Antidepression action of BDNF requires and is mimicked by Gαi1/3 expression in the hippocampus

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Stress-related alterations in brain-derived neurotrophic factor (BDNF) expression, a neurotrophin that plays a key role in synaptic plasticity, are believed to contribute to the pathophysiology of depression. Here, we show that in a chronic mild stress (CMS) model of depression the Gαi1 and Gαi3 subunits of heterotrimeric G-proteins are downregulated in the hippocampus, a key limbic structure associated with major depressive disorder. We provide evidence that Gαi1 and Gαi3 (Gαi1/3) are required for the activation of TrkB downstream signaling pathways. In mouse embryonic fibroblasts (MEFs) and CNS neurons, Gαi1/3 knockdown inhibited BDNF-induced tropomyosin-related kinase B (TrkB) endocytosis, adaptor protein activation, and Akt–mTORC1 and Erk MAPK signaling. Functional studies show that Gαi1 and Gαi3 knockdown decreases the number of dendrites and dendritic spines in hippocampal neurons. In vivo, hippocampal Gαi1/3 knockdown after bilateral microinjection of lentiviral constructs containing Gαi1 and Gαi3 shRNA elicited depressive behaviors. Critically, exogenous expression of Gαi3 in the hippocampus reversed depressive behaviors in CMS mice. Similar results were observed in Gαi1/Gαi3 double-knockout mice, which exhibited severe depressive behaviors. These results demonstrate that heterotrimeric Gαi1 and Gαi3 proteins are essential for TrkB signaling and that disruption of Gαi1 or Gαi3 function could contribute to depressive behaviors.

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Depression has also been reported to disrupt TrkB receptor downstream signaling (9). Binding of BDNF to TrkB results in its dimerization and tyrosine autophosphorylation and subsequent recruitment of adaptor proteins (10–14) that link TrkB to the activation of MAPK (11, 13, 15) and the PI3K–Akt–mammalian target of rapamycin (mTOR) pathways (10, 14, 15). Increasing evidence shows that the inhibitory alpha subunits of heterotrimeric guanine nucleotide-binding proteins (G proteins) play a key role in growth factor signaling (16, 17). G proteins were originally identified by their ability to inhibit adenyl cyclase and are members of four subclasses, Gs, Gi/o, Gq, and G12/13; the Gi/o includes Gαi (3), Go (2), and transducins (18). The Gαi subclass of heterotrimeric G proteins includes the highly similar Gαi1, Gαi2, and Gαi3 proteins encoded by the genes GNAI1, GNAI2, and GNAI3, respectively, with more than 94% sequence identity between Gαi1 and Gαi3 (19). Our studies have demonstrated that Gαi1 and Gαi3 (but not Gαi2) are required for EGF- and keratinocyte growth factor (KGF)-induced Akt–mTOR complex 1 (mTORC1) activation (16, 20). In the current study, we show that Gαi1 and Gαi3 (Gαi1/3) are required for BDNF-induced TrkB receptor signaling and the regulation of depressive behaviors.

**Results**

Double Knockout of Gαi1 and Gαi3 Inhibits BDNF-Induced Akt–mTORC1 and Erk Activation in Mouse Embryonic Fibroblasts. Mouse embryonic fibroblasts (MEFs) have been reported to express TrkB receptors and provide a valuable cell system to examine the underlying mechanisms of BDNF signaling (21). To begin to address the role of G proteins in TrkB signaling, we utilized a Gαi1 and Gαi3 double-knockout (DKO) MEF cell line (16, 20). Depletion of Gαi1 and Gαi3 in the DKO MEFs was confirmed by Western blot analysis (Fig. 1A), whereas Gαi2 expression was intact. TrkB

**Significance**

Heterotrimeric Gαi proteins are known to transduce G protein-coupled receptor signals. We have identified a role for Gαi proteins in mediating brain-derived neurotrophic factor (BDNF)–tropomyosin-related kinase B (TrkB) signaling. BDNF dysfunction contributes to the pathophysiology of depression. In a stress model of depression Gαi1 and Gαi3 proteins are downregulated in the hippocampus, a limbic structure associated with major depressive disorder. We show that Gαi1/Gαi3 proteins are required for TrkB downstream signaling, and knockout mice exhibited severe depressive behaviors with decreased dendritic morphology. Established stress-induced depressive behavior is corrected by intrahippocampal expression of Gαi3. These results demonstrate that heterotrimeric Gαi1 and Gαi3 proteins are essential for TrkB signaling and that disruption of Gαi1 or Gαi3 function could contribute to depressive behaviors.

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expression and BDNF-induced TrkB phosphorylation were comparable in WT and DKO MEFs. Significantly, BDNF-induced phosphorylation of Akt (at Ser-473 and Thr-308), S6 (Ser-235/240), and Erk1/2 (Tyr202/Thr-204) was significantly reduced in Gai1/3-DKO MEFs (P < 0.001 vs. WT MEFs) (Fig. 1 B and C), suggesting that Gai1 and Gai3 are required for Akt–mTORC1 and Erk–MAPK activation. In agreement with previous studies (16, 20), the expression of total Akt, S6, and Erk1/2 was equal in WT and DKO MEFs (Fig. 1 B and C). Furthermore, 7,8-Dihydroxyflavone (7,8-DHF), a selective TrkB agonist (22, 23), induced phosphorylation of Akt, S6, and Erk1/2 in WT MEFs but not in the DKO MEFs (Fig. 1D). In confirmation of the results in the MEF cell line, we tested isolated primary cultures of Gai1/3-DKO MEFs (16) and found that BDNF-induced Akt–mTORC1 and Erk–MAPK activation was similarly abolished (Fig. 1E), whereas PDGF-BB (25 ng/mL)-induced phosphorylation of Akt and Erk1/2 was unaffected (Fig. 1F). Immunofluorescence imaging further confirmed that BDNF-induced but not PDGF-BB-induced phosphorylation of Akt and Erk1/2 was blocked in Gai1/3-DKO MEFs (Fig. 1 G and H).

**Gai1 and Gai3 Have Redundant Roles in BDNF-Induced Akt–mTORC1 and Erk Activation in MEFs.** To investigate whether depleting Gai1 or Gai3 individually would disrupt BDNF signaling, Gai1 and Gai3 knockout (SKO) MEFs were utilized (16, 20). BDNF-induced phosphorylation of Akt (Ser-473 and Thr-308), S6 (Ser-235/236), and Erk1/2 (Tyr202/Thr-204) was only partially decreased in Gai1- or Gai3-SKO MEFs (P < 0.01, vs. WT MEFs) (Fig. 2A) and was intact in Gai2-SKO MEFs (P > 0.05, vs. WT MEFs) (Fig. 2B), whereas Gai1 and Gai3 DKO resulted in complete inhibition of BDNF signaling (Fig. 2C). These results suggest that expression of either Gai1 or Gai3 can take part in TrkB signaling. To demonstrate that expression of either Gai1 or Gai3 is sufficient for BDNF signaling, we tested whether exogenous expression of Gai1 or Gai3 would rescue signaling in DKO MEFs (16, 20). As shown in Fig. 2C, the exogenous expression of Gai1 or Gai3 in DKO MEFs was sufficient to restore BDNF-induced Akt and Erk activation. To exclude possible off-target effects from the genetically modified MEFs, we employed an siRNA strategy to knock down the Gai1 or Gai3 protein in WT MEFs. As shown in Fig. 2D, single knockdown of Gai1 or Gai3 by targeted siRNA in the WT MEFs resulted in a weak but significant inhibition of BDNF-induced phosphorylation of Akt, S6, and Erk1/2. In contrast, BDNF stimulation of WT MEFs depleted of both Gai1 and Gai3 using the CRISPR/Cas9 system showed complete inhibition of Akt, S6, and Erk1/2 phosphorylation (P < 0.001 vs. control cells) (Fig. 2D).
Gαi1/3 Are Required for TrkB Adaptor Complex Formation. TrkB autophosphorylation provides docking sites for adaptor proteins and the recruitment of downstream signaling molecules (24). We next examined whether depletion of Gαi1/3 would interfere with adaptor protein complex formation. BDNF-activated TrkB recruits the adaptor protein Shc, which in turn recruits Grb2. Grb2 then associates with Grb2-associated binder 1 (Gab1) to activate PI3K and downstream Akt–mTORC1 signaling (15). SHP2 recruitment is required for downstream MEK–Erk activation and is essential for neurite outgrowth and branching (25). We found that loss of Gαi1/3 in DKO MEFs abolished BDNF-induced TrkB recruitment and phosphorylation of adaptor proteins, including Shc and SHP2 (Fig. 3A) and Gab1 (Fig. 3B). Significantly, Gab1 was also part of the complex with Grb2, Gab1, and SHP2 (Fig. 3B). In agreement with the reduced recruitment of Gab1, BDNF-induced phosphorylation of Gab1, whose activation is required for downstream PI3K–Akt and Erk–MAPK activation, was inhibited in Gαi1/3-DKO MEFs (Fig. 3C) and in WT MEFs transfected with shRNA (Fig. 3D). Gαi1 or Gαi3 SKO inhibited Gab1 phosphorylation by BDNF, whereas Gab2 KO failed to affect Gab1 activation (Fig. 3C and E). Reexpression of Gαi1 or Gαi3 in the DKO MEFs restored Gab1 phosphorylation (Fig. 3F). In agreement with previous studies (25, 26), BDNF-induced phosphorylation of Akt, S6, and Erk1/2 was severely inhibited in Gab1-KO MEFs (Fig. 3G), in confirmation of the important role played by Gab1 in TrkB signaling.

Gαi1/3 Are Required for BDNF-Induced TrkB Signaling in Cerebellar Granule Neurons. In primary cerebellar granule neurons, BDNF signaling is required for migration (27) and survival (28). To examine the effect of neuronal knockdown of Gαi1/3 expression on BDNF signaling, primary murine cerebellar granule neurons were infected with lentiviral constructs expressing shRNA for Gαi1 and Gαi3 or a scrambled shRNA. Infection of neurons with lentiviral shRNA resulted in a 95% reduction of Gαi3 expression and an 80% reduction of Gαi1 expression (Fig. 4A). BDNF-induced phosphorylation of Akt, S6, and MEK1/2/Erk1/2 was greatly diminished in cerebellar granule neurons with Gαi1/3 shRNA (P < 0.001 vs. neurons with scrambled control shRNA) (Fig. 4A, Right).

To determine if Gαi1/3 proteins can be recruited to TrkB, we performed a coimmunoprecipitation assay. Results demonstrate that Gαi3 (Fig. 4B) and Gαi1 (Fig. 4D) communoprecipitated with TrkB in response to BDNF in cerebellar granule neurons. BDNF-induced TrkB immunoprecipitation with SHP2 and Gab1 was blocked by Gαi1/3 shRNA (Fig. 4B). Consequently, phosphorylation of SHP2 and Gab1 in response to BDNF was largely inhibited (Fig. 4C). We also found that TrkB communoprecipitated with APPL1 (a pleckstrin homology domain, phosphotyrosine-binding domain, and leucine zipper motif 1) (Fig. 4D), which is known to associate with TrkB and is required for signal transduction (29). These results in primary neurons, together with the MEF data, support a role for Gαi proteins in BDNF-induced TrkB adaptor complex formation and downstream signal transduction.
BDNF-induced TrkB phosphorylation were equivalent in control GFP neurons and Gαi1/3 shRNA neurons (Fig. 5A, Right). Significantly, TrkB endocytosis by BDNF was blocked by Gαi1/3 shRNA (Fig. 5A, Right), whereas EGF receptor (EGFR) surface levels were unaffected.

BDNF-TrkB activation is critical for hippocampal neuron development (30, 31), including dendritic outgrowth and spine formation (32). To examine whether knockdown of Gαi1/3 expression alters the morphology of hippocampal neurons, we infected hippocampal neurons at day 5 in vitro (DIV 5) with lentiviral Gαi1 or shGαi1/3 expression. Input control shows the expression of listed proteins in the total cell lysates. (C) WT MEFs, Gab1- or Gαi3-KO MEFs, and Gab1/3-DKO MEFs (C), WT MEFs or Gab1/3 MEFs transfected with shRNA or scr-shRNA (D), and WT and Gab1/2-KO MEFs were treated with BDNF and p-Gab1 and were tested by Western blot analysis. β-Actin served as the loading control. (F) DKO MEFs were transiently transfected with vector encoding EE-Gαi1-cDNA, EE-Gαi3-cDNA, or empty vector, and BDNF (25 ng/mL)-induced Gab1 phosphorylation was analyzed by Western blot. (G) WT or Gab1-KO MEFs were treated with BDNF (25 ng/mL) for the indicated time and were analyzed by Western blotting for the listed proteins. *P < 0.001 vs. WT MEFs.

Gαi1/3 Knockdown in the Hippocampus Produces Depressive Behavior. Chronic mild stress (CMS), a model of depression (33–35), decreases the expression of BDNF in the hippocampus, and this decreased expression is believed to contribute to dendrite and spine deficits in depression (36). We investigated whether CMS exposure could affect Gαi1/3 expression in the brain. CMS exposure for 14–56 d led to a significant decrease of Gαi1 and Gαi3 expression in the hippocampus (Fig. 6A) but not in the cortex (Fig. 6B), suggesting that Gαi1 and Gαi3 down-regulation could be associated with depressive behaviors. To test this hypothesis, we examined the consequences of bilateral hippocampal injection of lentiviral Gαi1 and Gαi3 shRNA on depression- and anxiety-like behaviors. Results show that Gαi1 shRNA or Gαi3 shRNA injection into the
hippocampus elicited depressive behaviors in which mice exhibited significantly longer immobility times in both a forced swim test (FST) \((P < 0.001\) vs. GFP control) (Fig. 6D) and a tail-suspension test (TST) \((P < 0.001\) vs. GFP control) (Fig. 6E). In a sugar-preference test, in which anhedonic behavior is inferred by a reduced preference for sugar water, mice showed decreased preference after hippocampal injection of G\(\text{a}1\) or G\(\text{a}3\) shRNA \((P < 0.001\) vs. GFP control) (Fig. 6F). Furthermore, injection of G\(\text{a}1\) shRNA and G\(\text{a}3\) shRNA lentivirus into the hippocampus intensified depressive behaviors (Fig. 6D–F) compared with the injection of G\(\text{a}1\) shRNA or G\(\text{a}3\) shRNA alone (Fig. 6D–F).

Based on the above results, we hypothesized that exogenous G\(\text{a}i\) overexpression in the hippocampus would induce anti-depressive behavior. To test this, we performed bilateral stereotactic delivery of an adeno-virus–G\(\text{a}i\)-3 construct (Ad-G\(\text{a}i\)-3) into the hippocampi of treatment-naive adult animals and assessed FST and TST behaviors. Immunoblotting of hippocampal tissue confirmed exogenous G\(\text{a}i\)-3 delivery into the hippocampus. Exogenous G\(\text{a}i\)-3 injection exhibited a significantly reduced duration of immobility in both the FST \((P < 0.001\) vs. Ad-GFP control) (Fig. 6D) and the TST \((P < 0.001\) vs. Ad-GFP control) (Fig. 6D).

If hippocampal G\(\text{a}i\) down-regulation is the cause of CMS-induced depression in mice, rather than a secondary effect, then restoring G\(\text{a}i\) expression should prevent depressive behavior. To test this hypothesis, we exogenously expressed G\(\text{a}i3\) by bilateral Ad-G\(\text{a}i\)-3 delivery into the hippocampus. Exogenous expression G\(\text{a}i3\) reversed CMS-induced depressive behavior (Fig. 6K–M). These results support the hypothesis that the level of G\(\text{a}i3\) expression in the hippocampus regulates TrkB signaling to influence depressive behavior.

Severe Depressive-Like Behaviors in G\(\text{a}i1/3\)-DKO Mice. To confirm the role of G\(\text{a}i1/3\) in depression, we generated G\(\text{a}i1/3\)-DKO mice. Western blotting of hippocampal lysates confirmed that G\(\text{a}i1\) and G\(\text{a}i3\) were depleted in the DKO mice, whereas G\(\text{a}i2\) was unaffected (Fig. 7A). TrkB expression and phosphorylation were equivalent in WT and DKO mice (Fig. 7A). However, p-Akt and p-Erk1/2 were decreased in the DKO mice (Fig. 7A). In behavioral testing, the DKO mice displayed depressive behaviors, with significantly increased immobility times in both the FST (Fig. 7B) and the TST (Fig. 7C) \((P < 0.001\) vs. WT mice). The DKO mice also exhibited a reduction in their preference for sugar \((P < 0.001\) vs. WT mice).

To examine the morphology of hippocampal CA1 neurons, G\(\text{a}i\)-expressing control lentivirus was microinjected into the hippocampus of DKO mice, and anti-GFP immunostaining was performed on sections 7 d postinjection. Analysis of confocal images of GFP antibody-stained neurons revealed that DKO mice displayed a reduced number of hippocampal pyramidal neurons \((P < 0.001\) vs. WT mice) (Fig. 7E and F). We also observed a severe reduction in dendrite complexity, as indicated by a reduction in the number of secondary dendrites extending from the perineuronal primary dendrite \((P < 0.001\) vs. WT mice, 40 neurons per group) (Fig. 7G). Furthermore, the number of dendritic spines was significantly reduced in the DKO mice \((P < 0.001\) vs. WT mice, 40 neurons per group) (Fig. 7H and I), whereas mean spine width was not significantly different between WT and DKO mice \((P > 0.05\) vs. WT mice) (Fig. 7J). These results show that knockout of G\(\text{a}i1/G\(\text{a}i3\) disrupts BDNF signaling, hypothesized to be required for the maintenance of pyramidal neuron morphology and the prevention of depressive behaviors.

Discussion
G\(\text{a}i\) proteins are well known to transduce signals between G protein-coupled receptors and their effectors (18). The major finding of this study is the unconventional role of G\(\text{a}i1\) and G\(\text{a}i3\) proteins in mediating downstream BDNF–TrkB signal transduction. The results suggest that G\(\text{a}i1\) and G\(\text{a}i3\) have redundant roles in TrkB signaling, as depletion of both G\(\text{a}i1\) and G\(\text{a}i3\) was required to completely block BDNF signaling (Figs. 2A and 5E), although disruption of G\(\text{a}i3\) had a greater impact. In hippocampal neurons, G\(\text{a}i3\) knockout more significantly reduced BDNF signaling (Fig. 5E) and dendrite morphology (Fig. 5 G and I). Furthermore, CMS decreased G\(\text{a}i3\) expression more than G\(\text{a}i1\) expression (Fig. 6A), and G\(\text{a}i3\) knockout produced larger depressive effects (Fig. 6 D–F).

In cerebellar and hippocampal neurons, in response to BDNF, G\(\text{a}i1\) or G\(\text{a}i3\) are recruited to TrkB (Fig. 4D) and are required for TrkB adaptor protein association (Fig. 4B) and downstream signaling (Figs. 4A and 5A). G\(\text{a}i1\) or G\(\text{a}i3\) proteins do not appear
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Fig. 5. Gαi1/3 are required for BDNF signaling, endocytosis, and dendrite outgrowth in the hippocampal neurons. (A) Lentiviral-mediated shRNA knockdown of Gαi1/3 disrupts downstream TrkB signaling and TrkB endocytosis in primary hippocampal neurons. Hippocampal neurons were infected with lentiviral Gαi1 shRNA plus lentiviral Gαi3 shRNA (shGαi1/3) or a lentiviral sc-rNA for 24 h. Neurons were stimulated with BDNF (25 ng/mL) and analyzed for the listed proteins in total and cell-surface lysates. (B and C) Lentiviral-mediated shRNA Gαi1/3 knockdown decreases dendritic morphology and TrkB endocytosis, and dendrite outgrowth in the hippocampal neurons. On day 5 after infection, neurons were stained for GFP expression (encoded by the lentiviral shRNA vectors), and the morphology of hippocampal neurons was quantified. (B) Representative images of anti-GFP staining are presented. Arrowheads indicate spines. (Scale bars: 25 μm; magnification: inset, 6.8×.) (C) Dendrite length, branching, soma area, and the number of dendritic spines were quantified. For spine analysis, 30-μm-long dendritic segments (50-80 μm from soma) were selected, and spines from 40 neurons were counted. (D) A representative image of GFP expression 7 d after hippocampal injection of a lentiviral GFP control virus shows a high efficiency of infection. (E) Intrahippocampal lentiviral shRNA-mediated knockdown of Gαi1/3 decreases Akt and Erk1/2 activity, as analyzed by Western blotting with phosphospecific antibodies. Hippocampi were isolated and tested by Western blotting of listed proteins (n = 5). (F–H) Intrahippocampal lentiviral shRNA-mediated knockdown of Gαi1/3 decreases the formation of secondary dendrites and spines. (F and H) Representative images showing hippocampal neuron morphology are presented for control and shRNA Gαi1/3-infected neurons stained for GFP. Arrowheads in H indicate spines. (Scale bars: 25 μm in F and 5 μm in H.) (G) The number of neurons and dendrites per neuron were counted. (I) Spines were counted from 30-μm-long dendritic segments (50-80 μm from soma) of 40 randomly selected neurons. *P < 0.001 vs. GFP. **P < 0.001.

to influence TrkB autophosphorylation but act downstream of TrkB and upstream of Gab1, resulting in phosphorylation of Gab1, SHP2, and She. Knockdown of Gαi1/Gαi3 inhibits BDNF-induced Gab1 activation and downstream PI3K–Akt–mTOR and Erk–MAPK activation. Our group reported a similar function for Gαi1 and Gαi3 in mediating EGFR signal transduction (16). Importantly, knockdown of Gαi1/3 blocks TrkB endocytosis in response to BDNF (Fig. S4), providing insight into the mechanism by which Gαi1/3 exhibits such a dominant effect on TrkB signaling. TrkB retrograde signaling is critical in the regulation of BDNF-induced survival and differentiation of neurons in the CNS (37–40). Significantly, we found that knockdown of Gαi1/3 disrupts the association of TrkB with the membrane adaptor protein APPL1 (Fig. 4D). Previous studies have reported that APPL1 binds to membrane receptors, including TrkA and TrkB, in endosomal fractions (41). APPL1 endosomes may serve as platforms for the assembly of TrkB adaptor signaling complexes regulating the Akt–mTORC1 and Erk–MAPK pathways (29, 41, 42). It is possible Gαi1/3 proteins play a role in TrkB sorting into distinct endosomal compartments essential for the activation of specific signaling cascades that modulate synapse formation and survival and the differentiation of neurons in the CNS.

BDNF is a key mediator of activity-dependent dendrite formation (43, 44) via PI3K and MAPK signaling (45). In support of the role for Gαi1/3 in BDNF signaling, Gαi1/3 knockdown in hippocampal neurons in vitro (Fig. 5C) and in vivo (Fig. 5 F and...
G) disrupted dendritic branching. BDNF is also required for the maintenance of mature synaptic spines, and blocking BDNF reduces spine density (46). In accordance, Gαi1/3 knockdown decreased the number of hippocampal dendritic spines in vitro (Fig. 5C) and in vivo (Fig. 5I and J). Significantly, in vivo knockdown of Gαi1/3 resulted in fairly rapid (within 7 d) morphological changes. Consistent with this observation, BDNF treatment has been shown to rapidly (within 24 h) regulate dendrite morphology (45) and spine density in hippocampal neurons (47–49). Depression is associated with a decrease in hippocampal spine density (50), and rapid BDNF-induced changes in dendrite complexity and spine density have been linked to the mechanism of fast-acting antidepressants (51, 52).

Given the role of BDNF signaling in the neuropathology of anxiety and depression, we examined if Gαi expression was associated with depression. Using the CMS model, we found a significant down-regulation of Gαi1 and Gαi3 expression in the hippocampus (Fig. 6A). Furthermore, mice subjected to lentiviral shRNA Gαi1/3 knockdown in the hippocampus and Gαi1/3-DKO mice presented with severe depressive-like behaviors, as indicated by prolonged immobility times in the FST and TST and decreased sugar preference (Fig. 6 D–F). Conversely, virally mediated expression of exogenous Gαi3 in the hippocampus prevented the antidepressive-like behaviors induced by CMS (Fig. 6K–M). Last, using the CRISPR-Cas9 method, we generated Gαi1/3-DKO mice, which exhibited depressive-like behavior identical to that demonstrated by mice with virally mediated hippocampal Gαi1/3 knockdown (Fig. 7).

BDNF deficiency is implicated in depression, and antidepressant drugs act to restore BDNF levels (53). The administration of the selective serotonin-reuptake inhibitor fluoxetine prevents stress-induced atrophy of dendrites and spines (54), and reduction of TrkB receptor signaling attenuates the antidepressant structural and behavioral actions of fluoxetine (55). Interestingly, in a postnatal mouse model, fluoxetine induced depression-like behaviors (56) that were associated with a decline in hippocampal Gαi1 (Gnai1) gene expression, as well as mTOR, protein kinase C gamma, and hyperpolarization-activated cyclic nucleotide-gated channel 1, via HDAC4-mediated transcriptional repression (56). Fluoxetine rescue of behavior was accompanied by normalization of Gαi1, Hdac4, and mTOR expression (56).

In summary, our results suggest a model in which Gαi1/3 proteins are required for BDNF-induced TrkB signaling. In response to BDNF, Gαi1 or Gαi3 associates with TrkB, resulting in TrkB internalization and endosomal trafficking, which is required for adaptor protein association and downstream signaling. In behavioral models of depression, the levels of Gαi1 and

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**Fig. 6.** Gαi1/3 knockdown in the hippocampus produces depressive behaviors. (A–C) CMS exposure decreases Gαi1 and Gαi3 expression in the hippocampus. Western blot analysis of Gαi1 and Gαi3 expression in the hippocampus (A) and cortex (B) of mice exposed to the CMS model for 14–56 d compared with control hippocampus (C). (D–F) Gαi1 shRNA or Gαi3 shRNA injection into the hippocampus elicited depressive behaviors. Immobility times in the FST (D) and TST (E) and sucrose water preference (F) were examined on day 7 after intrahippocampal injection of GFP or lentiviral Gαi1 shRNA and/or Gαi3 shRNA. (G–J Exogenous Gαi3 expression in the hippocampus induces anti-depressive behavior. On day 7 after intrahippocampal injection of Ad-GFP or Ad-Gαi3, immobility times in the FST (I) and TST (J) were tested, and then hippocampi were isolated and analyzed by Western blotting of the listed proteins (G and H). (K–M) Exogenous expression of Gαi3 reversed CMS-induced depressive behavior. The immobility times in the FST (K) and TST (L) and sucrose water preference (M) were tested in control and CMS mice with or without intrahippocampal injection of Ad-GFP or Ad-Gαi3. *P < 0.001 vs. GFP (D–F, I, and J). †P < 0.001 vs. control mice (K–M).
Gαi1/3 DKO mice. (A) Depletion of Gαi1 and Gαi3 in the DKO mice disrupts signaling. The expression of the listed proteins in the CA1 hippocampus of WT and Gαi1/3-DKO mice was examined by Western blot analysis. (B-D) DKO mice display depressive behaviors. For both WT and Gαi1/3-DKO mice, the FST (B), TST (C), and sucrose water preference test (D) were performed. (E-J) Analysis of DKO hippocampal CA1 neuronal morphology. (E and H) Representative images of CA1 pyramidal hippocampal neuronal morphology. Arrowheads indicate spines. (Scale bars: 25 μm in E and 5 μm in H.) (F) The number of neurons in randomly selected 200 × 200 μm fields was counted. (G and J) The number of secondary dendrites (G) and spines (J) in 40 random neurons were counted. Spines were analyzed from 30-μm-long apical dendritic segments (50–80 μm from soma). (J) The maximum spine width of 200 spines from 10 randomly selected neurons was measured by Image J software. *P < 0.001 vs. WT mice.

Materials and Methods
Reagents and Antibodies. BDNF and PDGF-BB were provided by Calbiochem. Puromycin was purchased from Sigma-Aldrich. The cell-culture reagents were provided by Gibco BRL. Tubulin, Erk1/2, Akt1/2, GSK3β, Shp2, Shc, Gab1, Grb2, Gαi1, Gαi2, Gαi3, goat anti-rabbit IgG-HRP, and goat anti-mouse IgG-HRP antibodies were obtained from Santa Cruz Biotechnology, and the mouse β-actin monoclonal antibody was from Sigma. p-Akt (Ser-473), p-Akt (Thr-308), p-Gab1 (Tyr-627), p-S6K (Thr-389), p-S6 (Ser-235/236), p-GSK3α/β (Ser-21/9), p-Erk1/2 (Thr-202/Tyr-204), and p-MEK1/2 antibodies were purchased from Cell Signaling Technology. TrkB and p-TrkB antibodies were purchased from Abcam.

MEFs. WT, Gαi1/3-DKO, and Gαi1-, Gαi2-, and Gαi3-SKO MEFs and WT and Gab1-KO MEFs were described previously (16, 20, 58).

Cerebellar Granule Neuron Culture. As described (59), cerebella were removed from 5-d-old mouse pups and placed in ice-cold HBSS, pH 7.4, with penicillin/streptomycin. Cerebella were diced into small chunks before incubation in 10 mL of trypsin (0.5 mg/mL) in HBSS at 37 °C for 10 min (with agitation every 2–3 min), followed by the addition of serum to stop protease digestion. Any chunks were pelleted and resuspended three times in growth medium (DMEM, 10% horse serum, 25 mM KCl, glucose (6 g/L), 2 mM glutamine) plus 10 U penicillin and streptomycin. The supernatant containing the dissociated cells was plated (2 million cells per well in six-well plates) into growth medium on laminin (Invitrogen)-coated plates.

Gαi1 and Gαi3 shRNA/siRNA of MEFs in Vitro. Lentivirus with shRNA targeting murine Gαi1 (sc-41751-V; Santa Cruz Biotechnology) or murine Gαi3 (sc-37255-V; Santa Cruz Biotechnology) were added for 18 h. For stable cell lines, MEFs infected with the lentiviral Gαi1/3 shRNA were selected with puromycin (1.0 μg/mL). The culture medium was replaced with fresh puromycin-containing culture medium every 2 d until resistant colonies were formed (7–8 d). Control MEFs or neurons were infected with scramble nonsense shRNA lentiviral particles or lentiviral GFP (Santa Cruz).
Primary Murine Hippocampal Neuron Culture and shRNA. Hippocampal cultures were prepared using a modified Banker culture protocol (60). Briefly, hippocampi from mice at postnatal day 4 (P4) were dissociated, dissected in a papain-enzyme solution (30 min at 37 °C), and plated into six-well plates at a density of 165 cells/mm² on poly-L-lysine−coated coverslips and were grown in Neurobasal plating medium containing B27 purchased from Invitrogen, penicillin/streptomycin, and 10% FBS. The lentiviral shRNA vectors PGLV3-U6/GFP/Puro (encoding a GFP marker under the control of a CMV promoter) expressing Gu3 shRNA (5′-AGAGTATGGCCAGGACTTA-3′) or the murine Gu1 shRNA (5′-GAGGAGTAGTGACTGTCGCA-3′) were generated by Genepharm. For in vitro studies, hippocampal neurons at DIV 5 were infected with lentiviral Gu1 shRNA plus lentiviral Gu3 shRNA (shGu1/3) or the lentiviral scrambled control shRNA (scr-shRNA, with GFP) (multiplicity of infection: 8). Five days after transfection (at DIV10), neurons were fixed, permeabilized, and labeled with a rabbit polyclonal anti-GFP (1:1,000; ab290; Abcam). The secondary antibody was goat anti-rabbit DyLight 488 (1:1,000). A confocal laser-scanning microscope (LSM700, Zeiss) was utilized to analyze the GFP staining. Forty randomly selected neurons per condition were analyzed for soma number and dendrite branching. For each measurement, at least 40 neurons were counted from randomly selected 200 × 200 μm fields. Spine numbers were counted manually from a 30-μm-long segment of a dendrite that was 50-80 μm away from soma. Spine head width, defined as the maximum width of the spine head, was measured using ImageJ software. At least 10 neurons and 20 spines per neuron were analyzed.

For adenovirus production Gu3 was amplified by RT-PCR. The primer sequences were as follows: P1, 5′-AGGTCGACTCTAGAGGAGACCCAACTG-3′; P2, 5′-CAATCTAAAAGAATGGTTGATGC-3′. The PCR fragment was subcloned into the BamH1/ AgeI site of the pDC315-Flag plasmid to produce pDC315-Gu3 with Flag fused to the C terminus of Gu3. HEK293 cells were transfected with the pDC315-Gu3-Flag using Lipofectamine 2000 and the pBHGloxα1,3 Cre plasmid (GenePharma) as the helper plasmid to generate the recombinant adenovirus Ad-Gu3-Flag, and the supernatant was harvested after 1 wk. After viral amplification (3×), the supernatant was purified using an Adeno-X Virus Purification kit (Clontech). To titer the virus, serially diluted adenovirus was used to transduce HEK293 cells. For each sample, the HEK293 cells were counted to calculate the viral titer (2.5 × 10⁶ pfu/mL).

CMS Depression Model. As described (63), the CMS procedure involves the sequential application of a variety of mild stressors, including forced-swim, restraint, water and food deprivation, housing in forced-swat, light/dark cycle reversal, and housing in constant illumination or darkness (33, 34). The CMS lasted for a total of 3 wk; a detailed protocol is provided in Table S1.

TST. Mice were suspended by the tail, using adhesive scotch tape, to a hook in a soundproof box. The test session was videotaped and observed by two independent observers (35, 63). The total duration of immobility during a 6-min test was calculated in seconds. Data collected were expressed as mean ± SEM.

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Western Blot Assay and Coimmunoprecipitation Assay. Assays were performed as described previously (16, 20, 58, 64–66). To test for proteins of similar size, we used primary antibodies raised in two different species, or the blot was stripped and reprobed. Alternatively we ran identical gels (sister gels) with the loading control for comparison.

Plasma Membrane Fractionation. The protocol for isolation of the plasma membrane fraction was based on a previous study (67), with minor modifications. Neuronal cultures were rinsed with HES buffer (20 mM Hapes (pH 7.4), 1 mM EDTA, 250 mM sucrose with protease inhibitors) and were scraped into cold HES buffer and passed through a 26-gauge needle. The nuclei were removed by centrifugation at 1,000 × g for 5 min. The supernatant was centrifuged at 100,000 × g for 60 min at 4 °C to separate the cytoplasmic fraction (supernatant) and membranous fraction (pellet). The pellet was resuspended in 1.2 M sucrose in HE buffer (20 mM Hapes (pH 7.4), 1 mM EDTA, 1X Halt protease inhibitor) and centrifuged at 100,000 × g for 60 min, yielding a white fluffy band at the interface (plasma membrane). The plasma membrane fraction was resuspended in HES buffer and pelleted at 40,000 × g for 20 min. The pellets were resuspended in sample buffer, and protein concentrations were quantified.

Statistical Analysis. All experiments were repeated at least three times, and data were expressed as means ± SD. Statistical differences were analyzed by one-way ANOVA followed by multiple comparisons performed with a post hoc Bonferroni test (SPSS version 20.0; IBM). Values of P < 0.01 were
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