Neuropsychiatric disorders are the leading cause of mental and intellectual disabilities worldwide. Current therapies against neuropsychiatric disorders are very limited, and very little is known about the onset and development of these diseases, and their most effective treatments. MIR137 has been previously identified as a risk gene for the etiology of schizophrenia, bipolar disorder, and autism spectrum disorder. Here we generated a forebrain-specific MIR137 knockout mouse model, and provided evidence that loss of miR-137 resulted in impaired homeostasis of potassium in mouse hippocampal neurons. KCC2, a potassium-chloride co-transporter, was a direct downstream target of miR-137. The KCC2 specific antagonist VU0240551 could balance the current of potassium in miR-137 knockout neurons, and knockdown of KCC2 could ameliorate anxiety-like behavior in MIR137 cKO mice. These data suggest that KCC2 antagonists or knockdown might be beneficial to neuropsychiatric disorders due to the deficiency of miR-137.

**Key words:** MIR137, KCC2, Anxiety, Potassium

Genome-wide association studies (GWAS) have identified MIR137 (encoding microRNA-137) as a risk gene for the etiology of schizophrenia [3], bipolar disorder [4], and autism spectrum disorders [5]. MIR137 is a key regulator in neurodevelopment, with deletion of Mir137 in the germline or nervous system resulting in embryonic or postnatal lethality [6]. Growing evidence supports the idea that microRNA-137 (miR-137) is a critical epigenetic modulator in neurogenesis, synaptogenesis, and synaptic plasticity [7-10]. Only a few important target genes of miR-137 that might be responsible for neuropsychiatric dysfunction, such as BDNF, CACNA1C, EZH2, PDE10A, TCF4, and ZNF804A, have been experimentally validated [11, 12].

Neural activity depends on electric signals that are transmitted from the presynaptic neuron to the postsynaptic cell via chemical signaling. The positive or negative change in membrane potential of the postsynaptic neuron is caused by the activation of postsynaptic receptors, which are ion channels whose activation alters...
permeability for specific ions [13]. Although genetic variation in genes coding for ion channels increases risk for psychiatric disorders [14-17], little is known about the function of miR-137 on ion channels in neurons.

In this study, we provide the first evidence that loss of miR-137 results in impaired homeostasis of potassium in neurons, both in vitro and in vivo. KCC2 (Slc12a5), a potassium-chloride co-transporter, was significantly upregulated in miR-137 knockout neurons. Importantly, knockdown of KCC2 could rescue anxiety-like phenotype in MIR137 cKO mice. These results suggest that miR-137 loss of function contributes to potassium efflux via KCC2, and targeting the miR-137-KCC2 pathway might have great therapeutic potential for treating neuropsychiatric disorders due to the deficiency of miR-137.

MATERIALS AND METHODS

Mice

All experiments involving animals were performed in accordance with the animal protocol approved by the Institutional Animal Care and Use Committee at the Institute of Zoology, Chinese Academy of Sciences. Mice were housed in groups of 3-5 animals under a 12 h light/12 h dark cycle, and were fed ad libitum on a standard mouse diet. The miR-137 flox/flox mice were generated as previously described [10]. The Emx1-Cre transgenic mice were bought from Jackson Laboratory (Stock No. 005628). The miR-137 conditional knockout mice were generated by breeding miR-137 flox/flox mice with Emx1-Cre transgenic mice, as described previously [19].

Primary hippocampal neuron culture

Hippocampal neurons were isolated from P0 MIR137 cKO and WT mice, and cultured on plates (1×10^4 cells per well in a 24-well plate) coated with poly-D-lysine (100 μg/ml). The dissected hippocampus tissue was digested with trypsin-EDTA for 10 min at 37°C, and then washed three times with MEM containing 10% FBS. Hippocampal neurons were then dissociated with the culture medium. Then neurons were grown in Neurobasal medium (Invitrogen) supplemented with 2% B27 (Invitrogen), 2 mM GlutaMAX (Invitrogen), and penicillin/streptomycin.

Dual luciferase assays

Approximately 300 base pairs around the predicted target site from the KCC2 3’ UTR was cloned into the pIS2 vector using the XhoI and NotI restriction sites in the multiple cloning region downstream of the luciferase reporter gene. Mutagenesis of the binding site on KCC2 3’UTR was performed using the Quick-Change II Site-directed Mutagenesis Kit (Stratagene, La Jolla, CA) according to the manufacturer’s protocol. All plasmid clones were then verified by sequencing.

Dual luciferase transfection assays were performed as previously described [20, 21]. In brief, HEK293 cells in 24-well plates were transfected with sh-miR-137 (pCR2.1 TOPO vector) and pIS2-3’UTR or mutated pIS2-3’UTR using Lipofectamine 2000 (Invitrogen). Meanwhile, pIS2 vector with no 3’UTR was cotransfected with U6-neg-shRNA (pCR2.1 TOPO vector) or sh-miR-137 to set up as a control. All Luciferase readings were recorded using Dual-Luciferase Reporter 1000 System (Promega) following manufacturer’s instructions.

Electrophysiological recordings

Whole-cell patch-clamp recordings were carried out using an Axopatch 700B amplifier (Axon Instruments, Union City, CA). The pClamp10.6 software was used for data acquisition and analysis. Patch pipettes (6–10 MΩ) were pulled from borosilicate glass capillaries with a micropipette puller (Sutter instrument, USA).

The internal pipette solution contained (in mM): 135 K-gluconate, 10 HEPES, 2 MgCl₂, 10 EGTA, 0.3 MgGTP, and 0.5 Na₂ATP (pH 7.3 with KOH). The membrane potential was held at -65 mV. Series resistances and cell capacitance compensation were carried out prior to recording. The recordings were included only in those with high resistance seal (>1 GΩ) and a series resistance <25 MΩ.

RNA extraction and qRT-PCR

Total RNA was extracted from hippocampus tissue or cultured neurons using TRIzol reagent (Invitrogen). Two micrograms of total RNA were reverse transcribed with either oligo (dT) primers or specific primers by a Transcriptor First Strand cDNA Synthesis Kit (Roche). For qRT-PCR analysis, 25 ng of cDNA and 0.5 μM primers were used in a final volume of 20 μl according to the manufacturer’s instructions (SYBR Green Master, Roche). Each reaction was run in triplicate and analyzed following the △△CT method using U6 or GAPDH as a normalization control. The following primers are used: KCC2 (forward: 5’-GGGCAGAGAGTACGATGGC-3’; reverse: 5’-TGGGGTAGGTTGGTGTAGTTG-3’), GAPDH (forward: 5’-AAGGTCATCCCAGAGCTGAA-3’; reverse: 5’-AGGAGACACCTGGTGCTCTA-3’).

Protein quantification

Hippocampal tissues or cultured neurons were lysed in a buffer containing 25 mM HEPES at pH7.9, 150 mM NaCl, 1 mM PMSF, 20 mM NaF, 1 mM DTT, 0.1% NP40, and protease inhibitor cocktails (Roche). Protein concentrations were determined by

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Folin phenol method with bovine serum albumin as protein standard. Twenty micrograms of protein were separated on 8–12% SDS-PAGE gels (Bio-Rad) and transferred to PVDF membranes (Millipore). The membranes were then blocked in 5% BSA in TBS-T with 0.05% Tween-20, and incubated with primary antibodies at 4°C overnights. Dilutions of primary antibodies were 1:1,000 for KCC2 (Millipore, #07–432), and 1:10,000 for β-actin antibody (Sigma). As for the secondary antibodies, we used HRP-linked goat anti-rabbit at 1:500. Enhanced chemiluminescence (ECL, Pierce) was used for detection. Quantifications of Western blots were determined using Quantity One V4.4.0 (BioRad).

Immunohistochemistry
Mice were anesthetized and transcardially perfused with cold PBS, followed by 4% PFA in PBS (pH 7.4). Brain tissue was dissected out, equilibrated in 30% sucrose, and sectioned into 40 μm-thick sections. The brain sections were washed in PBS for 15 min three times, and then blocked in a blocking solution (3% BSA, 0.3% Triton X-100, 0.2% sodium azide) at room temperature for 1 h. The primary antibodies we used were as follows: anti-KCC2 (1:1,000, Millipore, #07–432), anti-Map2 (1:1,000, Millipore, Mab3418). After overnight incubation with primary antibody at 4°C, the brain sections were washed with TBS for 30 min three times and then incubated with the secondary antibodies conjugated with Alexa Fluor 488 or 594 (1:500). Sections were finally stained with DAPI and mounted on glass-slides using adhesion anti-fade medium.

Lentivirus production and in vivo grafting
KCC2 shRNA sequence (CUACGAGAA GACAUUAGUA) [22] was inserted in the U6-shRNA lentiviral construct. Lenti-sh-KCC2 and lenti-sh-Neg (negative control) viruses were produced with titers at a range around 1×10^9 TU/ml as described previously [23, 24]. Lentivirus was grafted stereotaxically into the hippocampus of 8-week-old male MIR137 WT and cKO mice as described by Heldt et al. [25].

Behavioral tests
Mice were kept in groups of 4–5 animals on a 12:12 h light-dark cycle. The open field test and the light-dark preference test were performed during the light phase at week 3 after lentiviral injection as previously described [19]. Videos were recorded and analyzed by the software Smart V3.0.03 (Panlab, Barcelona, Spain).

Statistical analyses
Either unpaired Student’s two-tailed t tests or ANOVA with Tukey’s post hoc tests were conducted using IBM SPSS Statistics V26 software. Samples sizes were provided in each figure legend. All data were presented as mean±SEM. Differences were considered statistically significant when p<0.05.

RESULTS

Loss of miR-137 impairs the homeostasis of potassium in primary hippocampal neurons
We originally generated miR-137 conditional knockout mice that displayed dysregulated synaptic plasticity, repetitive behavior, anxiety-like behavior, and impaired learning and social behavior [10]. Since neurological and neuropsychological diseases are pathophysiologically linked to potassium channel dysfunction [26, 27], we speculated that loss of miR-137 may play a role in regulating K+ currents and thus result in neurodysfunction.

To examine the electrophysiological properties of primary hippocampal neurons isolated from MIR137 cKO and WT mice, we performed whole-cell patch-clamp recording of the electrical activity of neurons at 14 days in culture (Fig. 1A). We found that all primary hippocampal neurons elicited multiple action potentials upon the injection of depolarizing currents (Fig. 1B) (WT, n=9; cKO, n=10) and large fast-inactivating inward currents followed by outward potassium currents when evoked by a series of voltage steps (Fig. 1C) (WT, n=12; cKO, n=13). We observed no significant difference in spike frequency of action potential (Fig. 1D, resting membrane potential (RMP) (Fig. 1E), membrane resistance (Rm) (Fig. 1F), and membrane capacitance (Cm) (Fig. 1G) between MIR137 cKO and WT neurons. However, the peak of the voltage-gated K+ current was significantly higher in MIR137 cKO neurons (Fig. 1H and 1I). These data indicate that the deletion of MIR137 impairs the homeostasis of potassium in hippocampal neurons.

K+–Cl cotransporter 2 (KCC2) is a direct downstream target of miR-137
To get more insight into the molecular mechanism underlying the effect of miR-137 loss-of-function on voltage-gated K+ current, we performed a combined computational and experimental study to identify the downstream targets of miR-137 in the brain. We first used the TargetScan program to predict mRNA targets [28]. TargetScan analysis identified 15 potassium channel associated targets predicted to be responsive to miR-137 and conserved among species. Among these candidate targets, the K+–Cl cotransporter 2 (aka: KCC2 and SLC12A5) is known to play pivotal roles in the physiology of neurons, and its malfunction has been linked to multiple neurological diseases including seizures, epilepsy, and schizophrenia [29–33]. Indeed, there is a highly conserved binding site of miR-137 on the 3’-UTR sequence of mouse Kcc2 mRNA.

Statistical analyses
Either unpaired Student’s two-tailed t tests or ANOVA with Tukey’s post hoc tests were conducted using IBM SPSS Statistics V26 software. Samples sizes were provided in each figure legend. All data were presented as mean±SEM. Differences were considered statistically significant when p<0.05.
MiR-137-KCC2 Axis in K⁺ Homeostasis

Fig. 1. MiR-137 deficiency impairs K⁺ efflux of potassium in primary mouse hippocampal neurons. (A) Phase-contrast images of primary mouse hippocampal neurons during patch clamp recordings at DIV 14. (B) Representative traces of action potentials in response to step current injections in primary mouse hippocampal neurons at DIV 14. Membrane potential was maintained at approximately -40 mV. Step currents were injected from -50 pA to +250 pA in 50 pA increments (middle panel). All neurons elicited multiple action potentials upon the injection of depolarizing currents. (C) Representative traces of whole-cell currents in voltage-clamp mode. Primary neurons from MIR137 WT or cKO mice were held at -70 mV. Step depolarization from -80 mV to +60 mV at 10-mV intervals was delivered (middle panel). Insets were respective traces on an expanded scale. (D) Characterization of action potentials generation properties in terms of spikes frequency with current-pulse amplitude. Recordings from primary mouse hippocampal neurons of MIR137 WT or cKO mice (n=12 and 9, respectively). (E–G) Statistics of intrinsic membrane properties of MIR137 WT and cKO neurons (n =14 and 9, respectively). RMP, resting membrane potential (E); Rin, membrane input resistance (F); Cm, capacitance (G). (H) The relationship between voltage and K⁺ current (WT, n=12; cKO, n=8). (I) The peak amplitude of K⁺ current was elevated in MIR137 cKO neurons. *p<0.05.

(position 2229-2235; Fig. 2A). To determine whether miR-137 directly targets Kcc2 mRNA, we cloned the wildtype or mutated 3'-UTR of Kcc2 containing the predicted miR-137 target site into a Renilla luciferase reporter construct, which allowed us to examine KCC2 protein translation by measuring luciferase activities. We found that miR-137 could significantly repress the expression of Renilla luciferase through the 3'-UTR of Kcc2, while miR-137 had no effect on luciferase activity when the conserved binding site was mutated (Fig. 2B).

Next, we examined the expression levels of KCC2 in MIR137 cKO and WT brains. Elevated KCC2 mRNA expression levels were observed in the hippocampus and prefrontal cortex of MIR137 cKO mice compared to their WT littermates (Fig. 2C). Furthermore, our western blot analysis demonstrated that KCC2 protein levels were dramatically increased in the hippocampus and prefrontal cortex of MIR137 cKO mice compared to their WT littermates (Fig. 2D). Consistently, immunostaining of cultured hippocampal neurons at DIV 14 with antibodies against KCC2 revealed enhanced KCC2 immunoreactivity in MIR137 cKO neurons (Fig. 3A and 3B). Moreover, KCC2 immunohistochemistry analysis showed a stronger fluorescence intensity in brain sections including hippocampus (Fig. 3C and 3D) and cortex (Fig. 3E and 3F) of MIR137 cKO mice compared to their WT littermates. Taken together, these data support the idea that Kcc2 is a direct downstream target of miR-137 in neurons.
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KCC2 antagonist maintains potassium homeostasis in MIR137 cKO neurons

To further explore the role of KCC2 in the electrophysiological properties of neurons, we used a pharmacological agent, the KCC2 antagonist VU0240551, to intervene the function of KCC2 by preincubating neurons with the agent for 1 hour (10 μM), and performed the whole-cell patch-clamp recordings of primary hippocampal neurons at DIV 14. All primary hippocampal neurons elicited multiple action potentials upon the injection of depolarizing currents (Fig. 4A) and large fast-inactivating inward currents followed by outward potassium currents when evoked by a series of voltage steps (Fig. 4B). We then assessed membrane properties of hippocampal neurons that treated with VU0240551 by recording voltage-dependent currents in voltage-clamp mode. We found that maximum peak outward potassium amplitude of VU0240551-treated MIR137 cKO neurons were adjusted to the similar level of WT neurons (Fig. 4C and 4D). VU0240551 did not affect the resting membrane potential (RMP) (Fig. 4E), membrane resistance (Rin) (Fig. 4F), and membrane capacitance (Cm) (Fig. 4G) of hippocampal neurons. Therefore, these data indicate that KCC2 antagonist could maintain potassium homeostasis in MIR137 cKO neurons.

Knockdown of KCC2 ameliorates anxiety-like behavior in MIR137 cKO mice

Recently, we found that mice with forebrain-specific miR-137

Fig. 2. KCC2 is a direct target of miR-137. (A) Sequence alignment of miR-137 and the KCC2 3’-UTR, which contains a predicted conserved miR-137-binding site. The seed-recognizing site in the KCC2 3’-UTR is indicated in red, while the mutant KCC2 3’-UTR site is denoted in green. (B) KCC2 WT 3’-UTR-dependent expression of a Renilla luciferase was reduced, while mutation of the miR-137 binding site in the KCC2 3’-UTR did not affect the Renilla luciferase activity. (C) KCC2 mRNA expression levels were upregulated both in the hippocampus and in the prefrontal cortex of MIR137 cKO mice. (D) KCC2 protein expression levels were upregulated both in the hippocampus and in the prefrontal cortex of MIR137 cKO mice. Left panel, representative images of Western blot. Right panel, quantifications of Western blots with ImageJ. n=3 independent experiments; n.s non-significant. *p<0.05, **p<0.01.
loss-of-function can survive to adulthood, but exhibit anxiety-like behavior [19]. To examine whether knockdown of KCC2 might be beneficial to ameliorate anxiety-like behavior in MIR137 cKO mice, we injected lenti-sh-KCC2 virus into the hippocampus and performed behavioral assays at week 3 after grafting.

In the open field test, sh-KCC2 significantly ameliorates the anxiety-like behavior in MIR137 cKO mice, as indicated by increased numbers of entries (Fig. 5A) and time spent (Fig. 5B) in the central zone. In consistent with this, the light-dark box test showed that sh-KCC2 did improve entries (Fig. 5C) and time spent (Fig. 5D) in light compartment in MIR137 cKO mice.

DISCUSSION

Dysfunction of miR-137 has been linked with the pathogenesis of schizophrenia [3], bipolar disorder [4], anxiety and depression [19], and autism spectrum disorders [5, 10]. Fine-tuning the expression of miR-137 is critical in regulating neural development and synaptic plasticity [9, 20, 34]. Overexpression of miR-137 results in changes in synaptic vesicle pool distribution, impaired induction of mossy fiber long-term potentiation and deficits in hippocampus-dependent learning and memory [9]. He et al. [34] then confirmed these observed changes in synaptic transmission upon miR-137 overexpression. Although selective synaptic vesicle docking defects were not obtained, miR-137 overexpression had remarkable effects on docking, active zone length and total vesicle number [34]. Syt1, complexin-1 and neuroligin-3 are known miR-137 targets involved in synapse development [9, 34]. In contrast, a complete knockout of MIR137 in the mouse germline or nervous system leads to postnatal lethality [10, 35], while heterozygous knockout mice display synapse overgrowth, dysregulated synaptic plasticity, repetitive behavior, and anxiety-like behaviors [10, 19]. PDE10a and EZH2 are key experimentally validated targets of miR-137, and knockdown of PDE10a or EZH2 ameliorates the deficits observed in the heterozygous knockout mice [10, 19]. Here we provide the first evidence that miR-137 also plays an impor-
tant role in the homeostasis of potassium in neurons at least by targeting KCC2, which is a neuron-specific chloride–potassium cotransporter and is highly expressed in the mature CNS [36]. KCC2 mutations or dysfunctions have been identified as a critical component in the development of autism spectrum disorder [37], schizophrenia, epilepsy and seizures [38-42], neuropathic pain [43]. KCC2 has been well-known for its role in maintaining a low intracellular Cl\(^{-}\) concentration ([Cl\(^{-}\)]\(_i\)) essential for hyperpolarizing inhibition mediated by GABAA receptors [44], its loss-of-function results in enhanced [Cl\(^{-}\)], which thereby represses the

**Fig. 4.** KCC2 antagonist maintains potassium homeostasis in mouse MIR137 cKO neurons. (A) Representative traces of action potentials in response to step current injections in primary neurons at DIV-14. Membrane potential was maintained at approximately –65 mV. Step currents were injected from -50 pA to +300 pA in 50 pA increments (bottom panel). WT, n=21; cKO, n=32; cKO+VU0240551, n=26. (B) Representative traces of whole-cell currents in voltage-clamp mode. Primary neurons were held at -70 mV. Step depolarization from -80 mV to +60 mV at 10-mV intervals was delivered (bottom panel). Insets showing respective traces on an expanded scale. WT, n=23; cKO, n=28; cKO+VU0240551, n=33. (C) The relationship between voltage and K\(^{+}\) current. (D) The peak amplitude of K\(^{+}\) current was reduced to normal in MIR137 cKO neurons that were treated with VU0240551, a specific antagonist for KCC2. (E-G) Statistics of intrinsic membrane properties in MIR137 WT and cKO hippocampal neurons treated with or without VU0240551. RMP, resting membrane potential (E); Rin, membrane input resistance (F); Cm, capacitance (G). *p<0.05, **p<0.01.

**Fig. 5.** KCC2 knockdown rescues anxiety-like behaviors in MIR137 cKO mice. (A-B) In the open field test, MIR137 cKO mice with sh-KCC2 treatment dramatically increased the number of entries (A) and time spent (B) in the central zone (n=12–13 mice per group, *p<0.01, **p<0.005). (C, D) In the light/dark box test, sh-KCC2-treated mice demonstrated improved entries (C) and time spent (D) in light box (n=12–13 mice per group, *p<0.01, **p<0.005).
inhibitory strength of GABA and glycine, whose cognate receptors are ligand-gated ion channels permeable to Cl\(^-\) and HCO\(_3\)\(^-\) ions [45-47]. Besides its central role in hyperpolarizing inhibitory signaling based on chloride currents which are mediated by GABA- or glycine-gated receptor channels, KCC2 also acts a structural protein crucially involved in the maturation and regulation of excitatory glutamatergic synapses [48-51]. KCC2 is required for neuronal maturation by rendering GABA hyperpolarizing [49, 52-54], and overexpression of KCC2 enhances dendritic spines in the adult nervous system in mice [55]. Therefore, we speculate that the elevated expression of KCC2 may also contribute to dysregulated synaptic plasticity and altered behaviors in MIR137 cKO mice.

Potassium is essential for the proper function of all cells [56]. In neurons, the sodium-potassium flux generates the electrical potential that aids the conduction of nerve impulses [56]. Potassium channels participate in cell ionic balance and serve the fundamental function of supporting action potentials and electrical signal propagation along the neurons and their myelinated axons [57-59]. The increased outward K\(^+\) currents in MIR137 cKO mouse neurons were just indirectly measured by mediating voltage gated K\(^+\) channels in this study, lacking evidences showing the decreased intracellular Cl\(^-\) concentration. The increased K\(^+\) currents in MIR137 cKO neurons might reflect a direct modulation of leak K\(^+\) channels [60]. A recent study reported that KCC2 interacts with the leak K\(^+\) channel TASK-3, and promotes its membrane expression, which results in altered neuronal excitability in rat dentate granule cells [61]. The increased outward K\(^+\) currents in MIR137 cKO neurons might also reflect a direct modulation of leak K\(^+\) channels (e.g. TASK-3) by up-regulated KCC2, not only altered (i.e., decreased) intracellular K\(^+\) concentration.

Surprisingly, increased K\(^+\) currents in MIR137 cKO mouse neurons in our study did not result in a decrease in action potential firing in these cells as compared to the WT neurons. This could be explained by postulating that primary cultured hippocampal neurons constitute a developmentally and functionally heterogeneous cell population which might not be a good model to examine action potentials. For example, a recent study reported that the KCC2 inhibitor increases the firing rate of P0–P2 rat hippocampal CA3 pyramidal neurons in the absence of glutamatergic transmission, but no change in the output of interneurons synapsing was observed onto pyramidal neurons [62]. Furthermore, we cannot exclude the possibility that other unknown targets of miR-137 might play an opposite role in action potentials. For instance, NKCC1 (Slc12a2) is a predicted target of miR-137 by TargetScan algorithm [63]. In mature neurons, NKCC1 and KCC2 respectively mediate inward and outward cotransport of chloride and potassium ions under basal conditions [60]. Whether NKCC1 and other iron transporters also affect the homeostasis of potassium in MIR137 cKO neurons remains to be investigated.

In summary, we show here that miR-137 is a crucial player in the homeostasis of potassium by directly targeting KCC2, and treatment with the KCC2 antagonist can maintain potassium homeostasis in MIR137 knockout hippocampal neurons. Moreover, knockdown of KCC2 ameliorates anxiety-like behavior in MIR137 cKO mice. Considering drugs frequently have ‘hidden phenotypes’ that result from their binding to unknown targets or from unknown interactions between the intended drug target and other biochemical pathways [64], the safety and efficiency of targeting the miR-137-KCC2 pathway in treating neurological disorders should be the focus of future studies.

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