(1–717) could not be co-immunoprecipitated with G\textsubscript{i2} (Fig. 4A, 3rd lane). On the other hand, the truncated GRIN1-(1–717) mutant could be co-immunoprecipitated by MOR only when both were co-transfected together (Fig. 4B). Additionally, when MOR was uncoupled from G\textsubscript{i2} after PTX pretreatment, the interaction between MOR and GRIN1 decreased significantly (Fig. 4, C and D). In contrast, PTX pretreatment did not perturb the G\textsubscript{i2}-GRIN1 interaction. The amount of GRIN1 co-immunoprecipitated with the antibodies against G\textsubscript{i2} enhanced after PTX pretreatment (Fig. 4, E and F). The distinct GRIN1 domains involved in associating with MOR and G\textsubscript{i2} suggest GRIN1 serves as a tether in the association between MOR and G\textsubscript{i2}, thereby regulating the receptor interaction with the Ga.
agonist activation was examined. N2A cells were transfected with the GFP vector or GFP-GRIN1, with or without etorphine treatment. Eighty percent transfection efficiency was achieved as determined by GFP fluorescence under the microscope before cell lysis. To separate lipid raft from other cellular fractions, N2A cells were extracted and subjected to sucrose density gradient centrifugation as described under “Experimental Procedures.” The gradient was fractionated, and the fractions were analyzed for MOR, GRIN1, G\(_{\alpha}\)2, and non-raft marker transferrin receptor. The results are presented in Fig. 5A. Fractions 4 and 5 were reported to be raft fractions, whereas other fractions are referred to as non-raft fractions (29). Western blot analyses demonstrated that the non-raft marker transferrin receptor was present in fractions except fractions 4 and 5, although the G\(_{\alpha}\)2 level was highest in fractions 4 and 5, presumably due to the lipid raft fractions. In agreement with our previous report (20), activation of MOR with etorphine resulted in a translocation of the receptor to non-raft domains, which was not observed with morphine pretreatment (Fig. 5, A, panels c and e, and B). Surprisingly, overexpression of GRIN1 did not alter the microdomain distribution of MOR significantly, which might due to the abundance of endogenous GRIN1 (Fig. 5A, panel b).

However, when GRIN1 was overexpressed, the amount of MOR redistributed to the non-raft fraction after etorphine treatment was reduced (Fig. 5, A, panel d, and C). Because the etorphine-induced translocation of MOR did not involve the parallel translocation of G\(_{\alpha}\)2 (20), the increase in the amount of MOR within the lipid raft after GRIN1 overexpression suggested that GRIN1 tethered the receptor with G\(_{\alpha}\)2 and enhanced MOR distribution in the lipid rafts.

To demonstrate further the GRIN1-MOR interaction can affect MOR lipid raft location, lipid raft fractionation of MOR i33 mutant was also performed with or without etorphine treatment. The co-immunoprecipitation experiments suggested rafts, which resulted in pathway-selective signaling of these opioid agonists (20). Most intriguingly, G\(_{\alpha}\)2 was shown to involve the anchoring of MOR within these microdomains (20). As this study suggested GRIN1 could tether the receptor and G\(_{\alpha}\)2 together, whether GRIN1 could affect MOR translocation upon
that the third intracellular loop sequence, $^{267}$GSKEK$^{271}$, was involved in the GRIN1-MOR interaction (Fig. 2). The deletion of this sequence from MOR (i33 mutant) resulted in the increase in the non-raft distribution of the receptor mutant as compared with the wide type MOR (Fig. 5D, panels a and b). Overexpression of GRIN1 did not alter the i33 mutant distribution in the lipid raft because there was minimal interaction between i33 mutant and GRIN1 (Fig. 5D, panel c). Moreover, under the condition of PTX pretreatment that could further uncouple $G_{i}\alpha_{2}$ from the i33 mutant, a dramatic decrease of the receptor in lipid rafts was observed (Fig. 5D, panel d), which was not observed with the wild type MOR (20). Similarly, the deletion of the sequence $^{276}$RRITR$^{280}$ from the third intracellular loop of MOR (i35 mutant) uncoupled the receptor from $G_{i}\alpha_{2}$ that was also responsible for MOR anchoring in the lipid raft (31). MOR i35 mutant was minimally detected in the lipid raft fractions of the gradient (Fig. 5D, panel e). Overexpression of GRIN1 could increase slightly the level of MOR i35 mutant within the lipid raft, but no significant difference was observed (Fig. 5D, panels e and f). Probably, the ability of the i35 mutant to interact with GRIN1 enables some of receptors to be retained in the lipid rafts. This was further demonstrated with the GRIN1 mutant, GRIN1-(1–717), that could interact with MOR but not with $G_{i}\alpha_{2}$ (Fig. 4). In contrast to wild type GRIN1, overexpression of the GRIN1-(1–717) mutant did not alter the receptor distribution pattern within the gradient (Fig. 5D, panel g). These data suggest that by serving as a tether between MOR and $G_{i}\alpha_{2}$, GRIN1 can modulate MOR distribution among the various microdomains. The results obtained with rat brain fractionation also demonstrated that lentivirus-mediated GRIN1 siRNA (supplemental Fig. S1a, 1) expression could redistribute endogenous MOR out of the lipid raft (Fig. 5E, panel c), similar to PTX pretreatment (Fig. 5E, panel b). After PTX pretreatment of the brain slices infected with GRIN1 siRNA lentivirus, further redistribution of endogenous MOR outside of lipid raft was observed (Fig. 5E, panel d), indicating that the effect of GRIN1 on MOR distribution in lipid raft was due to the tethering of receptor-G protein in rat brain membrane and not limited to the overexpression cell models.

**GRIN1 Potentiates the MOR-mediated Regulation of Src Kinase Activity**—Because GRIN1 can stabilize the receptor complex within the lipid rafts, it is reasonable to suggest that GRIN1-MOR interaction can potentiate the signaling of the receptor. GRIN1 is known to induce neurite outgrowth. Thus, it is probable that GRIN1 can modulate the MOR-mediated regulation of the signaling pathway such as the Src kinase known to affect neurite extension (32, 33). Confocal microscopy experiment showed that PP2, a specific inhibitor for Src kinase, could reduce neurite outgrowth that was promoted by GRIN1 overexpression (Fig. 6C). Therefore, N2A cells stably expressing MOR (MOR-N2A) were transfected with GFP vector, GFP-GRIN1 or GFP-GRIN1 siRNA, with or without etorphine treatment, and Src kinase activities were determined. Cell lysates were subjected to lipid raft fractionation, and only the fractions (fractions 4 and 5) containing lipid raft were used in determining the Src kinase activity. Using the antibodies specific for the phospho-Tyr$^{416}$ Src to detect the activated kinase and the antibodies specific for total Src to determine percentage of Src
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being activated, a 10-min etorphine treatment was shown to activate Src in the N2A-MOR cells transfected with GRIN1 only. Meanwhile, etorphine was shown to decrease Src activity when the N2A-MOR cells were transfected with GRIN1 siRNA (Fig. 6A). At the same time, disruption of lipid raft by pretreatment of MβCD blocked the etorphine-induced Src kinase activation in the N2A-MOR cells overexpressed with GRIN1, suggesting that MOR location within the lipid raft was critical for Src activation (Fig. 6B).

**E**torphine-induced Neurite Outgrowth Is Mediated by GRIN1—Because GRIN1 was reported to induce neurite outgrowth (23, 24), whether the tethering of the receptor and G<sub>i</sub>,α<sub>2</sub> by GRIN1 could affect agonist-induced neurite outgrowth was investigated. N2A-MOR cells were transfected with GFP vector, GFP-GRIN1 or GFP-GRIN1 siRNA (GRIN1 siRNA number 3, GRIN1 siRNA efficiency to knock down endogenous GRIN1 level was demonstrated in Fig. 7A). The absence of effect exhibited by these siRNA constructs on cell viability could be demonstrated by examining percentage of cell death using the terminal dUTP nick end-labeling assay as shown in supplemental Fig. S1b. The cells were then exposed to 100 nm etorphine for 10 min. Afterward, the medium was removed, and neurites were induced by replacing the medium with serum-free DMEM. Live cell images of the same cells were recorded up to 48 h as described under “Experimental Procedures.” There was significant difference in the ratio of neurite outgrowth between cells transfected with GRIN1 and cells without GRIN1 overexpression. Approximately 70% of cells overexpressing with GRIN1 possessed long neurites or growing dendrites after a 48-h induction of differentiation (Fig. 7, B and D). In contrast, almost none of GFP-GRIN1 siRNA-transfected cells and only around 8% of GFP vector-transfected cells possessed long neurites or growing dendrites at 48 h after induction of differentiation (Fig. 7, C and D). These data indicate that overexpression of GRIN1 and etorphine treatment together could induce neurite outgrowth significantly, contrary to etorphine treatment alone. On the other hand, 100 nm morphine could induce neurite outgrowth regardless of GRIN1 overexpression. There was little difference in the morphine effect on neurite outgrowth between cells transfected with vector and those overexpressed with GRIN1, although the GRIN1-siRNA could attenuate the morphine response (Fig. 7E). The above data imply the selectivity of MOR agonist in inducing neurite outgrowth when GRIN1 is overexpressed. When the cells were pretreated with PTX or naloxone, the ability of etorphine to induce neurite outgrowth was blocked regardless of whether GRIN1 was overexpressed or not (supplemental Fig. S2), suggesting GRIN1-promoted neurite outgrowth was dependent on the association of activated MOR and G protein. Similarly, knocking down endogenous GRIN1 in N2A-MOR cells with GRIN1 siRNA blunted both etorphine- and morphine-induced long neurites or growing dendrites 48 h after serum removal (Fig. 7, D and E). The siRNA studies support the important role of GRIN1 in MOR-induced neurite outgrowth.
To further confirm the interaction between MOR and GRIN1 is crucial for MOR activation-induced neurite outgrowth, MORi33 mutant and MORi35 mutant were used for neurite outgrowth studies. In accordance with a previous report, MORi33 mutant showed decreased interaction with endogenous G\(_{\alpha_2}\) (Fig. 8A). Meanwhile, MORi33 mutant showed minimal interaction with endogenous GRIN1 (Fig. 2 and Fig. 8A). N2A cells differentiated in the absence of serum, and ~80% of cells possessed long neurites or growing dendrites 48 h later after removal of etorphine treatment when overexpressed with GRIN1. In contrast, although the deletion of 276RRITR280 resulted in the complete blockade of agonist-mediated inhibition of adenylyl cyclase activity (20, 31), around 7% of N2A cells transfected with the MORi35 mutant exhibited long neurite and growing dendrite 48 h after serum removal and etorphine treatment (Fig. 8B). On the other hand, even with no measurable difference in the ability of the agonist to regulate adenylyl cyclase activity in cells expressing the 267GSKEK271 deletion mutant (31), only 20% of MORi33 mutant-transfected cells possessed long neurites or growing dendrites at 48 h after serum removal and etorphine pretreatment (Fig. 8B). These data suggest the importance of the interaction between MOR and GRIN1 in the opioid agonist-induced neurite outgrowth.

**DISCUSSION**

In the management of chronic pain, the biggest clinical issue is that repeated opiate exposure can lead to the development of tolerance, sensitization, and physical dependence, which involves the change of neuron synaptic plasticity and neurogenesis, the brain reward circuit (34, 35). These phenomena can be considered adaptive processes similar to other experience-dependent changes in the brain, such as learning and neural development (34–38). Formation and extension of axons and dendrites, so-called neurite outgrowth, is a crucial event in neuronal differentiation and maturation during development of the nervous system (39, 40). Substantial evidence also showed that alteration of opioid receptor activity disturbed the neurobehavioral development of newborns (41), indicating the involvement of the opiate system in neuronal differentiation. Endogenous activity of opioid receptor could affect neurite outgrowth (42), which was demonstrated to be critical in other addiction progresses (43, 44). Meanwhile, it was also reported that etorphine and another \(\mu\)-selective ligand, [\(\text{d-Ala}^2,\text{N-MePhe}^4,\text{Gly}^5\text{-ol}\)]enkephalin, suppressed neurite outgrowth on cultured neurons (45, 46), but morphine at low concentrations enhanced neurite outgrowth of spinal and cortical neurons via a naloxone-independent mechanism (47). All these data suggested that activation of opioid receptor by different ligands could selectively affect neurite outgrowth.

Through mass spectrometry analysis and GST pulldown experiment, GRIN1 was found to be directly associated with MOR at its third intracellular loop (Figs. 1 and 2). Co-immunoprecipitation data demonstrated that MOR, G\(_{\alpha_2}\), and GRIN1 associated with each other and formed a distinctive signaling complex (Fig. 4). This particular protein-protein association suggested that GRIN1 had other functions when MOR was activated besides acting as a downstream signaling component with G\(_{\alpha}\). Within the membrane-targeting region of GRIN1 (772–827), potential palmitoylation sites were found at Cys\(_{818}\) and Cys\(_{819}\) that could facilitate GRIN1 translocation into lipid rafts (24), as observed in our current studies (Fig. 5). MOR signaling complex has been reported to be associated with G\(_{\alpha_2}\) in lipid rafts (48–50). Many signaling proteins are selectively distributed inside or outside lipid rafts (1), which are rich in cho-
lesterol and sphingolipids at the plasma membrane. One of the most important characteristics of lipid rafts is that they can retain or expel proteins to variable extents. Proteins possessing raft affinity include glycosylphosphatidylinositol-anchored proteins, doubly acylated proteins, such as Src kinase family or the β2-subunits of heterotrimeric G proteins, cholesterol-linked and palmitoylated proteins such as Hedgehog, and transmembrane proteins, particularly palmitoylated ones (32, 52–55).

Being a member of the GPCR superfamily, MOR was also demonstrated to be located in lipid rafts (29). However, when activated by etorphine, MOR translocated out of lipid raft due to the fact that agonist-receptor complex switched its interaction with G_{i/o} to β-arrestin (20). The translocation of the receptor from lipid rafts could be one of the determinants in the observed agonist-dependent pathway-selective signaling. By tethering the receptor with G_{i/o}, the GRIN1-stabilized MOR complex within the lipid rafts thereby altered the receptor signaling of those agonists that could translocate MOR out of lipid raft (i.e. etorphine). This was demonstrated by our current studies in which Src kinase activation and subsequent neurite outgrowth induced by etorphine were dramatically affected by GRIN1 expression. As expected, the activities of agonist such as morphine, which does not induce MOR translocation from lipid rafts, are not affected by the GRIN1 expression.

It was reported that the stability of lipid raft was very important in PC12 neurite outgrowth (56), which was consistent with our data that the stability of lipid raft also was crucial for MOR activation-induced neurite outgrowth (Fig. 9). Disrupting the structure of the lipid raft by MβCD could suppress neurite outgrowth, and recovery of lipid raft by adding cholesterol continued neurite outgrowth (Fig. 9). Thus, it is reasonable to suggest that MOR activation-induced neurite outgrowth take place in lipid raft. Overexpression of GRIN1 minimizes the removal of MOR out of lipid raft in the presence of etorphine and subsequently amplifies the signals involved in neurite outgrowth (Figs. 5 and 6). Once MOR was activated in lipid raft, c-Src kinase activity increased (Fig. 6), which could lead to a series of changes in downstream signal to induce neurite outgrowth (5, 57–61). The ability of PP2, a general Src kinase inhibitor and not PP3 to inhibit MOR-mediated neurite outgrowth supported the Src involvement in such a process (data not shown). Scanning GRIN1-binding motif, we found the possible proline-rich region at its C terminus, which suggested that GRIN1 might bind to the Src kinase and other proteins through the Src homology 2 region and Src homology 3 region (51, 62–64). Detailed mapping of GRIN1 for motifs involved in modulating neurite outgrowth needs to be demonstrated in the future.

Our current data depict a picture in which GRIN1 can increase the interaction between MOR and G_{i/o} in lipid rafts and further recruit downstream signaling molecules to amplify the receptor signals, including those involved in neurite outgrowth. The alteration in GRIN1 expression level could lead to variations in the formation of functional MOR signaling complex in lipid rafts, as demonstrated with current neurite outgrowth and Src kinase measurements and also with other receptor activities such as inhibition of adenylyl cyclase activity (data not shown). Thus, the selective influence of GRIN1 on the eventual microdomain location of the receptor signaling com-

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**FIGURE 9.** GRIN1-promoted etorphine-induced neurite outgrowth occurs within lipid raft. A, live cell images of neurite outgrowth in GRIN1-transfected cells when treated with saline as control, MβCD, cholesterol, or MβCD plus cholesterol 24 h after 100 nM etorphine treatment for 10 min. B, statistical analysis of cell number possessing neurite outgrowth in GRIN1-transfected cells calculated from >20 different vision fields of confocal images when treated with saline, MβCD, cholesterol, or MβCD plus cholesterol 24 h after etorphine treatment; **, p denotes <0.01.
plex observed with different opiate drugs such as etorphine and not with morphine may trigger differential cellular responses that are involved in the establishment of brain reward circuit and drug dependence. Thus, it is probable that depending on the GRIN1 expression level, the cellular adaptation during chronic drug treatment, will be agonist-dependent.

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