Appendix 1 to Vallès A, Martens GJM, De Weerd P, et al. MicroRNA-137 regulates a glucocorticoid receptor–dependent signalling network: implications for the etiology of schizophrenia. *J Psychiatry Neurosci* 2014

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Supplementary Methods

Animals, experimental procedure and tissue dissection

A subset of samples derived from a previous experiment involving genome-wide mRNA expression profiling after a brief period of enriched environment (EE) in the rat barrel cortex was used in this study.[1] Briefly, young adult male Long Evans rats (Harlan) were housed 2 per cage under standard conditions and divided in 2 experimental groups: control (n = 4) and EE (n = 8). On the test day, control animals remained in their home-cages and EE animals were allowed to explore a cage enriched with several tools and textures for 30 minutes. The rats were decapitated immediately after the end of the control or EE session, followed by brain extraction and barrel cortex dissection by micropunch.[1] Tissue samples were stored at –80 °C.

Neuronal cell cultures and transfections

Primary cultures of cortical neurons were prepared from embryonic day 18 rats[2] and maintained in a neurobasal medium supplemented with B27 (Invitrogen) and 2 mmol/L glutamine. Locked nucleic acid miR-137 inhibitor (anti-miR-137), as well as nontargeting control (NT) were obtained from Exiqon and were transfected into primary neurons at 6 DIV using lipofectamine 2000 (Invitrogen).

RNA Isolation

RNA from tissue samples was isolated with TRIzol reagent (Invitrogen) after homogenization of the tissue with a TissueLyser (Retsch GmbH). RNA from cell suspensions of primary cortical neurons was isolated using the NucleoSpin RNA II RNA isolation kit (Machery-Nagel), 24 hours after transfection with NTC or anti-miR-137. RNA concentration and quality was determined with a NanodropTM ND-1000 spectrophotometer (Thermo Fisher Scientific Inc.), and 1% agarose gel electrophoresis, respectively. The samples were kept at –80 °C until further analysis.

Quantitative Polymerase Chain Reaction (qPCR)

Two µg of DNAse-treated, total RNA from each sample was used for cDNA synthesis, using the RevertAid H Minus First Strand cDNA Synthesis kit (Fermentas Inc.) and the Qiagen miScript Reverse Transcription Kit II (Qiagen), for mRNAs and for precursor (pre-) and mature (mat-) miR-137, respectively. Prior to qPCR analysis, each cDNA sample was diluted 1:10 with MilliQ water. QPCR was performed according to previously described protocols[3,4] using standard cycling conditions, and performing a melting protocol to control for product specificity. The miscript miRNA expression analysis assay (Qiagen) was used for the quantification of mRNAs, pre- and mat-miR-137 levels. While mRNA and pre-miR-137 primers were designed by the investigators, using standard qPCR primer design strategies, we purchased pre-designed mat-miR-137 primers to assess mature miR-137 levels. All Ct values used for analyses were averaged from 2 to 3 replicates and those with high standard deviation (>1) were not included in the analyses. For pre- and mat-miR-137, relative expression was calculated using the comparative Ct method,[3] normalized to the expression of U6 snRNA. For all mRNAs, Ppia, Ywhaz or β-actin were measured as housekeeping genes and the 2 most constant (*Ppia* and *Ywhaz*) were selected with GNorm[5] for normalization.

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Supplementary Results

Effect of endogenous miR-137 silencing on the expression of EE-regulated putative miR-137 targets

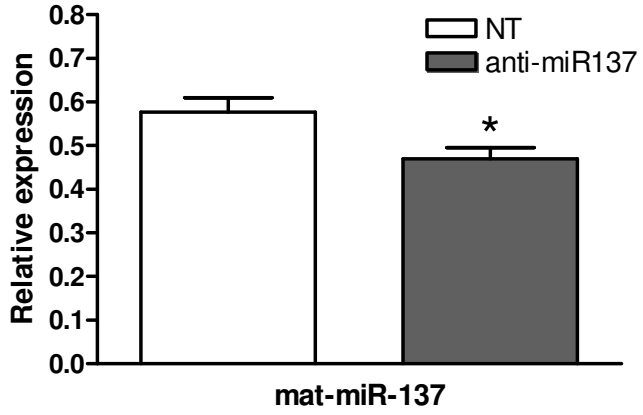

Fig. S1: Relative expression of mature miR-137 levels in primary cortical neurons, after transfection with non-targeting anti-miR control (NT) or specific anti-miR137 probes. The treatment with anti-miR137 significantly reduced mature miR-137 levels (two-tailed t-test, $*p < 0.05$)

Neuronal miR-137-regulated protein network

In this section, all proteins that were implicated in schizophrenia etiology through direct genetic evidence and/or expression data are indicated in bold. The confirmed miR-137 targets are underlined. The network is shown within a pyramidal neuron, the typical (post)synaptic neocortical neuron.

Glucocorticoids, secreted by the adrenal glands in response to stress, profoundly affect the structure and plasticity of neurons. Glucocorticoid action in neurons is mediated by the **glucocorticoid receptor** (**GR**) which, upon glucocorticoid binding, migrates from the cytoplasm to the nucleus where it functions as a transcription factor regulating the expression of a wide diversity of genes, including genes that are important for neuronal structure and plasticity.[6,7] Furthermore, the **GR** localizes to dendritic spines — including those from pyramidal neurons in the rat barrel cortex, the model of neuronal plasticity that was used in this study[8] — which implies that the **GR** mediates local glucocorticoid effects on synaptic/neuronal development and plasticity.[9,10]

In this respect, signalling in the network that is shown in Figure 2 of the main article centres around the nucleus where the **GR** acts as a transcription factor that upregulates the expression of **DUSP1**,[11] **EGR1**[12] and **SGK1**[13] and downregulates the expression of **BDNF**[14] and **COX2**.[15] **COX2**, **DUSP1** and **SGK1** are cytoplasmic proteins that are involved in regulating multiple cellular functions, including the modulation of synaptic plasticity.[16–19] **DUSP1** expression increases upon **COX2** activation,[20] whereas **COX2** expression is downregulated by **DUSP1**.[21] Moreover, **BDNF**, an extracellular growth factor that plays a key role in modulating synaptic plasticity,[22,23] is involved in activating **SGK1**[24] and upregulating **DUSP1** expression.[25] In addition, **BDNF** is involved in upregulating the expression of **GRIN1**,[26] the most important subunit of the NMDA glutamate receptor that has a crucial function in regulating synaptic plasticity.[27,28] Furthermore, after being activated through binding glutamate, the NMDA receptor composed of **GRIN1** and other subunits inhibits the entry of calcium ions into the neuron through the voltage-dependent L-type calcium channel **CACNA1C**,[29] which itself has been linked to the modulation of synaptic plasticity through NMDA receptor–independent signaling.[30] An increase in intracellular

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calcium concentration directly activates **SGK1**[31] and strongly inhibits the activity of **TCF4**,[32] a transcription factor that upregulates **BDNF** expression[33] and is itself upregulated by **EGR1**,[34] another transcription factor that has been implicated in regulating synaptic plasticity.[35,36] **EGR1** also downregulates **SGK1** expression[37] while it upregulates the expression of **DUSP4**,[38] a protein that belongs to the same family of phosphatases as **DUSP1** and is found in both the nucleus and cytoplasm, where it directly binds and interacts with **GRIN1** in NMDA receptor protein complexes.[39] Furthermore, **EGR2**, another synaptic plasticity-linked transcription factor,[35,36] upregulates **EGR1** expression,[40] and the expression of both **EGR1** and **EGR2** is positively regulated through **BDNF** signalling.[25]

Finally, **TCF4** is directly bound and functionally inhibited by DDIT3,[41] a transcription factor that negatively regulates synaptic plasticity[42] and is upregulated by the **ZNF804A** transcription factor.[43] Intriguingly, apart from within ***BDNF*** (see above), binding sites for **TCF4** have also been identified within or in close vicinity of ***DUSP4***, ***GRIN1*** and ***ZNF804A***,[44] which suggests that **TCF4** may regulate the expression of these three network genes as well.

Genetic evidence and/or mRNA/protein expression data implicating the genes encoding 12 proteins from the molecular network discussed above in schizophrenia etiology (*BDNF; CACNA1C; DUSP1; DUSP4; EGR1; EGR2; GRIN1; NR3C1*, which encodes the GR protein; *PTGS2*, which encodes the COX2 protein; *SGK1; TCF4;* and *ZNF804A*) is shown in Table S1.

Table S1: Genetic evidence and/or mRNA/protein expression data (part 1 of 2)

Gene	Genetic evidence	Expression data
BDNF	Ample evidence of genetic association with schizophrenia and altered *BDNF* expression in patients with schizophrenia (see recent reviews[45,46])	
CACNA1C	Genome-wide significant association with schizophrenia[47]	—
DUSP1	—	*DUSP1* expression is increased in peripheral blood mononuclear cells of treatment-naive patients with schizophrenia[48]
DUSP4	—	*DUSP4* expression is decreased in the postmortem cerebellum of patients with schizophrenia[49]
EGR1	—	*EGR1* expression is decreased in the postmortem prefrontal cortex of patients with schizophrenia[50,51]; *EGR1* expression is increased in whole blood of patients with schizophrenia in a highly delusional state[52]
EGR2	Genetic association with schizophrenia in female patients[53]	*EGR2* expression is decreased in the postmortem prefrontal cortex of patients with schizophrenia[50]; *EGR2* expression is increased in lymphoblastoid cell lines of female patients with schizophrenia[53]
GRIN1	Genetic association with schizophrenia[54,55]; *Grin1* knockdown mice constitute a validated animal model of schizophrenia[56]	—
NR3C1	—	*NR3C1* expression is decreased in several postmortem brain regions of patients with schizophrenia[57–59]
PTGS2	Genetic association with schizophrenia[60]	—
SGK1	—	*Sgk1* expression is increased in rat brain after administration of the commonly used antipsychotic drug clozapine[61]
TCF4	Genome-wide significant association with schizophrenia[62]; genetic association with a number of schizophrenia endophenotypes[63]	*TCF4* expression is increased in patients with schizophrenia and correlates with positive and negative schizophrenia symptom levels[63]
ZNF804A	Genome-wide significant association with schizophrenia in multiple studies (see a recent review[43])	—

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