Inhibition of the Schizophrenia-Associated MicroRNA miR-137 Disrupts Nrg1 alpha Neurodevelopmental Signal Transduction

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Graphical Abstract

Highlights
- miR-137 has multiple mRNA targets within the PI3K-Akt-mTOR pathway in neurons
- Inhibition of miR-137 ablates mTORC1-dependent responses to Nrg1α and BDNF
- Nrg1α stimulates GluA1 synthesis in dendrites by a miR-137-dependent mechanism
- miR-137 regulates Nrg1α- and BDNF-induced dendritic outgrowth

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In Brief
Thomas et al. show that the schizophrenia-associated microRNA miR-137 regulates neuronal responses to neuregulin-1α and BDNF signaling. miR-137 targets multiple components of the PI3K-Akt-mTOR pathway, which act downstream of both neuregulin and BDNF. Inhibition of miR-137 blocks stimulus-induced dendritic protein synthesis and outgrowth among other responses critical to neuronal development.
Inhibition of the Schizophrenia-Associated MicroRNA miR-137 Disrupts Nrg1α Neurodevelopmental Signal Transduction

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SUMMARY

Genomic studies have repeatedly associated variants in the gene encoding the microRNA miR-137 with increased schizophrenia risk. Bioinformatic predictions suggest that miR-137 regulates schizophrenia-associated signaling pathways critical to neural development, but these predictions remain largely unvalidated. In the present study, we demonstrate that miR-137 regulates neuronal levels of p55γ, PTEN, Akt2, GSK3β, mTOR, and rictor. All are key proteins within the PI3K-Akt-mTOR pathway and act downstream of neuregulin (Nrg)/ErbB signaling. Inhibition of miR-137 ablates Nrg1α-induced increases in dendritic protein synthesis, phosphorylated S6, AMPA receptor subunits, and outgrowth. Inhibition of miR-137 also blocks mTORC1-dependent responses to BDNF, including increased mRNA translation and dendritic outgrowth, while leaving mTORC1-independent S6 phosphorylation intact. We conclude that miR-137 regulates neuronal responses to Nrg1α and BDNF through convergent mechanisms, which might contribute to schizophrenia risk by altering neural development.

INTRODUCTION

Schizophrenia is a debilitating psychiatric disorder with no cure, poorly understood etiology, and complex underlying genetics. Recent genome-wide association studies show that genetic variants near and within MIR137, which encodes the microRNA (miRNA) miR-137, contribute to schizophrenia risk (Ripke et al., 2011, 2013, 2014). miRNAs are small non-coding RNAs that bind target mRNAs by complementary base pairing and inhibit mRNA translation or promote mRNA degradation. miR-137’s predicted targets are enriched within critical neurodevelopmental pathways, including the neuregulin (Nrg)/ErbB pathway (Wright et al., 2013). This suggests that dysregulation of miR-137 may contribute to schizophrenia etiology by altering neurodevelopmental signaling.

Genetic studies have linked mutations in the Nrg/ErbB pathway with schizophrenia and other psychiatric disorders (Mei and Nave, 2014). The Nrgs are a family of six transmembrane proteins (Nrg1–6) that may be cleaved to release soluble extracellular domains that interact with the ErbB family of receptors. Within the cortex and hippocampus, Nrg1 regulates dendrite outgrowth, synaptogenesis, and surface receptor levels (Mei and Nave, 2014).

In the present study, we test the hypothesis that miR-137 regulates Nrg/ErbB neurodevelopmental signaling. We report that miR-137 regulates target proteins within the phosphoinositide 3-kinase-Akt-mechanistic target of rapamycin (PI3K-Akt-mTOR) pathway, which acts downstream of ErbB receptors. Nrg1α increases phospho(Ser235/236)-S6 levels, mRNA translation, AMPA receptor levels, and outgrowth in the dendrites of primary neurons. Chronic inhibition of miR-137 reverses or abolishes the effects of Nrg1α signaling by all measures. Inhibition of miR-137 also abolishes dendritic outgrowth and mRNA translation induced by brain-derived neurotrophic factor (BDNF). Our data demonstrate a requirement for endogenous miR-137 in Nrg/ErbB and BDNF signaling and suggest a neurodevelopmental mechanism by which dysregulated miR-137 might contribute to schizophrenia etiology.

RESULTS

miR-137 Targets May Be Enriched within the Nrg/ErbB Pathway

We conducted a DAVID analysis to identify pathways in which predicted miR-137 targets were enriched. These pathways included Nrg/ErbB signaling (p = 0.00089), neurotrophin signaling (p = 0.019), and long-term potentiation (p = 0.033). Many predicted miR-137 targets fell within the PI3K-Akt-mTOR branch of Nrg/ErbB signaling (Figure 1A; Table S1).

miR-137 Targets mRNAs Encoding Proteins in the PI3K-Akt-mTOR Pathway

The PI3K-Akt-mTOR pathway is critical for neurite outgrowth, synaptic plasticity, and learning and memory (Crino, 2016). To
evaluate whether miR-137 regulates mTOR activity, we transfected primary cortical neurons with a plasmid to overexpress the miR-137 precursor (pre-miR-137 OE or miR-137 OE) and detected changes in mTOR by western blot. miR-137 OE increased miR-137 levels (Figure S1A) and reduced GluA1, a validated miR-137 target (Olde Loohuis et al., 2015) (Figures S1B and S1D). miR-137 OE reduced total mTOR without affecting Ser2448 phosphorylation (Figure 1B), where “phosphorylation” refers to the ratio of phospho- to total protein.

We next inhibited endogenous miR-137 using a locked nucleic acid inhibitor (miR-137 LNA). miR-137 LNA increased GluA1 and GluA2 protein levels (Figures S1E and S1F), as previously reported (Olde Loohuis et al., 2015), but had no effect on mTOR (Figure 1C). We conclude that miR-137 may regulate mTOR levels but not phosphorylation. The absence of miR-137 target sites within the mTOR 3’ UTR suggests that miR-137 does so through an indirect mechanism (Table S1).

We next examined predicted miR-137 targets encoding proteins that act upstream of mTOR. We conducted luciferase assays in Neuro2A cells and western blots in primary cortical neurons to validate the following predicted miR-137 targets: p55γ, PTEN, Akt2, GSK3β, and rictor. This list includes proteins that stimulate (i.e., p55γ and Akt2) and inhibit (i.e., PTEN, GSK3β, and rictor) mTOR complex 1 (mTORC1) signaling. Luciferase assays utilized human 3’ UTR sequences, while western blots measured endogenous protein levels in mouse primary cortical neurons.

*PIK3R3* mRNA encodes PI3K regulatory subunit p55γ, miR-137 OE reduced *PIK3R3* 3’ UTR reporter activity (Figure 1D), while miR-137 LNA increased activity (Figure 1E), consistent with prior reports (Sang et al., 2016). miR-137 OE had no effect on p55γ (Figure 1F), but miR-137 LNA increased p55γ (Figure 1G). We conclude that miR-137 regulates p55γ in neurons, likely by targeting the encoding mRNA.

PTEN is a potent inhibitor of PI3K signaling. miR-137 OE reduced *PTEN* 3’ UTR reporter activity (Figure 1H), while miR-137 LNA had no effect (Figure 1I). Similarly, miR-137 OE reduced PTEN (Figure 1J), while miR-137 LNA had no effect (Figure 1K). We conclude that miR-137 is capable of regulating PTEN but might not do so under basal conditions.

*AKT2* is a validated miR-137 target (Liu et al., 2014). miR-137 OE reduced Akt2 (Figure 1L), and miR-137 LNA increased Akt2 in neurons (Figure 1M).

Rictor is an obligate mTORC2 component. miR-137 OE had no effect on RICTOR 3’ UTR reporter activity (Figure 1N), but miR-137 LNA strongly increased activity (Figure 1O). By contrast, miR-137 OE reduced rictor protein levels in neurons (Figure 1P), while miR-137 LNA had no effect (Figure 1Q), suggesting that miR-137 might not target rictor in neurons under basal conditions.

GSK3β 3’ UTR reporters were purchased in two overlapping fragments: the first containing nucleotides 11–2,510 and the second containing nucleotides 2,293–4,835. Both reporters showed reduced activity with miR-137 OE (Figure 1R), but neither was affected by miR-137 LNA (Figure 1S). miR-137 OE reduced GSK3β (Figure 1T), and miR-137 LNA increased GSK3β without affecting Ser9 phosphorylation in neurons (Figure 1U).

In summary, miR-137 targets the 3’ UTRs of *PIK3R3, PTEN, RICTOR*, and *GSK3β* mRNAs and regulates mTOR, p55γ, PTEN, Akt2, rictor, and GSK3β protein levels (Table S1). Inconsistencies between experiments may be due to experimental variables such as: (1) differences in miR-137 affinity for the human and mouse 3’ UTR sequences, (2) differences in mRNA abundance between the overexpressed luciferase reporter mRNAs and endogenous target mRNAs, or (3) differences in miR-137 binding site accessibility in Neuro2A cells and primary neurons (e.g., due to the presence of competing miRNAs or RNA-binding proteins). Nonetheless, the data show that
miR-137 regulates multiple proteins within the PI3K-Akt-mTOR pathway. These results prompted us to investigate whether miR-137 regulates mTOR-dependent responses to Nrg/ErbB signaling in neurons.

**Nrg1α Increases Dendritic Phospho-S6 and mRNA Translation by a miR-137-Dependent Mechanism**

To evaluate Nrg/ErbB signaling integrity when miR-137 is inhibited, we transduced primary hippocampal neurons on DIV2 with mTORC1 inhibitors: anisomycin (Anis), a phospho-S6 (P-S6) and puromycin (Puro), (A) Experimental timeline for puromycylation experiments. (B–D) Representative neurons and dendritic regions of interest (ROIs) are shown in (B). Puromycylation (D), but not phospho-S6 (P-S6) (C), was sensitive to anisomycin and dependent on puromycin (Puro) (D). ****p < 0.0001, one-sample t test, test value = 1; n = 18 cells per condition from three biological replicates. Experiments were conducted in parallel with those in (E)–(G). (E–G) miR-137 sponge blocks Nrg1α-induced increases in dendritic phospho(Ser235/236)-S6 and protein synthesis in DIV7–9 primary hippocampal neurons. Representative neurons and dendritic ROIs for both measurements are shown in (E). Phospho(S235/236)-S6 is shown in (F) (two-way ANOVA: p [sponge] = 0.0070, and ***p [interactive] = 0.0001; Sidak’s test: ****p < 0.0001; n = 60 cells per condition from three biological replicates; ns, not significant). Puromycylation is shown in (G) (two-way ANOVA: p [sponge] = 0.0038, p [Nrg] = 0.0136, and ***p [interactive] = 0.0027; Sidak’s test: ****p < 0.0001; n = 60 cells per condition from three biological replicates).

(H–J) Rapamycin blocks Nrg1α-induced increases in dendritic phospho(Ser235/236)-S6 and protein synthesis in DIV6–7 hippocampal neurons. Representative neurons and dendritic ROIs are shown in (H). Phospho-S6 is shown in (I) (two-way ANOVA: p [rapamycin] < 0.0001, p [Nrg] = 0.0005, and **p [interactive] = 0.0027; Sidak’s test: ****p < 0.0001; n = 60 cells per condition from three biological replicates). Puromycylation is shown in (J) (two-way ANOVA: p [Nrg] = 0.0099, and ****p [interactive] < 0.0001; Sidak’s test: ****p < 0.0001; n = 60 cells per condition from three biological replicates).

Data are shown as mean ± SEM. Scale bars, 25 μm. See also Figure S2.
Figure 3. Inhibition of miR-137 Blocks Nrg1α-Induced GluA1 Synthesis

(A–C) Stimulation of dendritic GluA1 and GluA2 levels by Nrg1α is ablated by miR-137 sponge in DIV6 primary hippocampal neurons. Representative neurons and dendritic ROIs are shown in (A). GluA1 and GluA2 are quantified in (B) and (C), respectively. (B) Sidak’s test: *p < 0.05; n = 79–80 cells per condition from four biological replicates. ns, not significant. (C) Two-way ANOVA: p (sponge) = 0.0003, p (Nrg) = 0.0184, and ***p (interactive) = 0.0003; Sidak’s test: **p < 0.0001; n = 79–80 cells per condition from four biological replicates. Scale bars, 25 μm for whole-cell and 2 μm for dendrite ROIs.

(D) Nrg1α increases GluA1 and GluA2 protein levels in DIV6 primary cortical neurons (Sidak’s test: **p < 0.01; n = 5). Representative western blots are shown at the left.

(E) Nrg1α does not affect Gria1 or Gria2 mRNA levels in DIV6 primary cortical neurons (Sidak’s test: ns; n = 5).

(F–J) Inhibition of miR-137 blocks Nrg1α-induced GluA1 synthesis in the dendrites of DIV6–7 primary hippocampal neurons. Schematic for PLA detection of newly synthesized GluA1 is shown in (F). Representative neurons and dendritic ROIs are shown in (G). PLA puncta are quantified in (H) (two-way ANOVA: p (sponge) = 0.0003, p (Nrg) = 0.0184, and ***p (interactive) = 0.0003; Sidak’s test: **p < 0.0001; n = 79–80 cells per condition from four biological replicates. Scale bars, 25 μm for whole-cell and 2 μm for dendrite ROIs).
Mutation of the miR-137 target site reduces miR-137 and miR-92a association with mouse Gria1 Schematic shows the relative positions of miR-137, miR-92a, and miR-128 predicted binding sites in the Nrg1 (Loohuis et al., 2015), and (qIF) (Figure 2A).

Ribosomal protein S6 is phosphorylated downstream of mTORC1 (Roux et al., 2007). Nrg1α consistently increased dendritic phospho(Ser235/236)-S6 (Figures 2E, 2F, 2H, 2I, and S2C–S2N). Whether this was primarily due to increased phosphorylation or total S6 varied across experiments. Therefore, we chose dendritic phospho-S6 as our primary measure of Nrg1α signaling integrity. Nrg1α increased dendritic phospho-S6 by stimulating ErbB receptors (Figures S2A–S2F). Nrg1α did not increase dendritic phospho-S6 in neurons expressing the miR-137 sponge (Figures 2E, 2F, and S2G–S2J).

S6 phosphorylation is often correlated with mRNA translation (Roux et al., 2007). We used puromycylation to label newly synthesized proteins which were then detected by qIF with a puromycin-specific antibody (Figure 2A). Puromycylation was translation and puromycin dependent (Figures 2B–2D). Nrg1α increased dendritic protein synthesis in control neurons but not in neurons expressing the miR-137 sponge (Figure 2G).

We next used rapamycin to inhibit mTORC1 prior to Nrg1α treatment. Rapamycin reduced dendritic phospho-S6 by 80% (Figures 2H, 2I, and S2K–S2N). Total S6 was not affected (Figure S2M). Rapamycin ablated Nrg1α-induced increases in phospho-S6 (Figures 2H, 2I, and S2K–S2N) and protein synthesis (Figure 2J). We conclude that both mTORC1 and miR-137 are required for Nrg1α to stimulate dendritic phospho-S6 and protein synthesis.

Inhibition of miR-137 Blocks Nrg1α-Induced GluA1 Synthesis

Nrg1 signaling increases surface AMPA receptor (AMPA) levels (Cahill et al., 2013), but whether Nrg1 stimulates AMPAR subunit synthesis is unknown. Nrg1α increased dendritic GluA1 and GluA2, and this response was blocked by miR-137 (Figure 3F) sponge (Figures 3A–3C). Nrg1α increased total GluA1 and GluA2 protein (Figure 3D) without affecting mRNA levels (Figure 3E). We next used puromycylation, followed by proximity ligation assay (PLA) to detect endogenous, newly synthesized GluA1 (Figure 3F). (See tom Dieck et al., 2015, for a detailed method description.) We chose to focus on GluA1 rather than GluA2 because GluA1 is encoded by a validated miR-137 target (Olde Loohuis et al., 2015), and GRIA1 has been linked to schizophrenia with genome-wide significance (Ripke et al., 2014). Nrg1α increased dendritic GluA1 synthesis in control neurons, but the miR-137 sponge ablated Nrg1α-induced GluA1 synthesis (Figures 3G and 3H). The PLA signal was translation and puromycin dependent (Figures 3I and 3J). We conclude that Nrg1α regulates AMPAR subunits by a miR-137-dependent mechanism.

Surprisingly, the miR-137 sponge did not increase dendritic GluA1 or GluA2 (data not shown) or dendritic GluA1 synthesis under basal conditions (Figure 3H). GluA1 was validated as a miR-137 target using the human GRIA1 3' UTR sequence and rat primary neurons (Olde Loohuis et al., 2015). We conducted luciferase assays to confirm that miR-137 targets mouse Gria1 mRNA. miR-137 OE reduced Gria1 3' UTR reporter activity (Figure S3A), miR-137 LNA increased reporter activity (Figure S3B), miR-137 bound Gria1 mRNA in vitro (Figure 3K). Mutation of the miR-137 binding site reduced miR-137 association with Gria1 mRNA in vitro (Figure 3K) and blocked the effects of miR-137 LNA on the Gria1 3' UTR reporter (Figure S3B). We conclude that miR-137 directly targets the mouse Gria1 3' UTR. Conflicts between the imaging and western blot data may indicate that miR-137's effects on mTORC1 signaling may be more important than direct mRNA targeting for regulating GluA1 synthesis at this developmental time point (DIV6–7).

Nrg1α Does Not Alter miR-137 Levels, Localization, or Association with Gria1 mRNA

Nrg1α might also regulate protein synthesis by altering miR-137 activity. However, Nrg1α had no effect on total or dendritic miR-137 levels (Figures S3C–S3E) or on miR-137 association with Gria1 mRNA in vitro (Figure 3L).

Nrg1α Regulates Dendritic Outgrowth by a miR-137- and mTOR-Dependent Mechanism

The PI3K-Akt-mTOR pathway, Nrg1, and miR-137 regulate dendritic outgrowth (Gerecke et al., 2004; Kumar et al., 2005; Smrt et al., 2010). We predicted miR-137 would also regulate Nrg1α-induced dendritic outgrowth. We treated primary neurons on DIV2 with 10 nM Nrg1α or vehicle control and assessed dendritic complexity on DIV4 by Sholl analysis (Figures 4A–4C).

Nrg1α promoted dendritic outgrowth in primary cortical and hippocampal neurons (Figures 4D–4L and S4A–S4I). Concurrent treatment with PD158780 (a pan-ErbB inhibitor) or rapamycin blocked Nrg1α-induced dendritic outgrowth (Figures 4D–4F and S4A–S4C), miR-137 sponge increased dendritic outgrowth; however, Nrg1α reduced dendritic outgrowth in neurons expressing the miR-137 sponge (Figures 4G–4I and S4D–S4F), miR-137 sponge also blocked Nrg1β-induced dendritic outgrowth (Figures S4G–S4I). Nrg1α-induced dendritic outgrowth

ANOVA: p (spponge) = 0.0001; Sidak’s test: *p < 0.05; n = 70–71 cells per condition from four biological replicates). The sum of the intensities of the PLA puncta was normalized to the dendritic volume within each ROI. The PLA signal was sensitive to anisomycin and dependent on puromycin (L and J) (one-sample t test, test value = 1; ***p = 0.0001; n = 18 cells per condition from three biological replicates). Scale bars, 25 μm for whole-cell and dendritic ROIs.

(k) Mutation of the miR-137 target site reduces miR-137 and miR-92a association with mouse Gria1 mRNA when measured by a biotinylated mRNA pull-down assay. Two-way ANOVA; p (3' UTR) = 0.0007, p (miRNA) = 0.1323, and p (interactive) = 0.0009; Sidak’s test: *p < 0.05 and ***p < 0.001; n = 4 biological replicates. Schematic shows the relative positions of miR-137, miR-92a, and miR-128 predicted binding sites in the Gria1 3' UTR. See Figures S3A and S3B for luciferase assays with Gria1 3' UTR reporters.

(L) Nrg1α has no effect on miR-137's ability to bind Gria1 mRNA in vitro when measured by biotinylated mRNA pull-down assay (ns, paired t test; n = 4). Bound miR-137 was normalized to miR-137 input as well as Gria1 mRNA pull-down. Data are shown as mean ± SEM. See also Figure S3.
was not significant in neurons overexpressing miR-137 (Figures 4J–4L). We conclude that Nrg1α stimulates dendritic outgrowth by an ErbB-, mTOR-, and miR-137-dependent mechanism. Nrg1α did not affect miR-137 levels (Figure S4J).

Acute Nrg1α increased dendritic MAP2 in DIV6 neurons in a miR-137-dependent manner (Figures S4K and S4M). MAP2 promotes dendritic outgrowth (Harada et al., 2002), suggesting that MAP2 may contribute to Nrg1α-induced outgrowth. miR-137 sponge did not affect dendrite diameter (Figure S4L).

miR-137 Regulates BDNF-Induced Dendritic Outgrowth

We hypothesized that miR-137 targets within the PI3K-Akt-mTOR pathway would affect neuronal responses to neurotrophins, such as BDNF (Figure 1A). BDNF increased dendritic outgrowth in control neurons but not in neurons treated concurrently with rapamycin (Figures 5A–5C) or in neurons expressing miR-137 sponge (Figures 5D–5F) or miR-137 OE constructs (Figures 5G–5I). We conclude that BDNF stimulates dendritic outgrowth by an mTOR- and miR-137-dependent mechanism.

By contrast, neither rapamycin (Figures 5J–5M) nor the miR-137 sponge (Figures 5N–5Q) blocked BDNF-induced S6 phosphorylation in dendrites. BDNF may stimulate S6 phosphorylation through an alternative pathway (Roux et al., 2007), which may be unaffected by miR-137.

BDNF stimulates mRNA translation in neurons by an mTORC1-dependent mechanism (Schraff et al., 2004). BDNF stimulated mRNA translation in control DIV14 hippocampal neurons but not in neurons expressing miR-137 sponge (Figures 5R and 5S).

We conclude that miR-137 regulates mTOR-dependent responses to Nrg1 and BDNF through a shared mechanism.

DISCUSSION

We tested the hypothesis that miR-137 regulates Nrg/ErbB signaling in neurons. We found that inhibition of miR-137 blocked Nrg1α-induced increases in dendritic phospho-S6, mRNA translation, GluA1 synthesis, and outgrowth in primary neurons. Inhibition of miR-137 also blocked mTORC1-dependent responses to BDNF—specifically, mRNA translation and dendritic outgrowth—while leaving mTORC1-independent S6 phosphorylation intact. Together, our data suggest that miR-137 regulates responses to multiple signaling ligands but may selectively regulate the PI3K-Akt-mTOR branch of Nrg/ErbB and BDNF signaling.

The EGF (epidermal growth factor)-like domain of Nrg exists in alpha and beta forms. We utilized a soluble form of the EGF-like domain of Nrg1α to stimulate Nrg/ErbB signaling. To date, the majority of research concerning the role of Nrg1 in neurons has focused on Nrg1α, leaving the α form largely unstudied (Bernstein and Bogerts, 2013). Nrg1α is highly expressed in pyramidal neurons and interneurons of the cortex and hippocampus of the human pre- and perinatal brain (Bernstein et al., 2006). Nrg1α expression declines over the course of brain maturation, but Nrg1α-positive interneurons remain evident in adult cortex and hippocampus (Bernstein et al., 2006; Connor et al., 2009). Post-mortem studies suggest that the density of Nrg1α-positive neurons is increased in schizophrenia patients, while the density of Nrg1α-positive neurons is significantly reduced (Bernstein et al., 2013; Bertram et al., 2007). Nrg1α-induced activation of PI3K signaling is also impaired in lymphoblastoid cell lines derived from schizophrenia patients (Law et al., 2012). This suggests that Nrg1α plays important roles both in normal brain development and in schizophrenia etiology (Bernstein and Bogerts, 2013) and that Nrg1α signaling may be disrupted in schizophrenia in a manner similar to that described in the present study.

Inhibition of miR-137 also disrupted mRNA translation, glutamate receptor expression, and dendritic outgrowth in ways that may be relevant to schizophrenia etiology. Inhibition of miR-137 blocked Nrg1α- and BDNF-induced protein synthesis in neurons. A recent study reported reduced protein synthesis in olfactory cells derived from schizophrenia patients (English et al., 2015), and the antipsychotic haloperidol stimulates mTORC1 signaling and mRNA translation in primary striatal neurons (Bowling et al., 2014). miR-137 may also contribute to
dysregulated glutamate signaling by at least two mechanisms: by directly targeting glutamate receptor mRNAs and by disrupting stimulus-induced glutamate receptor synthesis. GRIA1 and GRIK2A genetic variants have been linked to schizophrenia with genome-wide significance (Ripke et al., 2014), and miR-137 directly targets GRIA1 and GRIK2A mRNAs (Olde Loohuis et al., 2015; Zhao et al., 2013). Inhibition of miR-137 also blocked stimulus-induced dendritic outgrowth. Reduced dendritic complexity has been reported in subpopulations of neurons within the cerebral cortex of schizophrenia patients (Broadbent et al., 2002; Kalus et al., 2002; Rosoklija et al., 2000). Furthermore, the antipsychotics haloperidol and olanzapine promote neurite outgrowth in primary neurons (Bowling et al., 2014; Zhang et al., 2016). Additional studies in schizophrenia model systems are needed to determine whether dysregulation of miR-137 contributes to deficits in protein synthesis, glutamatergic signaling, or dendritic complexity in schizophrenia.

EXPERIMENTAL PROCEDURES

Bioinformatic Analysis
miR-137 targets were identified using TargetScan (v6.2). The Functional Annotation Tool within DAVID (Database for Annotation, Visualization, and Integrated Discovery, v6.7) was used to identify signaling pathways in which miR-137 targets were enriched. Pathways of interest were visualized using KEGG (Kyoto Encyclopedia of Genes and Genomes) pathway maps within DAVID. Target sites within mRNAs of interest were identified using TargetScan (v6.2) or miRmap (http://mirmap.ezlab.org/app/).

Cell Culture and Drug Treatments
Neuro2A cells, a mouse neuroblastoma cell line, were cultured as previously described (Williams et al., 2016). Primary hippocampal and cortical neuron cultures were prepared from C57BL/6J mouse embryos of either sex on embryonic day 17 as described by Williams et al. (2016). Animal protocols were approved by the Institutional Animal Care and Use Committee at Emory University. For additional details, see the Supplemental Experimental Procedures.

Figure 5. miR-137 Regulates mTOR-Dependent Responses to BDNF
(A–C) Rapamycin co-treatment blocks BDNF-induced dendritic outgrowth in DIV4 primary hippocampal neurons. Representative neurons are shown in (A). Dendritic complexity is quantified by Sholl analysis in (B) intersections versus distance from cell center; Sidak’s test: *p < 0.05 for DMSO/BDNF versus DMSO/control, and #p < 0.05 for rapamycin/BDNF versus rapamycin/control; n = 60 cells per condition from three biological replicates). (C) Total intersections, normalized to DMSO/control; two-way ANOVA: *p [rapamycin] < 0.0001, and **p [interactive] < 0.0041; Sidak’s test: ***p < 0.01; n = 60 cells per condition from three biological replicates).

(D–F) miR-137 sponge blocks BDNF-induced dendritic outgrowth in DIV4 primary hippocampal neurons. Representative neurons are shown in (D). Dendritic complexity is quantified in (E) intersections versus distance from cell center; Sidak’s test: *p < 0.05 for control/BDNF versus control/control [vehicle]; #p < 0.05 for miR-137 sponge/control [vehicle] versus control/control [vehicle], and p < 0.05 for miR-137 sponge/BDNF versus control/control [vehicle]; n = 59–60 cells per condition from three biological replicates) and (F) total intersections, normalized to control (GFP/vehicle); two-way ANOVA: ***p [interactive] = 0.0004; Sidak’s test: *p < 0.05, and **p < 0.01; n = 59–60 cells per condition from three biological replicates). ns, not significant.

(G–I) miR-137 OE blocks BDNF-induced dendritic outgrowth in DIV4 primary hippocampal neurons. Representative neurons are shown in (G). Dendritic complexity is quantified in (H) intersections versus distance from cell center; Sidak’s test: *p < 0.05; control/BDNF versus control/control [vehicle]; n = 60 cells per condition from three biological replicates) and (I) total intersections, normalized to control [GFP/vehicle]; two-way ANOVA: *p [interactive] = 0.0334; Sidak’s test: *p < 0.05; n = 60 cells per condition from three biological replicates).

(J–M) BDNF-induced S6 phosphorylation (Ser235/236) is miR-137 independent in DIV7 primary hippocampal neurons. Representative neurons are shown in (J). Phospho-S6 is quantified in (K) (Student’s t test: ***p < 0.0001; n = 58–60 cells per condition from three biological replicates). Total S6 is quantified in (L) (Student’s t test: ns; n = 58–60 cells per condition from three biological replicates). The ratio of phospho- to total dendritic S6 is quantified in (M) (Student’s t test: ***p < 0.0001; n = 58–60 cells per condition from three biological replicates). Total S6 is quantified in (P) (two-way ANOVA: ns; n = 59–60 cells per condition from three biological replicates). The ratio of phospho- to total dendritic S6 is quantified in (Q) (two-way ANOVA: p [BDNF] < 0.001; Sidak’s test: *p < 0.05, and **p < 0.01; n = 59–60 cells per condition from three biological replicates).

(R and S) miR-137 sponge blocks BDNF-induced dendritic protein synthesis in DIV14 primary hippocampal neurons. Representative neurons and dendritic ROIs are shown in (R). Puromycylation is quantified in (S) (two-way ANOVA: ***p [interactive] = 0.0007; Sidak’s test: *p < 0.05; n = 60 cells per condition from three biological replicates).

Data are shown as mean ± SEM. Scale bars, 25 μm.
Statistical Analyses
For each experiment, data were derived from a minimum of three independent biological replicates. Each biological replicate represents neurons obtained from the pooled embryos of one pregnant mouse, i.e., three biological replicates represent cultures from three different dams. All experimental conditions were represented within each biological replicate. Statistical analyses and graphs were prepared in GraphPad Prism (v.7). The Venn diagram was prepared using Gliffy (https://www.gliffy.com). For all experiments, \( z \) was set at 0.05. See the figure legends for specific statistical tests. For luciferase assays, outliers were identified using Grubb’s test and removed before further analysis.

SUPPLEMENTAL INFORMATION
Supplemental Information includes Supplemental Experimental Procedures, four figures, and one table and can be found with this article online at http://dx.doi.org/10.1016/j.celrep.2017.06.038.

AUTHOR CONTRIBUTIONS

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