

Haploinsufficiency of *Cyfp2* Causes Lithium-Responsive Prefrontal Dysfunction

Seung-Hyun Lee, PhD,^{1#} Yinhua Zhang, BSc,^{2,3#} Jina Park, PhD,⁴ Bowon Kim, PhD,⁴
Yangsik Kim, MD, PhD,^{5,6} Sang Hoon Lee, PhD,⁷ Gyu Hyun Kim, MSc,⁷
Yang Hoon Huh, PhD,⁸ Bokyoung Lee, MSc,² Yoonhee Kim, MSc,^{2,3}
Yeunkum Lee, PhD,^{2,3} Jin Yong Kim, MD, PhD,^{3,9} Hyojin Kang, PhD,¹⁰
Su-Yeon Choi, PhD,^{5,6} Seil Jang, PhD,^{5,6} Yan Li, PhD,^{5,6} Shinhyun Kim, MSc,^{2,3}
Chunmei Jin, MSc,^{2,3} Kaifang Pang, PhD,^{11,12} Eunjeong Kim, PhD,¹³ Yoontae Lee, PhD,¹³
Hyun Kim, MD, PhD,^{3,9} Eunjoon Kim, PhD,^{5,6} Jee Hyun Choi, PhD,⁴ Jeongjin Kim, PhD,⁴
Kea Joo Lee, PhD,^{7,14} Se-Young Choi, PhD,¹ and Kihoon Han, PhD^{2,3}

Objective: Genetic variants of the cytoplasmic FMR1-interacting protein 2 (*CYFIP2*) encoding an actin-regulatory protein are associated with brain disorders, including intellectual disability and epilepsy. However, specific *in vivo* neuronal defects and potential treatments for *CYFIP2*-associated brain disorders remain largely unknown. Here, we characterized *Cyfp2* heterozygous (*Cyfp2*^{+/-}) mice to understand their neurobehavioral phenotypes and the underlying pathological mechanisms. Furthermore, we examined a potential treatment for such phenotypes of the *Cyfp2*^{+/-} mice and specified a neuronal function mediating its efficacy.

Methods: We performed behavioral analyses of *Cyfp2*^{+/-} mice. We combined molecular, ultrastructural, and *in vitro* and *in vivo* electrophysiological analyses of *Cyfp2*^{+/-} prefrontal neurons. We also selectively reduced *CYFIP2* in the prefrontal cortex (PFC) of mice with virus injections.

Results: Adult *Cyfp2*^{+/-} mice exhibited lithium-responsive abnormal behaviors. We found increased filamentous actin, enlarged dendritic spines, and enhanced excitatory synaptic transmission and excitability in the adult *Cyfp2*^{+/-} PFC that was restricted to layer 5 (L5) neurons. Consistently, adult *Cyfp2*^{+/-} mice showed increased seizure susceptibility and auditory steady-state responses from the cortical electroencephalographic recordings. Among the identified prefrontal defects, lithium selectively normalized the hyperexcitability of *Cyfp2*^{+/-} L5 neurons. RNA sequencing revealed reduced expression of potassium channel genes in the adult *Cyfp2*^{+/-} PFC. Virus-mediated reduction of *CYFIP2* in the PFC was sufficient to induce L5 hyperexcitability and lithium-responsive abnormal behavior.

Interpretation: These results suggest that L5-specific prefrontal dysfunction, especially hyperexcitability, underlies both the pathophysiology and the lithium-mediated amelioration of neurobehavioral phenotypes in adult *Cyfp2*^{+/-} mice, which can be implicated in *CYFIP2*-associated brain disorders.

ANN NEUROL 2020;88:526–543

View this article online at [wileyonlinelibrary.com](https://www.wileyonlinelibrary.com). DOI: 10.1002/ana.25827

Received Oct 7, 2019, and in revised form May 22, 2020. Accepted for publication Jun 14, 2020.

Address correspondence to Professor Lee, Neural Circuits Research Group, Brain Research Institute, Daegu 41062, South Korea. E-mail: relaylee@kbri.re.kr; or Professor Choi, Department of Physiology, Dental Research Institute, Seoul National University School of Dentistry, Seoul 03080, South Korea.

E-mail: sychoi@snu.ac.kr; or Professor Han, Department of Neuroscience, College of Medicine, Korea University, Seoul 02841, South Korea.

E-mail: neurohan@korea.ac.kr

[#]Seung-Hyun Lee and Yinhua Zhang share first authorship.

From the ¹Department of Physiology, Dental Research Institute, Seoul National University School of Dentistry; ²Department of Neuroscience, College of Medicine, Korea University; ³Department of Biomedical Sciences, College of Medicine, Korea University; ⁴Center for Neuroscience, Korea Institute of Science and Technology, Seoul; ⁵Center for Synaptic Brain Dysfunctions, Institute for Basic Science, Daejeon; ⁶Department of Biological Sciences, Korea Advanced Institute of Science and Technology, Daejeon; ⁷Neural Circuits Research Group, Korea Brain Research Institute, Daegu; ⁸Center for Electron Microscopy Research, Korea Basic Science Institute, Chungcheongbuk-do; ⁹Department of Anatomy, College of Medicine, Korea University, Seoul; ¹⁰Division of National Supercomputing, Korea Institute of Science and Technology Information, Daejeon, South Korea; ¹¹Department of Pediatrics, Baylor College of Medicine; ¹²Jan and Dan Duncan Neurological Research Institute, Texas Children's Hospital, Houston, TX; ¹³Department of Life Sciences, Pohang University of Science and Technology, Pohang; and ¹⁴Department of Brain and Cognitive Sciences, Daegu Gyeongbuk Institute of Science & Technology, Daegu, South Korea

Additional supporting information can be found in the online version of this article.

Two evolutionarily conserved members of the cytoplasmic FMR1-interacting protein (CYFIP) gene family, *CYFIP1* and *CYFIP2*, are associated with various brain disorders.^{1,2} Specifically, deletions and duplications of *CYFIP1*, which is located within the 15q11.2 breakpoints BP1–BP2 of the Angelman/Prader–Willi syndrome region,³ are associated with intellectual disability (ID), autism spectrum disorders (ASDs), and schizophrenia (SCZ).^{2,4} Regarding *CYFIP2*, interstitial deletions of the chromosomal region 5q33.3–q35.1 harboring *CYFIP2* (ie, haploinsufficiency) were identified in individuals with developmental delay, mild to severe ID, and seizures.^{5,6} Moreover, recent whole-exome and whole-genome sequencing studies identified de novo *CYFIP2* missense variants in patients with developmental delay, early-onset epileptic encephalopathy, and severe ID.^{7–10}

CYFIP1 and *CYFIP2* have high sequence homology and are considered to have similar functions at the molecular level. Both proteins were originally identified as direct binding partners of the fragile X mental retardation protein (FMRP),¹ an RNA-binding protein whose loss causes fragile X syndrome (FXS), the most common inherited cause of ID.¹¹ Moreover, *CYFIP1/2* is a key component of the heteropentameric Wiskott–Aldrich syndrome protein family verprolin-homologous protein (WAVE) regulatory complex (WRC) that regulates actin cytoskeleton dynamics in diverse cellular compartments, including neuronal synapses.¹²

Despite these molecular functions, in vivo neurobiological mechanisms underlying *CYFIP*-associated brain disorders remain largely unknown and have thus far been investigated mainly for *CYFIP1*.^{13–15} Moreover, *CYFIP1* and *CYFIP2* have distinct expressions and functions in vivo,^{16–19} strongly encouraging further investigation of *CYFIP2* in brain function and dysfunction. To address this, we combined molecular, ultrastructural, and in vitro and in vivo electrophysiological analyses in *Cyfp2* heterozygous (*Cyfp2*^{+/-}) mice to identify specific brain regions, neuronal subtypes, and their functions involved in abnormal behavior and seizure susceptibility of the mice. Furthermore, we examined a potential treatment for such neurobehavioral phenotypes of *Cyfp2*^{+/-} mice and identified a neuronal function that mediated its efficacy.

Materials and Methods

Mice

Cyfp2^{+/-} mice have been described previously.²⁰ Floxed *Cyfp2* (*Cyfp2*^{fl/fl}) mice were generated by injecting *Cyfp2*^{tm1a(EUCOMM)Wtsi} embryonic stem cells (European Mutant Mouse Archive [EMMA]). All mice were maintained on a C57BL/6J background according to the Korea University College of Medicine

Research Requirements, and procedures were approved by the Committees on Animal Research at Korea University College of Medicine (KOREA-2016-0066). Unless otherwise specified, only male mice were used for experiments.

Behavioral Assays

All data acquisition and analyses were performed blinded to genotype. The open field test (OFT) was performed as described previously.²⁰ To evaluate lithium response, mice received an intraperitoneal injection of saline or 50mg/kg lithium 30 minutes before measurement of activity. The elevated plus maze (EPM) test was performed as described previously.²¹ The tail suspension test (TST) was performed as described previously.²²

In Situ Hybridization

Hybridization probes for *Cyfp1* and *Cyfp2* mRNAs were prepared by cloning of the nucleotides 4108–4557 of *Cyfp1* (NM_011370.3) and nucleotides 3113–4063 and 4215–4766 of *Cyfp2* (NM_001252460.1) into the pGEM-T Easy vector (Promega), respectively. Antisense riboprobes were generated using ³⁵S-UTP and the Riboprobe system (Promega).

RNA Sequencing and Analysis

RNA sequencing of the prefrontal cortex (PFC) and bioinformatic analyses were performed as described previously.²³ Illumina HiSeq 2000 was used for sequencing to generate 101-bp paired-end reads (Table S1). Raw data were submitted to the gene expression omnibus (GEO) repository under accession number GSE129121.

RNA Purification and qRT-PCR

Real-time quantitative reverse transcription PCR (qRT-PCR) was performed as described previously.²³ Primers were as follows: *Actb*, 5'-AAGACCTGTACGCCAACACA-3' and 5'-GCAGT GATCTCCTTCTGCATC-3'; *Cyfp1*, 5'-GGTTATGGCAGG AAGTTTGC-3' and 5'-GATCGTGGCTCCCTGATTT-3'; *Cyfp2*, (exon 6–7) 5'-GCGGTCTTGGACGAGCTAAAG-3' and 5'-GGGTGATCCTGTTGTGATTGG-3'; *Cyfp2*, (exon 8–9) 5'-AGATGTACCTGACGCCAGT-3' and 5'-TGAC ATTTCCGTCCATCAGA-3'; *Gapdh*, 5'-GGCATTGCTCT CAATGACAA-3' and 5'-CCCTGTTGCTGTAGCCGTAT-3'; *Kcnd1*, 5'-GCGCTTTGAGACCTGGAAAAA-3' and 5'-GGCCAGTGCGATAAAAAGTTTCAG-3'; *Kcng4*, 5'-CGCAG CCATGAGGAGATCAC-3' and 5'-GCCAGGAAGCTCACG ATCAC-3'; *Kcnh4*, 5'-GGGGTTTTCCCATCGTCTACT-3' and 5'-AGCAGATTTTCAGTTCGGTGTTC-3'; *Kcnip3*, 5'-TG AAGGCATCAGATGGCAACC-3' and 5'-CCGTGGATAACT CCAGTTCACT-3'; *Kcnk2*, 5'-CCGAGGCTCTCATTCTCC TCA-3' and 5'-AGGACGACCACCAGGAAAAATC-3'; and *Wasf1*, 5'-GAAAGTGCCAAGAGCACCTC-3' and 5'-CACGT ATGTCTGTGGCCTTG-3'.

Western Blotting

Mouse brain whole lysates and subcellular fractions were prepared as described previously.²⁰ Antibodies against β -actin (Santa Cruz, sc-47778, 1:5000), phospho-Akt (Cell Signaling Technology, #2965, 1:1000), Akt (Cell Signaling Technology, #4691, 1:1000), phospho-Cofilin (Cell Signaling, #3311, 1:1000), Cofilin (Abcam, AB42824, 1:1000), CYFIP1 (Millipore, AB6046, 1:1000), CYFIP2 (Abcam, ab95969, 1:2000), phospho-ERK (Cell Signaling Technology, #4370, 1:2000), ERK (Cell Signaling Technology, #4695, 1:1000), GAPDH (Cell Signaling, #2118, 1:3000), phospho-GSK3 (Cell Signaling Technology, #9336, 1:1000), GSK3 (Millipore, 05-412, 1:2000), Kcnd1 (Alomone Labs, APC-119, 1:400), Kcnp3 (NeuroMab, 75-005, 1:1000), phospho-PAK (Abcam, AB40795, 1:5000), PAK (Abcam, AB196834, 1:500), PSD-95 (ThermoFisher Scientific, MA1-046, 1:1000), Rac1 (Sigma-Aldrich, #05-389, 1:1000), phospho-WAVE1 (ECM Biosciences, WP1771, 1:1000), and WAVE1 (NeuroMab, 75-048, 1:1000) were used. Images were acquired by ChemiDoc Touch Imaging System (Bio-Rad) and quantified using ImageJ.

Immunohistochemistry

Brain sections were prepared as described previously.²⁴ Antibodies against β -galactosidase (Promega, Z3781, 1:1000), CTIP2 (Abcam, ab18465, 1:250), CYFIP2 (Abcam, ab95969, 1:200), GFP (Abcam, ab13970, 1:1000), MBP (Biolegend, 808401, 1:2000), NeuN (Millipore, MAB377, 1:1000), NF-H (Abcam, ab8135, 1:1000), parvalbumin (Swant, PV235, 1:5000), Alexa Fluor 488 phalloidin (Invitrogen, A12379, 1:40), and Alexa Fluor-conjugated secondary antibodies (Jackson Immuno-research, 111-545-003 and 115-585-003, 1:500) were used. Confocal microscopy (Zeiss, LSM800) was used to acquire images of the prelimbic medial PFC (mPFC; 3.00–1.77mm anterior to bregma) and of the somatosensory cortex (0.95mm posterior to bregma) from coronal sections. Entire regions were obtained by tile scanning, and each frame was taken in Z-stacks of 5–10 slices (45–55 μ m thickness in total). Tiled Z-projection images were aligned and converted into a single flattened image using ZEN software from Zeiss. From each tiled image, three regions of interests (ROIs; 150 μ m $\times$ 150 μ m) were randomly selected from each cortical layer and analyzed using ImageJ software. All quantifications were performed by researchers who were blinded to the genotype and were involved in neither staining nor image acquisition processes.

Electron Microscopy

Brain tissue from two pairs of wild-type (WT) and *Cyfp2*^{+/-} mice was imaged to produce eight serial block-face scanning electron microscopy (SB-SEM) datasets; four sets in layer 2 (L2) and four sets in layer 5 (L5). Mice were perfused with 2% paraformaldehyde and 2.5% glutaraldehyde in 0.15 M cacodylate buffer (pH 7.4). The brains were sliced into 150 μ m-thick coronal sections and dissected into the prelimbic mPFC. The slices were postfixed in 2% OsO₄/1.5% potassium ferrocyanide for 1 hour. Samples were then immersed in 1% thiocarbonylhydrazide (TCH; Ted Pella) in ddH₂O for 20 minutes followed by 2% OsO₄ for

30 minutes. Tissues were incubated in 1% uranyl acetate at 4°C overnight and lead aspartate solution at 60°C for 30 minutes as described previously.²⁵ Samples were dehydrated using a series of ethanol followed by acetone for 10 minutes. Tissues were embedded in 7% (w/v) conductive Epon 812 resin (EMS, Hatfield, PA, USA) mixed with Ketjen black powder.²⁶ Tissue blocks were imaged with a Merlin VP field-emission scanning electron microscopy (SEM; Carl Zeiss) equipped with 3View2 (Gatan). Serial images were acquired with a 30 μ m aperture, high vacuum, voltage of 2.5kV, image size of 5,000 $\times$ 5,000 pixels, and x-y resolution of 12nm at a nominal thickness of 50nm. The section thickness was estimated using the cylindrical diameters method as described previously²⁷ and did not differ from the nominal thickness (50 $\pm$ 1.9nm). Two stacks of 210 serial images were obtained per mouse (37,800 μ m³ per stack), one in L2 and another in L5 of the prelimbic mPFC. Images were processed with ImageJ and Fiji plugins (<http://fiji.sc/wiki/index.php/Fiji>). TrakEM2 was used to assemble images for 3-dimensional (3D) reconstruction.²⁸ We randomly selected spiny dendritic segments with lengths ranging between 10 and 15 μ m. To ensure that our analysis was restricted to pyramidal neurons, we avoided all dendritic segments with few or no spines. A synapse was defined by the presence of a presynaptic bouton with ≥ 3 synaptic vesicles and a postsynaptic density (PSD). All protrusions were divided into spines with synapses and spines lacking synapses ($\sim 13\%$ of all protrusions). In total, 19 dendritic branches were segmented in L5 (10 WT and 9 *Cyfp2*^{+/-}) and 24 in L2 (12 dendrites per group). Dendritic profiles were segmented manually in the reconstruct software (<https://synapseweb.clm.utexas.edu/software-0>) by four annotators blinded to genotype.

Electron Tomography and 3D Reconstruction of Presynaptic Vesicles

Tissues were sectioned (250nm thickness) for electron tomography of the synapses in L5 of the prelimbic mPFC. Sections were imaged under a high-voltage electron microscope at 1,000 kV (JEM-1000BEF; JEOL, Tokyo, Japan). Excitatory synapses were randomly selected, and synapses were tilted from +60 to -60° in increments of 2°. A total of 61 tilt images were recorded by TEM Recorder software (JEOL System Technology, Tokyo, Japan), and digitized tilting images were aligned and tomographically reconstructed using Composer and Visualizer-Kai software (TEMography.com, system in Frontiers Inc., Tokyo, Japan). Virtual slices were extracted from the 3D tomogram, and the boundaries of synaptic vesicles were traced. Surface rendering and 3D modeling were performed using AMIRA software (FEI, Hillsboro, OR).

Stereotaxic Surgery and Microinjection

Adeno-associated virus (AAV)-hSyn-EGFP (Addgene, #50465) and AAV-hSyn-EGFP-Cre (Addgene, #105540) with 1×10^{12} vector genomes/ml titers were injected bilaterally into the mPFC (1.97mm anteroposterior, ± 0.25 mm mediolateral and -2.0mm dorsoventral to bregma). A total volume of 0.2 μ l AAV vectors per side was delivered at a rate of 3nl/second with a glass pipette injector connected to a Micro4 micro-syringe pump

(World Precision Instruments, Sarasota, FL). The injection sites were verified at the end of the experiments.

Slice Electrophysiology

Coronal slices of the mPFC or transverse slices of the hippocampus were prepared (each 300 μ m thick) as described previously.^{29,30} After decapitation, the brains were quickly removed and placed in ice-cold, oxygenated (95% O₂ and 5% CO₂), low-Ca²⁺/high-Mg²⁺ dissection buffer containing (millimolar): 5 KCl, 1.23 NaH₂PO₄, 26 NaHCO₃, 10 dextrose, 0.5 CaCl₂, 10 MgCl₂, and 212.7 sucrose. Slices were transferred to a storage chamber in an incubator containing oxygenated artificial cerebrospinal fluid (ACSF) containing (millimolar): 124 NaCl, 2.5 KCl, 1.23 NaH₂PO₄, 2.5 CaCl₂, 1.5 MgCl₂, 26 NaHCO₃, and 10 dextrose at 28–30°C for $\geq$ 30 minutes before recording. Slices were transferred to a recording chamber, where they were perfused with oxygenated ACSF (23–25°C) at a flow rate of 2 ml/minute. Slices were equilibrated for 5 minutes before recordings. Patch pipettes (4–6 M Ω) were filled with (millimolar): 107 CsMeSO₄, 10 CsCl, 3.7 NaCl, 5 TEA-Cl, 20 HEPES, 0.2 EGTA, 1 Qx-314, 4 Mg-ATP, 0.3 Na-ATP at pH 7.3 and 300–305 mOsm for the miniature excitatory postsynaptic current (mEPSC) experiments; 130 CsCl, 1.1 EGTA, 2 MgCl₂, 0.1 CaCl₂, 10 NaCl, 10 HEPES, and 2 ATP-Na for the miniature inhibitory postsynaptic current (mIPSC) experiments; and 145 potassium gluconate, 5 NaCl, 10 HEPES, 0.2 EGTA, 1 MgCl₂, 2 ATP-Mg, and 0.1 GTP-Na for the excitability experiments at pH 7.4 and 280–290 mOsm. The extracellular recording solution consisted of ACSF supplemented with (micromolar): 100 picrotoxin, 1 TTX, and 50 DL-AP5 for the mEPSC experiments; 1 TTX, 20 CNQX and 50 DL-AP5 for the mIPSC experiments; and 100 picrotoxin for the excitability experiments. Experiments with acute Li⁺ treatment were performed by using ACSF supplemented with 10 mM LiCl or equimolar NaCl. Data were acquired using an EPC-8 amplifier (HEKA), filtered at 3 kHz, digitized at 10 kHz with Digidata 1550B (Axon Instruments), and analyzed using pClamp v.10 (Molecular Devices). Only cells with access resistance <20 M Ω and input resistance >100 M Ω were studied. Cells were discarded if the input or access resistance changed by >20%.

Pentylenetetrazole-Induced Seizures

After intraperitoneal injection of Pentylenetetrazole (PTZ; Sigma; 60 mg/kg for adult mice and 50 mg/kg for juvenile mice), behaviors were video recorded for 15 minutes to analyze seizure stages, which were defined as follows: stage 1, immobility, ear twitching, short jerk; stage 2, straub tail, convulsive twitching; stage 3, myoclonic jerk, rearing; stage 4, jumping; stage 5, tonic-clonic seizure; and stage 6, death. The maximum stage that each mouse displayed during the 15 minute monitoring period was used to calculate *p* values. We categorized seizure stages into non-severe (stages 1–4) and severe (stages 5 and 6) and performed Fisher's exact test. To evaluate the response to lithium, mice received an intraperitoneal injection of saline or 50 mg/kg lithium 30 minutes before PTZ injection.

Electroencephalogram/Electromyogram Measurements of PTZ-Induced Seizures

To implant electrodes, postnatal day (P)70 mice were anesthetized with 1.5% isoflurane and held in a stereotaxic device (David Kopf Instruments or Neurostar). Epidural electrodes for electroencephalogram (EEG) recording were implanted in the right frontal cortex and left temporal cortexes (right frontal: 1.0 mm anteroposterior, 1.0 mm lateral; and left temporal: –2.4 mm anteroposterior, –2.4 mm lateral) with grounding over the cerebellum (–5.0 mm anteroposterior). For electromyogram (EMG) recording, two Teflon-coated tungsten electrodes (diameter, 0.002 and 0.004 mm without and with Teflon coating, respectively; A-M Systems Inc.) were inserted into the nuchal musculature. All connectors were fixed to the skull with Super-Bond (Sun Medical Co., Japan) and dental cement (Stoelting).

After 1 week of recovery, the EEG/EMG signals and video recordings were acquired for 3 hours for the basal state and 1 hour for PTZ, during which time the mice were free to explore in the EEG chamber. All electrodes for EEG and EMG recordings were then connected to an electrode interface board (EIB-16; Neuralynx), which was connected to a Digital Lynx acquisition system (Neuralynx). At the same time, behavioral changes in mice were recorded using an action camera (SQ12, China). The EEG and EMG signals were acquired using a Neuralynx amplifier and Cheetah program. EEG and EMG spikes were bandpass filtered at 0.5–50 and 30–500 Hz, respectively. Both signals were digitized using Spike Extractor software (Neuralynx). Interictal and ictal spikes were counted manually using Neuroview software (Neuralynx). Representative traces of EEG, EMG, and spectrogram data were obtained using Neuroexplorer (Nex Technologies). To detect peaks and measure the amplitude of induced seizure spikes, the relative domain-specific maxima detection algorithm was implemented using MATLAB software (R2019b; MathWorks).

EEG Measurements for Auditory Steady-State Responses

P56 mice were used for auditory steady-state response (ASSR) recording. The detailed procedures were described previously.³¹ In brief, three microscrews were implanted on the skull above the right frontal lobe (2 mm anteroposterior and 1 mm medolateral to bregma) for the frontal EEG and above the left and right cerebellum for ground and reference electrodes in anesthetized mice (ketamine/xylazine cocktail (intraperitoneal injection), 120 and 6 mg/kg, respectively). After 1 week of recovery, the EEG was recorded at a sampling rate of 2 kHz using a Synamps2 amplifier and a SCAN v.4.5 data-acquisition system (Neuroscan, Inc., El Paso, TX). The baseline EEG was recorded during 10 minutes of habituation, after which ASSR EEGs were recorded with steady-state auditory stimulation. The stimulus consisted of 1 second of an 80 dB sound pulse train consisting of 1 millisecond pulses at five different repetition rates (10, 20, 30, 40, and 50 Hz) with an interstimulus interval of 1–1.5 seconds. Each frequency was repeated 200 times, and the order of frequencies was randomized.

Epochs consisting of 0.5 seconds pre- and post-stimulus and 1 second stimulus periods were extracted for each frequency and baseline corrected by subtracting the averaged prestimulus EEG. The event-related potentials (ERPs) of ASSR stimulations were obtained by averaging 200 epochs with the same frequency of stimulation. For spectrogram analysis, a short-time Fourier transformation was conducted with 200 millisecond time bins and 10% overlap. To scale a gross level of neural synchrony, the intertrial locking was calculated with the phase locking value (PLV) across trials, which is defined by $PLV(f) = \frac{1}{N} \sum_{n=0}^{N-1} e^{i\Delta\phi_n(f)}$, where N is the total number of trials and $\Delta\phi_n(f)$ is the phase of the EEG signal filtered by a bandpass filter (cut-off frequency = $[f - 2, f + 2]$ Hz) with respect to the sound onset in the n^{th} trial. For all EEG data, we performed a normality test using the Kolmogorov–Smirnov test for goodness of fit, followed by a multiple comparison test with a repeated-measures analysis of variance (ANOVA) to test the effect of groups (WT and *Cyfp2*^{+/-}), stimulation frequencies, and their interaction. In ANOVA, the violation of sphericity was tested using Mauchly's test, and all p values were corrected using the Huynh–Feldt epsilon. After the multiple comparison test, we performed post-hoc analysis using a Wilcoxon signed-rank test. All spectral, synchrony, and statistical analyses were performed in Matlab (v.R2015a; The Mathworks, Natick, MA).

Results

Lithium-Responsive Abnormal Behaviors in Adult *Cyfp2*^{+/-} Mice

We previously showed that *Cyfp2*^{+/-} mice exhibit behavioral phenotypes similar to those observed in fragile X mental retardation 1 (*Fmr1*)-null mice, an animal model of FXS.²⁰ We characterized behaviors and drug responses further in adult (P56) *Cyfp2*^{+/-} mice (Fig 1). In the OFT, both male and female *Cyfp2*^{+/-} mice showed increased locomotor activity and speed. Given that lithium treatment ameliorates several behavioral phenotypes, including hyperactivity, in *Fmr1*-null mice,³² we also examined its effects on *Cyfp2*^{+/-} mice. Acute injection of lithium (50mg/kg) mitigated hyperactivity and the speed of male and female *Cyfp2*^{+/-} mice in the OFT, whereas the same treatment had no significant effect on WT mice. We found that *Cyfp2*^{+/-} mice showed reduced anxiety in the EPM test. The reduced anxiety in male *Cyfp2*^{+/-} mice in the EPM test was also reversed by lithium treatment. In the TST measuring behavioral despair with immobility time, the duration of immobility in *Cyfp2*^{+/-} mice was shorter than that in WT littermates, and the behavior was reversed by lithium in male *Cyfp2*^{+/-} mice. Importantly, with the higher dose (100mg/kg) of lithium, the locomotor activity of WT mice was also decreased, suggesting that lithium has general effects on locomotor activity. Moreover, the above results showing greater effects of lithium (50mg/kg) in *Cyfp2*^{+/-} mice compared with those in

WT mice might also include a floor effect of WT mice, and thus should be interpreted cautiously. In contrast to the adult mice, juvenile (P21) *Cyfp2*^{+/-} mice showed normal locomotor activity and speed in the OFT and normal anxiety levels in the EPM test.

Layer-Specific Molecular Changes in the mPFC of Adult *Cyfp2*^{+/-} Mice

Next, we tried to identify the brain regions that are potentially involved in the abnormal behaviors of *Cyfp2*^{+/-} mice. Considering the high sequence homology between CYFIP1 and CYFIP2, we hypothesized that brain regions with high *Cyfp2* but low *Cyfp1* expression were more likely to be affected in *Cyfp2*^{+/-} mice. The in situ hybridization and qRT-PCR analyses of mouse brains suggested the cortex as one such brain region (Fig 2). The BrainSpan database (<http://www.brainspan.org>) confirmed that *CYFIP2* transcripts were more abundant than *CYFIP1* in the human neocortex as well. At the protein level, CYFIP1 was evenly expressed in different brain regions, whereas CYFIP2 and WAVE1 were relatively enriched in the mouse cortex, striatum, and hippocampus.

Among the cortical subregions of *Cyfp2*^{+/-} mice, we focused on the PFC because of its roles in top-down control over various brain regions that regulate multiple types of behaviors³³ and its defects in various brain disorders.³⁴ Moreover, CYFIP2 expression was decreased in the post-mortem PFC of SCZ patients,³⁵ suggesting a potential association between brain disorders and lower PFC CYFIP2 levels. The results from fluorescent immunohistochemistry (IHC) against β -galactosidase (β -gal) expressed in *Cyfp2*^{+/-} mice, which represents *Cyfp2* promoter activity, indicated *Cyfp2* expression in multiple layers of the prelimbic mPFC (Fig 2). Furthermore, the β -gal signals colocalized with COUP-TF-interacting protein 2 (CTIP2)-positive excitatory neurons and parvalbumin-positive inhibitory neurons of the mPFC.

In adult *Cyfp2*^{+/-} mice, we confirmed a reduction in CYFIP2 and WAVE1 in mPFC synaptosomes (Fig 2), consistent with previous studies showing that WAVE of WRC becomes active but less stable without CYFIP.^{20,36} There was no change in phosphorylated or total levels of other actin-regulatory or signaling proteins. Notably, a recent study showed that CYFIP1 protein expression was decreased only in a subset of brain regions in *Cyfp1*^{+/-} mice.³⁷ Therefore, we also examined layer-specific CYFIP2 changes in the adult *Cyfp2*^{+/-} mPFC. Intriguingly, CYFIP2 was significantly decreased only in L5, but not in layer 2/3 (L2/3). Based on these findings, we also investigated layer-specific F-actin levels, which are expected to be negatively regulated by CYFIP2.³⁶ Indeed, F-actin levels were significantly increased in layers 1 and 5

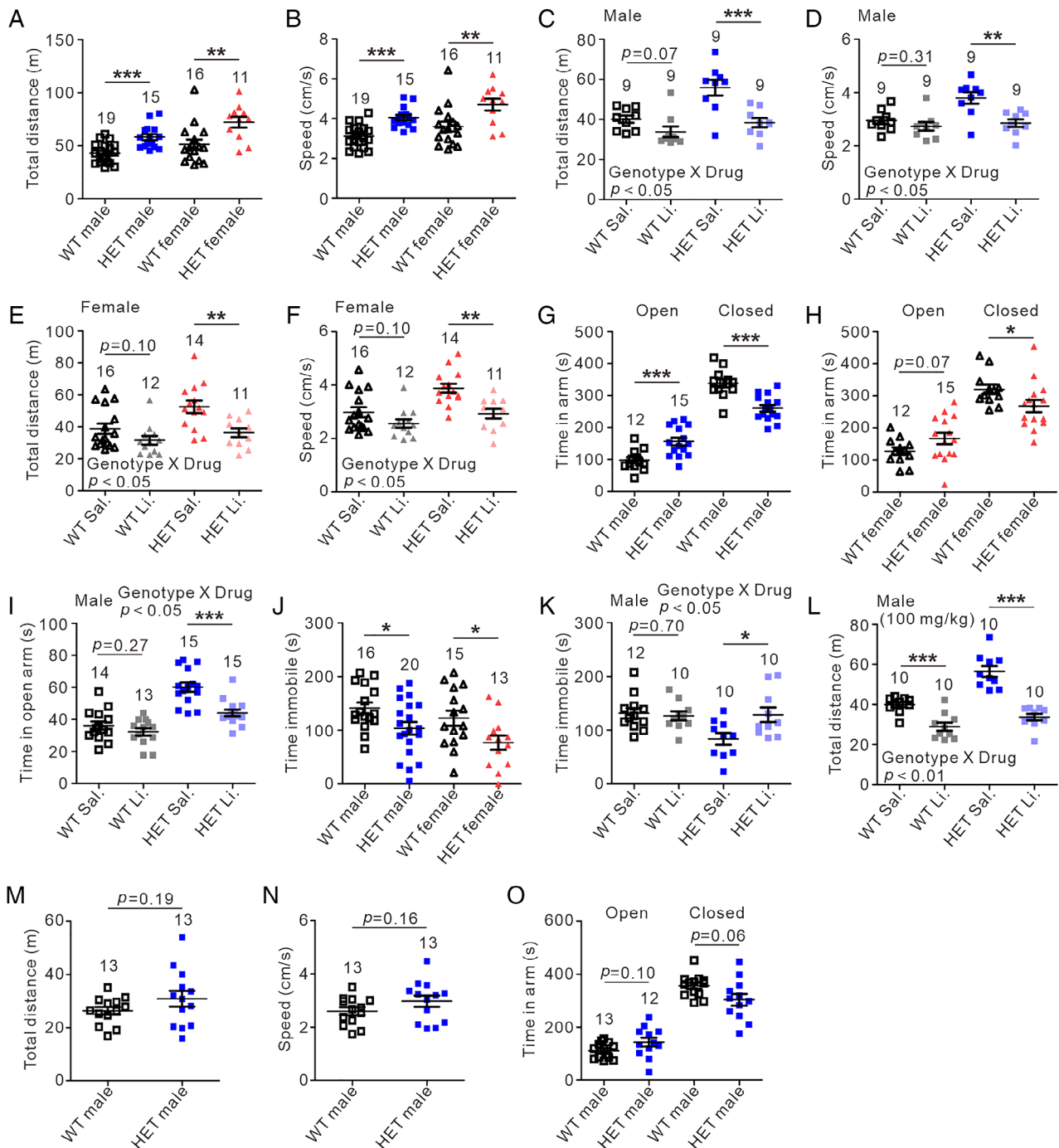


FIGURE 1: Lithium-responsive abnormal behaviors of adult *Cyfp2*^{+/-} mice. (A) Locomotor activity of adult wild-type (WT) and *Cyfp2*^{+/-} mice in the open field test (OFT). Numbers of mice used for the test are indicated (Student unpaired two-tailed t test). HET, heterozygous. (B) Movement speed of WT and *Cyfp2*^{+/-} mice during the OFT. (C, D) Locomotor activity and speed of male WT and *Cyfp2*^{+/-} mice after saline (Sal.) or lithium (Li.) injection (two-way ANOVA with Bonferroni post-hoc test). (E, F) Locomotor activity and speed of female WT and *Cyfp2*^{+/-} mice after saline or lithium injection. (G, H) Time spent in the open and closed arms during the elevated plus maze (EPM) test for WT and *Cyfp2*^{+/-} mice. (I) Time spent in the open arms during the EPM test for male WT and *Cyfp2*^{+/-} mice after saline or lithium injection. (J) Time spent immobile for WT and *Cyfp2*^{+/-} mice during the tail-suspension test (TST). (K) Time spent immobile for male WT and *Cyfp2*^{+/-} mice during the TST after saline or lithium injection. (L) Locomotor activity of male WT and *Cyfp2*^{+/-} mice after saline or high-dose (100mg/kg) lithium injection. (M, N) Locomotor activity and speed of juvenile WT and *Cyfp2*^{+/-} mice in the OFT. (O) Time spent in the open and closed arms during the EPM test for juvenile WT and *Cyfp2*^{+/-} mice. Data are represented as the mean ± standard error of the mean. **p* < 0.05; ***p* < 0.01; ****p* < 0.001.

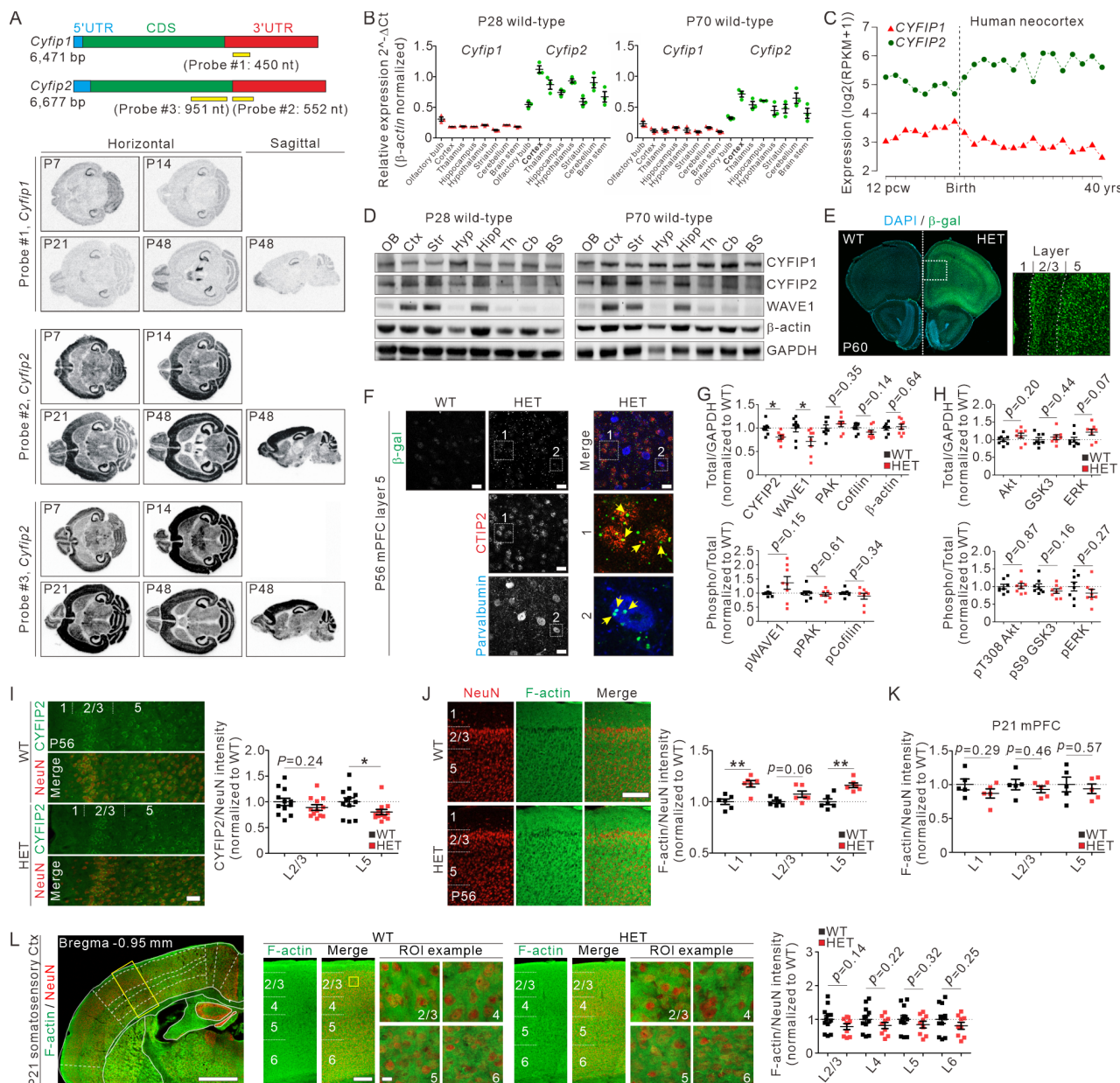


FIGURE 2: Layer-specific CYFIP2 decrease and F-actin increase in the adult *Cyfp2*^{+/-} (HET) medial prefrontal cortex (mPFC). (A) In situ hybridization of *Cyfp1* and *Cyfp2* transcripts in developing mouse brains. bp, base pair; CDS, coding sequence; P, postnatal day; nt, nucleotide; UTR, untranslated region. (B) qRT-PCR analysis of *Cyfp1* and *Cyfp2* transcripts in the brain regions of P28 and P70 wild-type (WT) mice ($n = 3$ mice per age group). (C) Analysis on the BrainSpan database for the expression levels of *CYFIP1* and *CYFIP2* in the developing human neocortex. pcw, postconception weeks; RPKM, reads per kilobase of transcript per million mapped reads; yrs, years. (D) Western blot analysis of brain regional expression of *CYFIP1*, *CYFIP2*, and *WAVE1* proteins in P28 and P70 WT mice. BS, brain stem; Cb, cerebellum; Ctx, cortex; Hipp, hippocampus; Hyp, hypothalamus; OB, olfactory bulb; Str, striatum; Th, thalamus. (E) Fluorescent immunohistochemistry (IHC) of *β-gal* antibodies in the mPFC of WT and *Cyfp2*^{+/-} mice. DAPI (4',6-diamidino-2-phenylindole) was used as a nuclear counterstain. (F) Fluorescent IHC of *β-gal*, COUP-TF-interacting protein 2 (CTIP2), and parvalbumin antibodies in the mPFC layer 5 of WT and *Cyfp2*^{+/-} mice. Scale bars: 20μm. (G, H) Western blot analysis of phosphorylated and total levels of actin-regulatory and signaling proteins in the mPFC synaptosome of WT and *Cyfp2*^{+/-} mice ($n = 8$ mice per genotype; Student unpaired two-tailed t test). (I) Fluorescent IHC of *CYFIP2* and NeuN antibodies in the mPFC of WT and *Cyfp2*^{+/-} mice ($n = 12$ mice per genotype). Scale bar: 50μm. (J) Fluorescent IHC of NeuN antibodies and Alexa Fluor-conjugated phalloidin in the mPFC of WT and *Cyfp2*^{+/-} mice ($n = 6$ mice per genotype). Scale bar: 200μm. (K) Quantification of F-actin intensity in the mPFC of juvenile WT and *Cyfp2*^{+/-} mice ($n = 5$ mice per genotype). (L) Quantification of F-actin intensity in the somatosensory cortex of juvenile WT and *Cyfp2*^{+/-} mice ($n = 5$ mice per genotype, with two sections from each mouse). Yellow boxes represent regions of interest (ROIs) for quantification. Scale bars: 1mm (left panel), 200μm (middle panel) and 20μm (right panel). * $p < 0.05$; ** $p < 0.01$. Data are represented as the mean $\pm$ standard error of the mean.

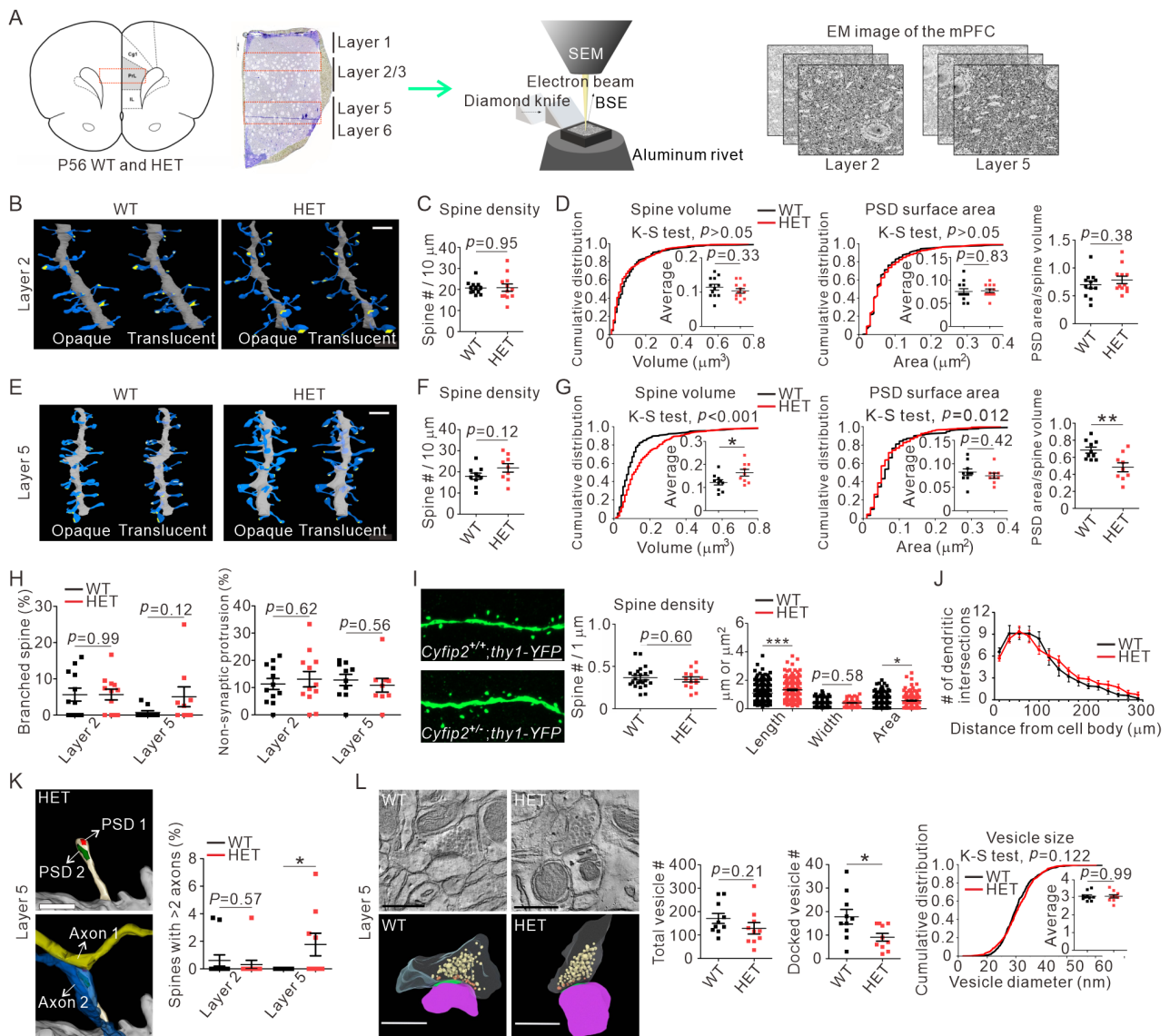


FIGURE 3: Abnormal excitatory synaptic structures of medial prefrontal cortex (mPFC) layer 5 (L5) neurons in adult *Cyfp2*^{+/-} (HET) mice. (A) Summary of procedures for serial block-face scanning electron microscopy on the basal dendritic segments of prelimbic mPFC layer 2 (L2) and L5 neurons. BSE, back-scattered electron. (B) Representative images of a 3-dimensional (3D) reconstruction of dendritic segments from wild-type (WT) and *Cyfp2*^{+/-} mPFC L2 neurons. Scale bar: 2μm. (C) Quantification of dendritic spine density in mPFC L2 neurons (n = 12 dendritic segments per genotype; Student unpaired two-tailed t test). (D) Quantification of dendritic spine volume, postsynaptic density (PSD) surface area, and PSD area-to-spine volume ratio of mPFC L2 neurons (n = 390 and 317 dendritic spines of WT and *Cyfp2*^{+/-} mice, respectively; Kolmogorov-Smirnov test; Student unpaired two-tailed t test). (E) Representative images of a 3D reconstruction of dendritic segments from mPFC L5 neurons. Scale bar: 2μm. (F) Quantification of dendritic spine density in mPFC L5 neurons (n = 10 and 9 dendritic segments for WT and *Cyfp2*^{+/-} mice, respectively). (G) Quantification of dendritic spine volume, PSD surface area, and PSD area-to-spine volume ratio of mPFC L5 neurons (n = 273 and 277 dendritic spines of WT and *Cyfp2*^{+/-} mice, respectively). (H) Quantification of branched spine and non-synaptic protrusion frequencies from mPFC L2 and L5 neurons. (I) Representative confocal images showing apical dendritic spines of WT and *Cyfp2*^{+/-} L5 neurons. Scale bar: 5μm. Quantification of dendritic spine density and morphology of mPFC L5 neurons (n = 299 and 171 spines from 23 WT and 14 *Cyfp2*^{+/-} neurons, respectively). (J) Sholl analysis of the biocytin-infused mPFC L5 neurons (n = 10 and 14 neurons from 3 WT and 3 *Cyfp2*^{+/-} mice, respectively). (K) Representative 3D images and quantification of multiple axonal contacts on a single dendritic spine of *Cyfp2*^{+/-} mPFC L5 neurons. Scale bar: 1μm. (L) Representative 3D images and quantification of presynaptic vesicles on the dendritic spines of mPFC L5 neurons (n = 10 synapses per genotype). Scale bars: 0.5μm. Data are represented as the mean ± standard error of the mean. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

of the adult *Cyfp2*^{+/-} mPFC, where apical and basal dendrites, respectively, of L5 neurons mainly arborize.³⁸ In contrast, L2/3 showed an increase in F-actin, but it was not statistically significant. Moreover, F-actin levels were normal in all layers of juvenile *Cyfp2*^{+/-} mPFC and somatosensory cortex. Myelination levels, as measured by fluorescent IHC of myelin basic protein (MBP) and neurofilament-H (NF-H), were normal in the mPFC and corpus callosum of adult *Cyfp2*^{+/-} mice (data not shown).

Layer-Specific Changes of Synaptic Structures in Adult *Cyfp2*^{+/-} mPFC

Given that F-actin is the key cytoskeletal component of excitatory synapses,¹² we investigated the synaptic morphologies of mPFC neurons in adult *Cyfp2*^{+/-} mice (Fig 3). To understand whether the synaptic morphologies were also altered in a layer-specific manner, we investigated the basal dendrites of L2 and L5 pyramidal neurons separately in the prelimbic mPFC. The images were obtained by SB-SEM and processed further for 3D reconstruction of dendritic segments. Dendritic spine density, spine volume, PSD area, and PSD area-to-spine volume ratio were normal in L2 neurons of the *Cyfp2*^{+/-} mPFC. In L5 neurons of the *Cyfp2*^{+/-} mPFC, dendritic spine density was normal; however, there was a significant increase in spine volume compared with that in WT neurons. Notably, even with enlarged dendritic spines of *Cyfp2*^{+/-} L5 neurons, the PSD area, which is normally correlated with spine volume,³⁹ was comparable to that of WT neurons, leading to a significant decrease in PSD area-to-spine volume ratio in *Cyfp2*^{+/-} L5 neurons (see Fig 3G, right). In both L2 and L5 neurons, the frequencies of branched spines and non-synaptic protrusions were not different between WT and *Cyfp2*^{+/-} mice. We also investigated the dendritic spines of apical dendrites of L5 neurons by crossing *Cyfp2*^{+/-} mice with *Thy-1-YFP-H* mice.⁴⁰ A normal dendritic spine density was observed, but with increased length and head area, from the apical dendrites of *Cyfp2*^{+/-} L5 neurons. Meanwhile, dendritic branching was comparable between L5 neurons of WT and *Cyfp2*^{+/-} mice.

We also found that a subset of dendritic spines of L5 neurons in adult *Cyfp2*^{+/-} mPFC had multiple axonal contacts (Fig 3), which are normally observed only transiently during synaptic development and markedly decrease in number with maturation.⁴¹ In contrast, spines with multiple axonal contacts were scarcely observed in either L2 neurons in the *Cyfp2*^{+/-} mPFC or in L2 and L5 neurons in WT mPFC. Therefore, we evaluated the presynaptic structures of mPFC L5 neurons further using 3D electron tomography and found that the number of docked vesicles in a single presynaptic terminal was lower

in *Cyfp2*^{+/-} mice compared with WT mice. The numbers of total vesicles and vesicle size were comparable between WT and *Cyfp2*^{+/-} mice.

Layer-Specific Functional Changes of Neurons in the Adult *Cyfp2*^{+/-} mPFC

To validate the layer-specific synaptic changes in the adult *Cyfp2*^{+/-} mPFC, we performed electrophysiological analyses on acute brain slices (Fig 4). In L2 neurons of *Cyfp2*^{+/-} mPFC, excitatory synaptic transmission (mEPSCs) was normal. Furthermore, there was no difference in the amplitude and frequency of mIPSCs between WT and *Cyfp2*^{+/-} L2 neurons. In contrast, in L5 neurons of *Cyfp2*^{+/-} mPFC, the frequency, but not the amplitude, of mEPSCs was significantly increased compared with that in WT neurons. Meanwhile, neither the amplitude nor the frequency of mIPSCs was altered in L5 neurons of *Cyfp2*^{+/-} mPFC. Moreover, the α -amino-3-hydroxy-5-methyl-4-isoxazole propionate receptor (AMPA) to *N*-methyl-D-aspartic acid receptor (NMDAR) current ratio was comparable between WT and *Cyfp2*^{+/-} L5 neurons. Ion channel dysfunction and neuronal hyperexcitability are strongly associated with ID, especially FXS, and epilepsy.⁴² Therefore, we examined the changes in excitability of mPFC neurons by performing current-clamp recordings in response to a series of current injections. Similar to the layer-specific changes of mEPSCs, the number of action potentials was significantly increased only in L5, but not in L2, mPFC neurons in *Cyfp2*^{+/-} mice compared with that in WT mice. In contrast to the adult mPFC, mEPSCs and excitability were normal in L5 neurons of juvenile *Cyfp2*^{+/-} mPFC. Moreover, in the adult hippocampus of *Cyfp2*^{+/-} mice, excitability of cornu ammonis 1 (CA1) pyramidal and dentate gyrus (DG) granule neurons was normal.

Increased Seizure Susceptibility and ASSRs of Adult *Cyfp2*^{+/-} Mice

Based on the hyperexcitability of *Cyfp2*^{+/-} neurons, we investigated the seizure susceptibility of *Cyfp2*^{+/-} mice, which might potentially be implicated in epilepsy commonly seen in patients with *CYFIP2* deletions and mis-sense variants.^{5,7-10} We induced seizures in mice by injecting PTZ and assessed seizure behaviors. We found increased seizure susceptibility in adult *Cyfp2*^{+/-} mice compared with WT littermates (Fig 5A). Approximately 56% (5 of 9) of adult *Cyfp2*^{+/-} mice died during the 15 minute monitoring period after PTZ injection, whereas no (0 of 10) WT mice died. In contrast, juvenile *Cyfp2*^{+/-} mice unexpectedly exhibited reduced seizure susceptibility compared with that of WT mice (see Fig 5B).

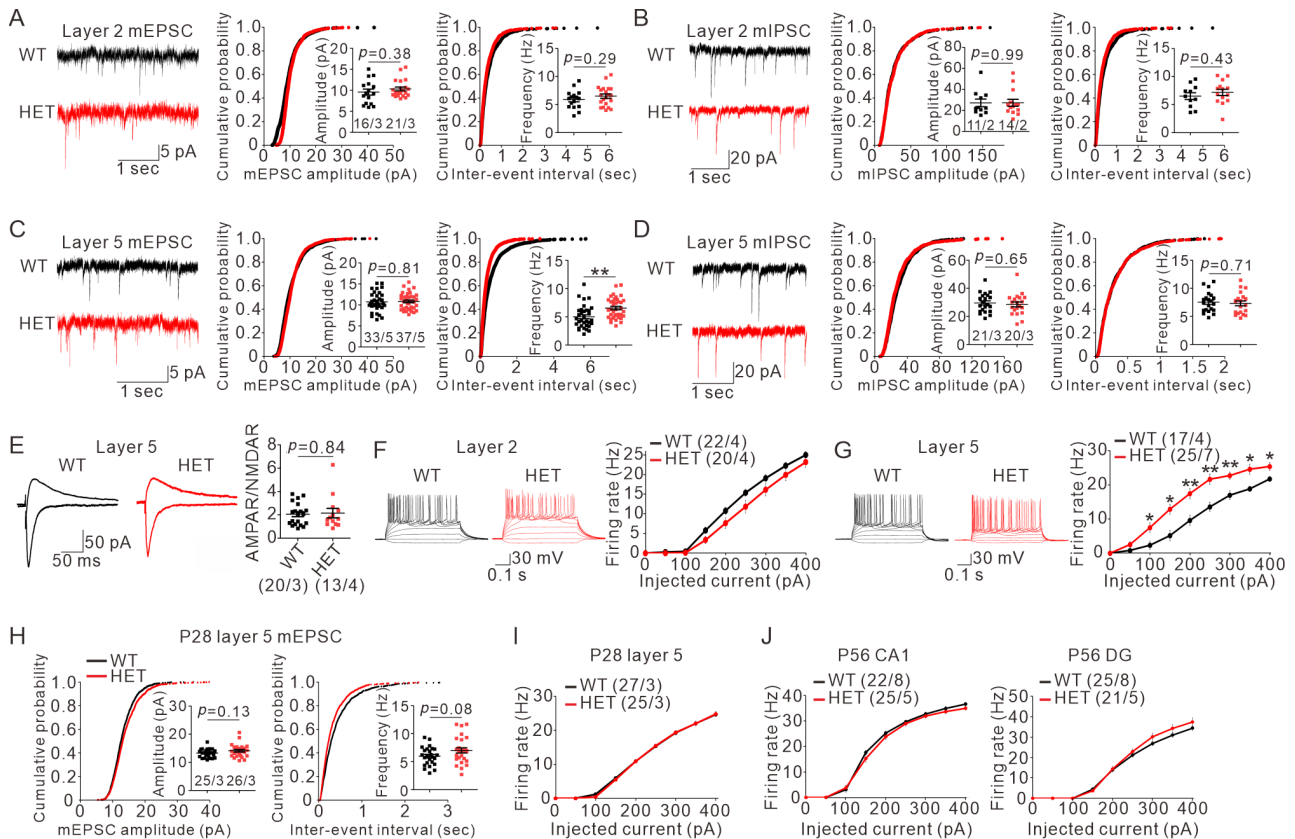


FIGURE 4: Altered excitatory synaptic transmission and excitability of medial prefrontal cortex (mPFC) layer 5 (L5) neurons in adult *Cyfp2*^{+/-} (HET) mice. (A) Representative traces, cumulative/mean amplitude, and frequency of miniature excitatory postsynaptic currents (mEPSCs) of mPFC layer 2 (L2) neurons. Numbers of cells and mice used for the experiments are indicated (Student unpaired two-tailed t test), WT, wild-type. (B) Amplitude and frequency of miniature inhibitory postsynaptic currents (mIPSCs) of mPFC L2 neurons. (C) Amplitude and frequency of mEPSCs of mPFC L5 neurons. (D) Amplitude and frequency of mIPSCs of mPFC L5 neurons. (E) α -Amino-3-hydroxy-5-methyl-4-isoxazole propionate receptor (AMPA) to *N*-methyl-D-aspartic acid receptor (NMDAR) current ratio of mPFC L5 neurons. (F) Intrinsic excitability of mPFC L2 neurons measured as firing rates against injected current. (G) Intrinsic excitability of mPFC L5 neurons. (H) Amplitude and frequency of mEPSCs of juvenile mPFC L5 neurons. (I) Intrinsic excitability of juvenile mPFC L5 neurons. (J) Intrinsic excitability of cornu ammonis 1 (CA1) and dentate gyrus (DG) neurons in the adult hippocampus. Data are represented as mean $\pm$ standard error of the mean. * $p < 0.05$; ** $p < 0.01$.

We assessed PTZ-induced seizures of adult *Cyfp2*^{+/-} mice further by performing simultaneous EEG and EMG recordings (Fig 5). Consistent with behavioral observation, faster and stronger responses with muscle twitching were detected from *Cyfp2*^{+/-} mice compared with WT mice. Specifically, the number of seizure spikes during 150 seconds after PTZ injection increased and the latency to the first seizure spike decreased in *Cyfp2*^{+/-} mice. The overall seizure intensity measured by the amplitude of seizure spikes was also increased in *Cyfp2*^{+/-} mice. Additionally, basal power spectral analysis showed that stronger seizure generation changed basal brain oscillatory status in *Cyfp2*^{+/-} mice.

We next investigated activities of the *Cyfp2*^{+/-} frontal cortex in a more physiological context. ASSRs are electrophysiological responses of neural circuits to periodic auditory stimuli and are commonly measured by EEG recordings. Alterations in ASSR phase locking and power in the gamma range (30–80Hz) have been observed in patients with

multiple brain disorders.^{31,43} To measure ASSRs, we applied five different randomly ordered frequencies with a pulse width of 1 millisecond (Fig 5). Adult *Cyfp2*^{+/-} mice showed significantly increased PLV and ASSR power at 40 and 50Hz, whereas no significant group differences were observed at 10, 20, and 30Hz. We computed the power spectra of baseline oscillations using the prestimulation periods; however no difference was observed between WT and *Cyfp2*^{+/-} mice.

Lithium Selectively Rescues Hyperexcitability of L5 Neurons in the *Cyfp2*^{+/-} mPFC

Next, we investigated the potential mechanisms underlying the observed effect of lithium on *Cyfp2*^{+/-} mouse behavior (Fig 1) by examining whether lithium could normalize any molecular or electrophysiological changes observed in the adult *Cyfp2*^{+/-} mPFC. Initially, we asked whether an acute injection of lithium could affect the levels of CYFIP1/2 and WAVE1 or F-actin in the mPFC

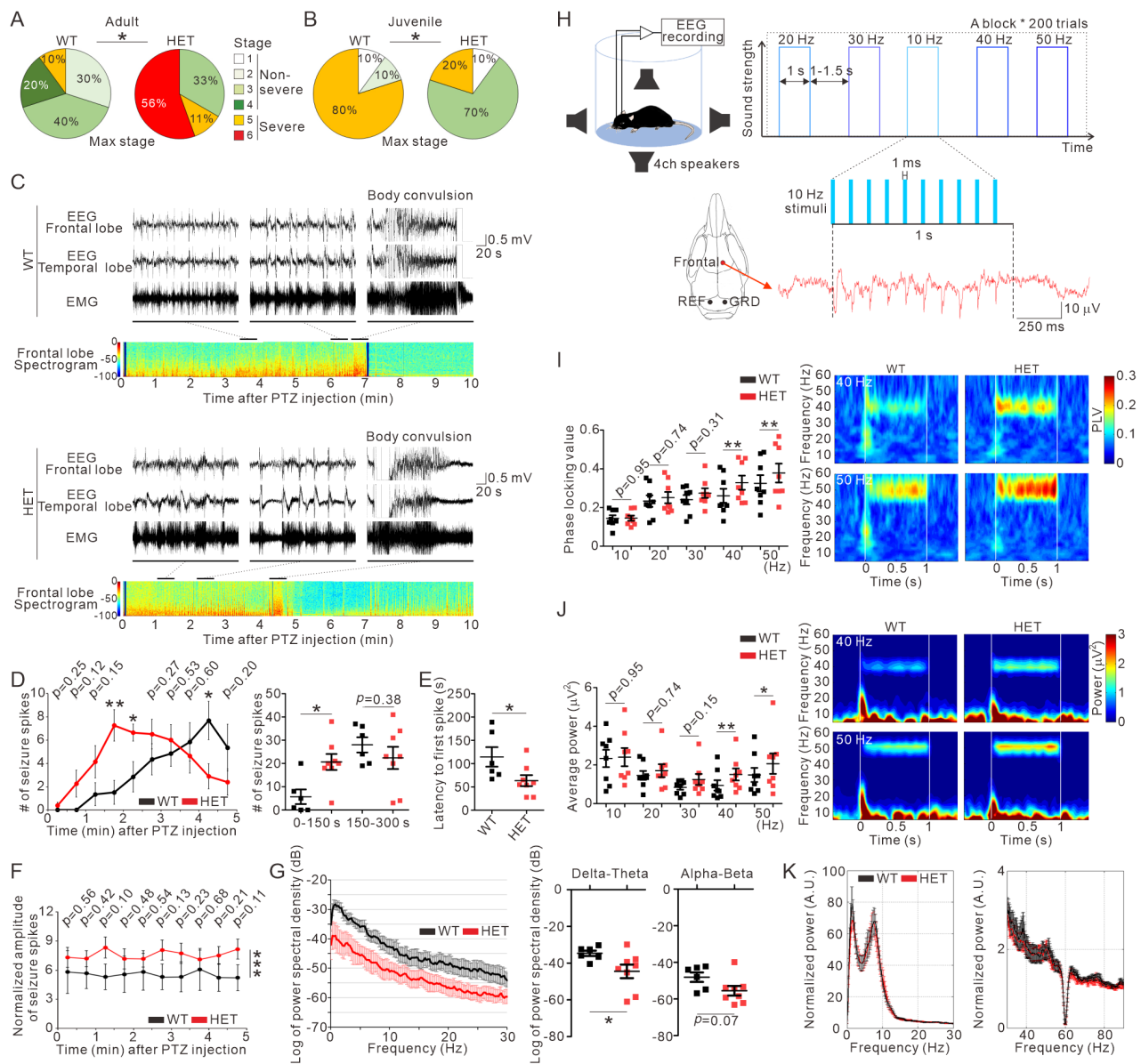


FIGURE 5: Increased seizure susceptibility and auditory steady-state responses (ASSRs) in the frontal cortex of adult *Cyfip2*^{+/-} (HET) mice. (A) Pentylentetrazole (PTZ)-induced behavioral seizure scores in adult wild-type (WT) and *Cyfip2*^{+/-} mice ($n = 10$ and 9 mice for WT and *Cyfip2*^{+/-}, respectively; Fisher's exact test). (B) PTZ-induced behavioral seizure scores in juvenile WT and *Cyfip2*^{+/-} mice ($n = 10$ and 10 mice for WT and *Cyfip2*^{+/-}, respectively). (C) Representative traces of electroencephalogram (EEG) and electromyogram (EMG) recordings after PTZ injection in adult WT and *Cyfip2*^{+/-} mice. Representative spectrograms of frontal lobe are also displayed. Spectrograms are shown for 0–50 Hz. (D) Numbers of seizure spikes from WT and *Cyfip2*^{+/-} mice at different time points after PTZ injection ($n = 6$ and 8 mice for WT and *Cyfip2*^{+/-}, respectively; two-way ANOVA and Student unpaired two-tailed t test). (E) Latency to the first seizure spike after PTZ injection for WT and *Cyfip2*^{+/-} mice. (F) Normalized amplitudes of seizure spikes from WT and *Cyfip2*^{+/-} mice at different time points after PTZ injection (two-way ANOVA and Student unpaired two-tailed t test). (G) Power spectral density of EEG recorded from the frontal cortex 5 minutes after PTZ injection. Comparison of the relative power in each EEG frequency band between WT and *Cyfip2*^{+/-} mice. Low-frequency (delta, 0.5–4 Hz; theta, 4–8 Hz) oscillatory power and high-frequency (alpha, 8–13 Hz; beta, 13–30 Hz) oscillatory power spectral densities are displayed. (H) Schematic diagram of experimental procedures for measurement of ASSRs. ch, channel; GRD, ground; REF, reference. (I) Quantification showing ASSR phase-locking values of WT and *Cyfip2*^{+/-} mice at different stimulation frequencies ($n = 8$ mice per genotype; Wilcoxon signed-rank test). Phase locking factor spectrogram of EEG recordings from WT and *Cyfip2*^{+/-} mice showing synchrony across time. The vertical bars indicate the onset and cession of sound stimuli. PLV, phase locking value. (J) Quantification showing ASSR power of WT and *Cyfip2*^{+/-} mice at different stimulation frequencies. Mean trial power spectrogram for WT and *Cyfip2*^{+/-} mice showing the magnitude of response across time. (K) Average power spectrum of spontaneous activity of frontal EEG. Data are represented as the mean $\pm$ standard error of the mean. $*p < 0.05$; $**p < 0.01$; $***p < 0.001$.

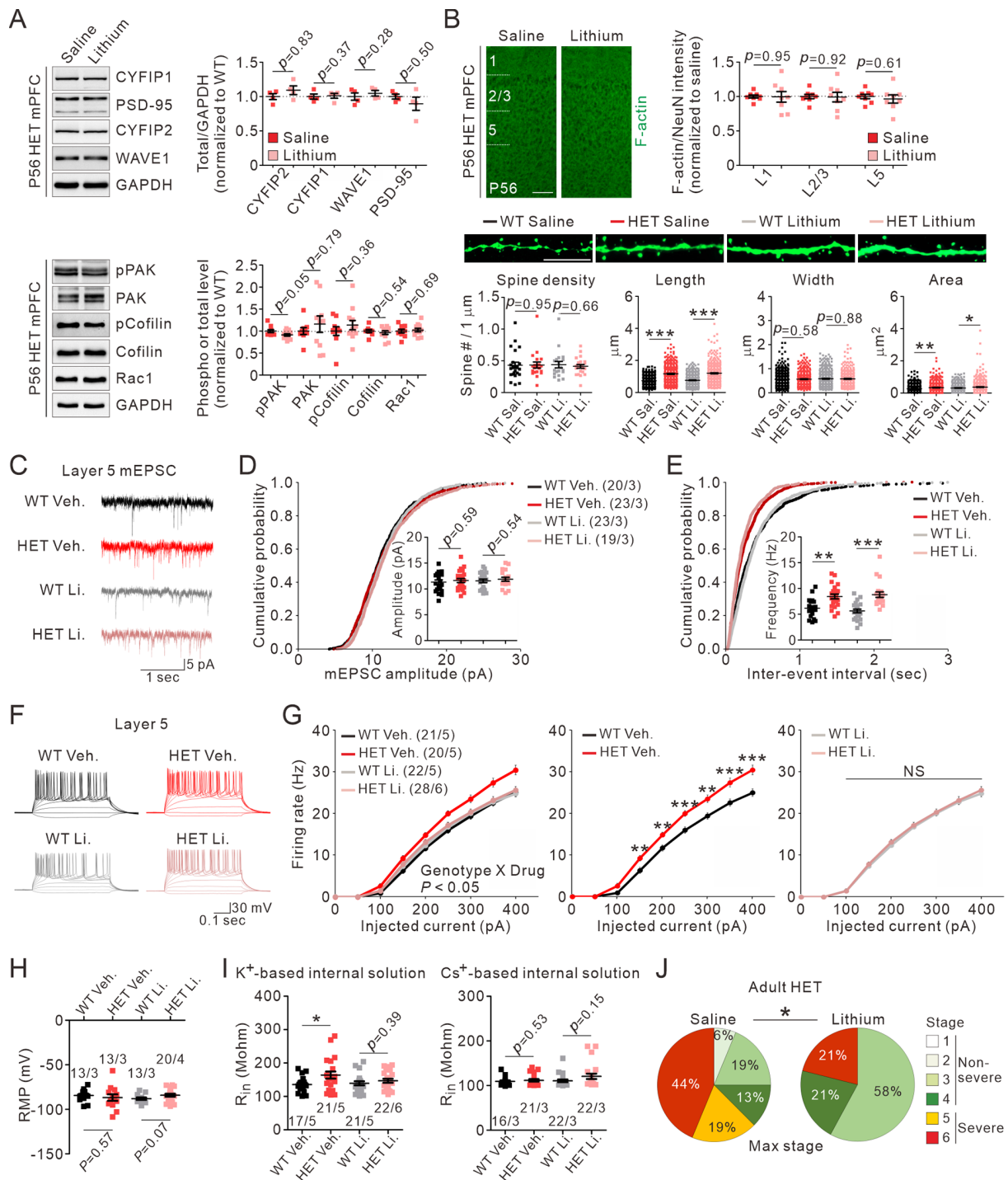


FIGURE 6: Lithium selectively rescues hyperexcitability of layer 5 (L5) neurons in the *Cyfp2*^{+/-} (HET) medial prefrontal cortex (mPFC). (A) Western blot analysis of CYFIP1/2, WAVE1, PSD-95, and Rac1 signaling components in the mPFC of *Cyfp2*^{+/-} mice injected with saline or lithium (n = 4–12 mice per condition; Student unpaired two-tailed t test). (B) F-actin levels (n = 8 mice per condition) and apical dendritic spines (n = 450 and 400 spines from 25 saline- and 24 lithium-treated neurons from 3 *Cyfp2*^{+/-} *Thy-1-YFP* mice for each condition; two-way ANOVA with Bonferroni post-hoc test) of L5 neurons in the mPFC of *Cyfp2*^{+/-} mice injected with saline or lithium. Scale bars: 200 (upper panel) and 10μm (lower panel). (C) Representative traces of miniature excitatory postsynaptic currents (mEPSCs) of WT and *Cyfp2*^{+/-} mPFC L5 neurons in vehicle (Veh.) and lithium-treated conditions. (D) Cumulative and mean amplitude of mEPSCs. Numbers of cells and mice used for the experiments are indicated. (E) Cumulative and mean frequency of mEPSCs. (F) Representative traces of intrinsic excitability of WT and *Cyfp2*^{+/-} mPFC L5 neurons in vehicle- and lithium-treated conditions. (G) Intrinsic excitability of mPFC L5 neurons measured as firing rates against injected current. NS, not significant. (H) Quantification of resting membrane potential (RMP) of mPFC L5 neurons in vehicle- and lithium-treated conditions. (I) Quantifications of input resistance (R_{in}) of the mPFC L5 neurons in vehicle- and lithium-treated conditions. (J) Pentylentetrazole (PTZ)-induced behavioral seizure scores in adult *Cyfp2*^{+/-} mice injected with saline or lithium (n = 16 and 19 mice for saline and lithium, respectively; Fisher's exact test). Data are represented as the mean ± standard error of the mean. *p < 0.05; **p < 0.01; ***p < 0.001.

of adult *Cyfp2*^{+/-} mice, but observed no difference between saline- and lithium-injected conditions (Fig 6). Consistently, phosphorylated and total levels of Rac1 signaling components and apical dendritic spines of L5 neurons in the mPFC of adult *Cyfp2*^{+/-} mice were not

altered by lithium treatment. In lithium-treated conditions, dendritic spine length and area were still increased in the *Cyfp2*^{+/-} neurons compared with WT neurons.

Therefore, we next examined the effects of lithium on the functional changes of L5 neurons in the mPFC by

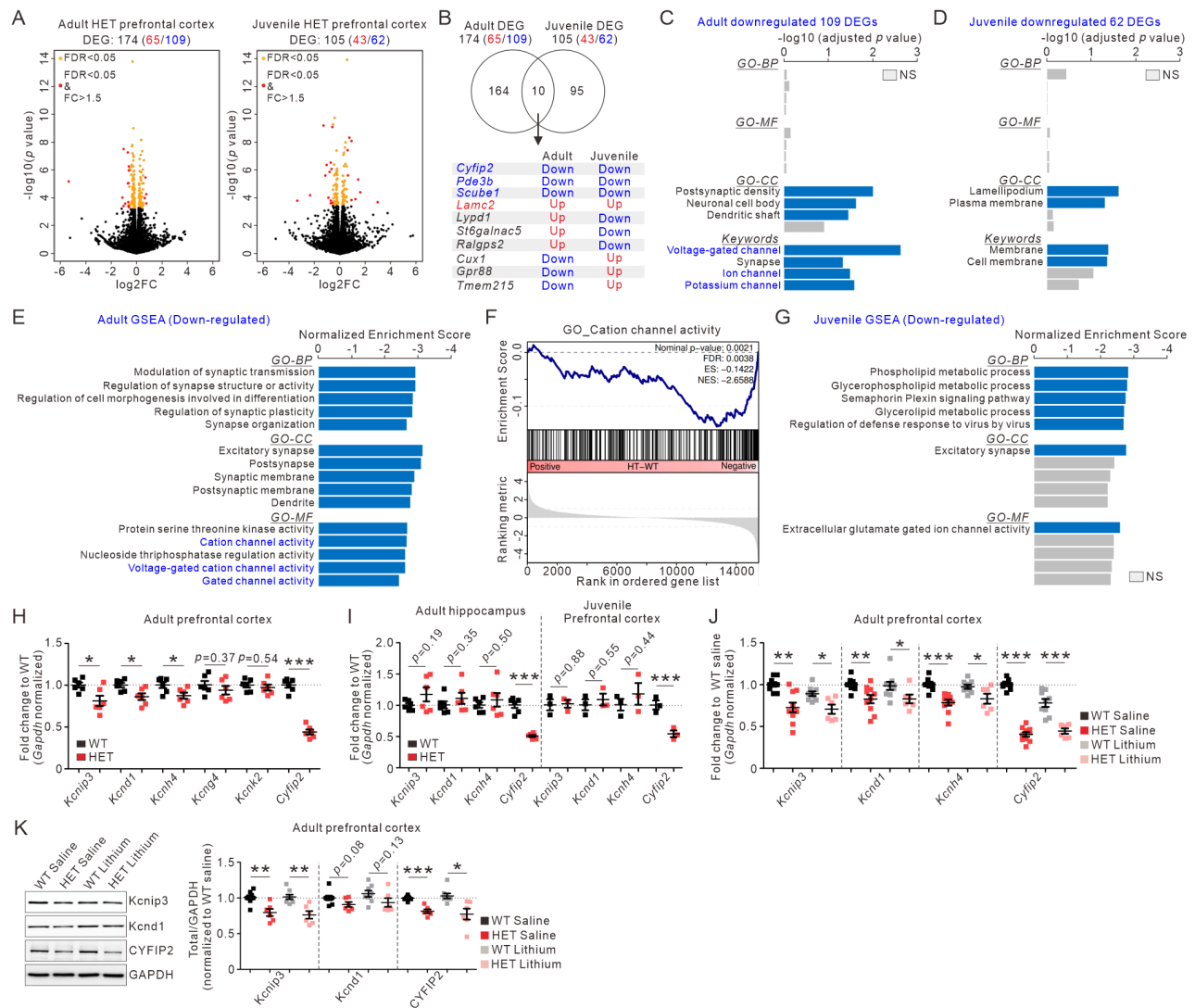


FIGURE 7: Reduced expression of potassium channel genes in the prefrontal cortex (PFC) of adult *Cyfp2*^{+/-} (HET) mice. (A) Volcano plots for the adult and juvenile PFC RNA-sequencing analyses of *Cyfp2*^{+/-} mice. Differentially expressed genes (DEGs), defined by a false-discovery rate (FDR) < 0.05, are shown as orange (FC ≤ 1.5) or red (FC > 1.5) circles. FC, fold change. (B) Venn diagram showing the number of common DEGs between adult and juvenile *Cyfp2*^{+/-} PFC. The 10 common DEGs and their directional expression changes in the adult and juvenile *Cyfp2*^{+/-} PFC are shown. (C) Database for annotation, visualization and integrated discovery (DAVID) functional analysis of the downregulated DEGs of the adult *Cyfp2*^{+/-} PFC. Only significant terms (Benjamini-adjusted *p* value < 0.05) are shown. BP, biological process; CC, cellular component; MF, molecular function; NS, not significant. (D) DAVID functional analysis of the downregulated DEGs of the juvenile *Cyfp2*^{+/-} PFC. (E) Normalized enrichment scores of the gene set enrichment analysis (GSEA) on the gene ontology (GO) gene sets for the adult *Cyfp2*^{+/-} PFC RNA-sequencing analysis. (F) The enrichment plot of GSEA of adult *Cyfp2*^{+/-} PFC for the cation channel activity gene set. (G) GSEA on the GO gene sets for the juvenile *Cyfp2*^{+/-} PFC RNA-sequencing analysis. (H) qRT-PCR analysis of potassium channel genes in the adult PFC of wild-type (WT) and *Cyfp2*^{+/-} mice (*n* = 6 mice per genotype; Student unpaired two-tailed *t* test). (I) qRT-PCR analysis of potassium channel genes in the adult hippocampus and juvenile PFC (*n* = 3–6 mice per genotype). (J, K) qRT-PCR and western blot analyses of potassium channel genes in the medial prefrontal cortex (mPFC) of WT and *Cyfp2*^{+/-} mice injected with saline or lithium (*n* = 6–10 mice per condition). Data are represented as the mean ± standard error of the mean. **p* < 0.05; ***p* < 0.01; ****p* < 0.001.

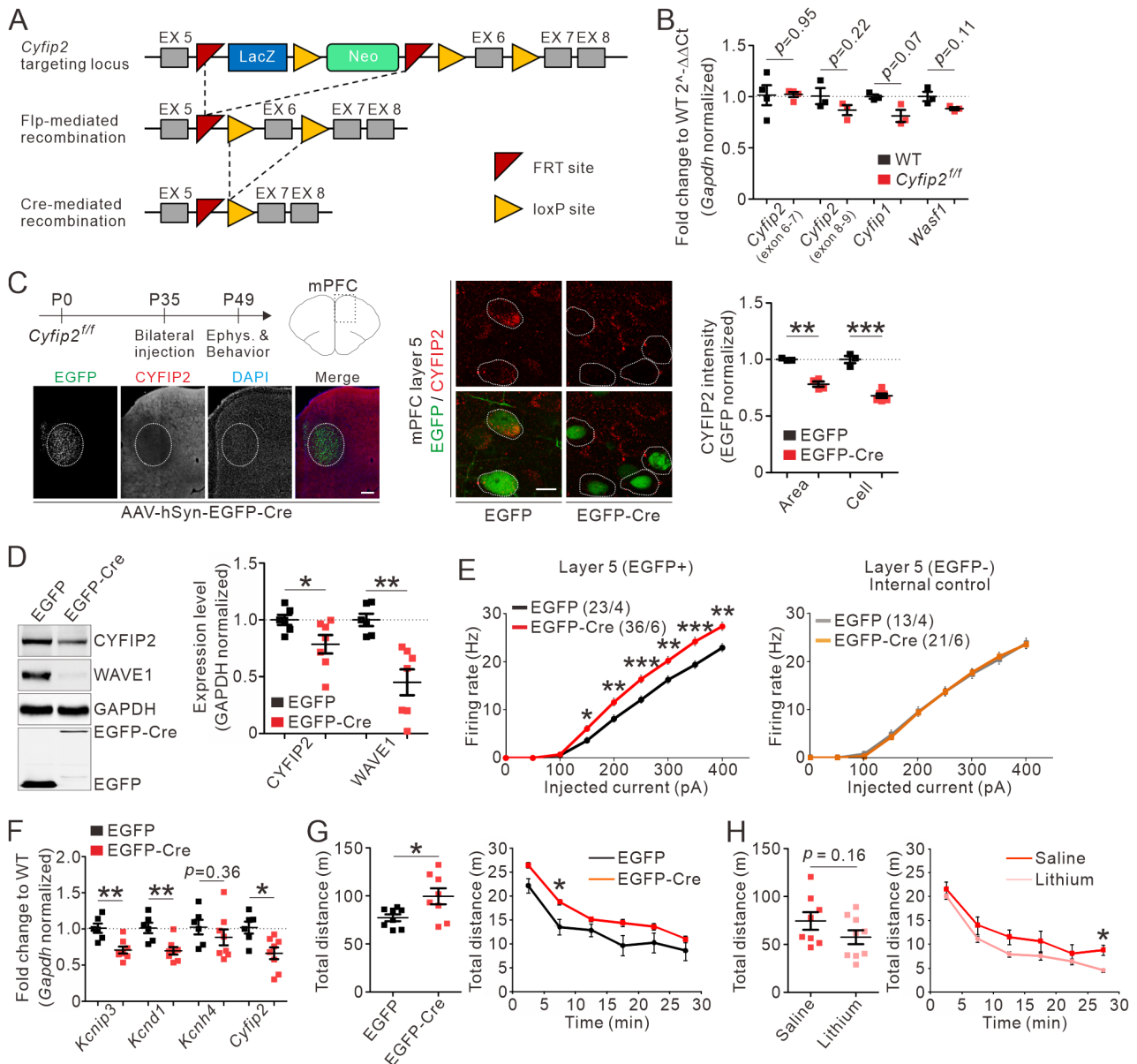


FIGURE 8: CYFIP2 reduction in the medial prefrontal cortex (mPFC) is sufficient to induce neuronal hyperexcitability and behavioral hyperactivity. (A) Schematic diagram showing Cre-mediated removal of exon 6 of the *Cyfp2* gene. EX, exon. (B) qRT-PCR analysis of *Cyfp1*, *Cyfp2*, and *Wasf1* transcripts in the prefrontal cortex (PFC) of wild-type (WT) and *Cyfp2*^{fl/fl} mice (n = 3 mice per genotype; Student unpaired two-tailed t test). (C) Experimental time line of virus injection, electrophysiology (Ephys.), and behavioral assays. Fluorescent immunohistochemistry (IHC) of CYFIP2 and EGFP antibodies in the mPFC of *Cyfp2*^{fl/fl} mice injected with AAV-hSyn-EGFP or AAV-hSyn-EGFP-Cre. CYFIP2 intensities were quantified in an area-based (low-magnification) and a cell-based (high-magnification) manner. Note that EGFP-Cre proteins localize to the nucleus. Scale bars: 200 (left panel) and 10μm (right panel). Quantification of CYFIP2 intensity in the mPFC (n = 3 and 4 mice for EGFP and EGFP-Cre injections, respectively). (D) Representative western blot images and quantification of CYFIP2 and WAVE1 in the mPFC of *Cyfp2*^{fl/fl} mice expressing EGFP or EGFP-Cre (n = 6 and 7 mice for EGFP and EGFP-Cre injections, respectively). (E) Intrinsic excitability of mPFC layer 5 (L5) neurons from virus-injected *Cyfp2*^{fl/fl} mice. Numbers of cells and mice used for the experiments are indicated. (F) qRT-PCR analysis of potassium channel genes in the mPFC of *Cyfp2*^{fl/fl} mice expressing EGFP or EGFP-Cre (n = 6 and 8 mice for EGFP and EGFP-Cre injections, respectively). (G) Locomotor activity of virus-injected *Cyfp2*^{fl/fl} mice in the open field test (OFT). Left panel, total distance of 30 minute measurement; n = 8 mice for condition. Right panel, 5 minute time bins for distance traveled in the OFT (two-way ANOVA with Bonferroni post-hoc test). (H) Locomotor activity of EGFP-Cre-injected *Cyfp2*^{fl/fl} mice after saline or lithium treatment (n = 8 and 9 mice for saline and lithium treatment, respectively). Data are represented as the mean ± standard error of the mean. *p < 0.05; **p < 0.01; ***p < 0.001.

performing electrophysiological analyses on acute brain slices incubated in extracellular solution containing LiCl or NaCl (as the vehicle control). We found that lithium affected neither the amplitude nor the frequency of mEPSCs of L5 neurons in both WT and *Cyfp2*^{+/-} mice (Fig 6). In lithium-treated conditions, the frequency of mEPSCs was still increased in the *Cyfp2*^{+/-} neurons compared with WT neurons. However, we found that lithium fully ameliorated the increased excitability of L5 neurons in the *Cyfp2*^{+/-} mPFC. Excitability was increased in *Cyfp2*^{+/-} neurons compared with that in WT neurons in vehicle-treated conditions, but lithium decreased the excitability of *Cyfp2*^{+/-} neurons to levels comparable to those in WT neurons.

To understand how lithium normalized hyperexcitability, we measured the intrinsic properties of L5 neurons (Fig 6). There was no difference in the resting membrane potential (RMP) between WT and *Cyfp2*^{+/-} mice in both vehicle- and lithium-treated conditions. However, input resistance was increased in *Cyfp2*^{+/-} neurons compared with that in WT in vehicle-treated conditions, suggesting that the amount of current injection required to induce the same voltage change was less in *Cyfp2*^{+/-} neurons compared with WT, thus explaining the hyperexcitability of *Cyfp2*^{+/-} neurons (see Fig 6I, left). Moreover, the input resistance of *Cyfp2*^{+/-} neurons became comparable to that of WT neurons in lithium-treated conditions. Intriguingly, when we changed the K⁺-based internal solution to a Cs⁺-based solution during measurement, there was no difference in input resistance between WT and *Cyfp2*^{+/-} neurons even in vehicle-treated conditions (see Fig 6I, right). Consistent with the decrease in neuronal excitability, acute injection of lithium ameliorated PTZ-induced behavioral seizures in adult *Cyfp2*^{+/-} mice.

Reduced Expression of Potassium Channels in the Adult *Cyfp2*^{+/-} PFC

Input resistance of *Cyfp2*^{+/-} L5 neurons increased in the presence of a K⁺-based, but not a Cs⁺-based, internal solution (Fig 6I), suggesting that changes in potassium channels might mediate hyperexcitability of these neurons. To understand the molecular changes, we performed RNA sequencing on both adult and juvenile PFC of *Cyfp2*^{+/-} mice and their WT littermates. We identified 174 and 105 differentially expressed genes (DEGs) from the adult and juvenile *Cyfp2*^{+/-} PFC, respectively (Fig 7; Tables S2 and S3). Only 10 genes, including *Cyfp2*, were common between the 2 DEG lists. Database for Annotation, Visualization and Integrated Discovery (DAVID) functional annotation analysis of the DEGs in adult *Cyfp2*^{+/-} PFC suggested that ion channel- and ribosome-related terms were significant for the down- and upregulated DEGs, respectively (Table S4). In contrast, the same analyses on the DEGs of juvenile *Cyfp2*^{+/-} PFC suggested that cell membrane-related terms

were significant for the downregulated DEGs (Table S5). There was no significant term for the upregulated DEGs of juvenile *Cyfp2*^{+/-} PFC.

Gene set enrichment analysis (GSEA) also indicated that ion channel- and synapse- related terms were significantly enriched in the downregulated gene profile of the adult *Cyfp2*^{+/-} PFC (Fig 7; Table S6). In contrast, GSEA on the juvenile *Cyfp2*^{+/-} PFC indicated that metabolic process-related terms were significantly enriched in the downregulated gene profile (Table S7). To validate these findings, we performed qRT-PCR analysis on five potassium channel genes categorized in the ion channel-related terms that were enriched in the adult *Cyfp2*^{+/-} PFC. Three of them (*Kcnip3*, *Kcnd1*, and *Kcnb4*) had significantly decreased expression in the adult PFC, but not in the adult hippocampus or juvenile PFC, of *Cyfp2*^{+/-} mice. Nevertheless, acute lithium treatment did not normalize mRNA levels of the potassium channel genes in the adult *Cyfp2*^{+/-} mPFC. At the protein level, *Kcnip3* had significantly decreased expression in the adult *Cyfp2*^{+/-} mPFC, but lithium treatment did not normalize it.

mPFC-Specific CYFIP2 Reduction Is Sufficient to Induce Neuronal Hyperexcitability and Behavioral Hyperactivity

Next, we investigated whether mPFC-specific reduction of CYFIP2 was sufficient to induce the electrophysiological and behavioral changes observed in *Cyfp2*^{+/-} mice (Fig 8). We generated floxed *Cyfp2* mutant (*Cyfp2*^{fl}) mice possessing loxP sites flanking exon 6 of the *Cyfp2* gene. The loxP sites themselves did not affect expression of *Cyfp2*, *Cyfp1*, and *Wasf1* (encoding WAVE1). We bilaterally injected an adeno-associated virus (AAV9, human Synapsin 1 promoter), expressing either EGFP or EGFP-Cre, into the mPFC of *Cyfp2*^{fl} mice at P35 and performed electrophysiological and behavioral experiments at P49. We confirmed that EGFP-Cre expression reduced CYFIP2 and WAVE1 in the *Cyfp2*^{fl} mPFC compared with EGFP expression. We found that *Cyfp2*^{fl} L5 neurons expressing EGFP-Cre showed hyperexcitability compared with those expressing EGFP (see Fig 8E, left). As an internal control, we also measured the excitabilities of nearby EGFP-negative L5 neurons in both conditions, which showed comparable excitabilities (see Fig 8E, right). Furthermore, EGFP-Cre expression reduced potassium channel gene expression. At the behavioral level, EGFP-Cre-injected *Cyfp2*^{fl} mice displayed hyperactivity compared with EGFP-injected mice. Furthermore, the hyperactivity of EGFP-Cre-injected mice was mildly ameliorated by acute lithium injection.

Discussion

Defects in the PFC have been identified in various brain disorders, including ID, ASDs, and SCZ.³⁴ The PFC is

composed of morphologically and functionally heterogeneous neuronal populations in a multilayered architecture.³⁸ The superficial L2/3 neurons are the primary input neurons receiving afferent projections from limbic and cortical regions, whereas the deepest layer 5/6 neurons are the main output neurons targeting various subcortical nuclei, such as the nucleus accumbens, dorsal raphe nucleus, and ventral tegmental area.³³ Therefore, abnormal synaptic transmission and excitability of L5 neurons in *Cyfip2*^{+/-} mice might drive circuit-level dysfunction in these brain regions, thereby causing behavioral changes. Moreover, hyperexcitability of L5 neurons can also promote increased seizure susceptibility in adult *Cyfip2*^{+/-} mice.

Beyond increased seizure susceptibility, the hyperexcitability of mPFC L5 neurons might contribute to the enhanced ASSR of *Cyfip2*^{+/-} mice by stimulating the cortical interneuron–pyramidal neuron network.⁴⁴ The enhanced ASSR and abnormal behavior of *Cyfip2*^{+/-} mice somewhat contradict numerous reports on deficits of ASSR in psychosis patients and also contrast with the positive role of prefrontal gamma oscillations in top-down control of attention.⁴⁵ However, some investigators have reported disparate findings, such as increased ASSR in psychosis patients,⁴⁶ positive correlations between hallucinations and gamma oscillations,⁴⁷ and state-dependent ASSR switch.⁴⁸ In addition, a recent study of graded doses of ketamine has presented a dose-dependent biphasic ASSR,⁴⁹ suggesting that the ASSR abnormalities include both decreased and increased gamma activity. Therefore, it is also possible that the enhanced ASSR of *Cyfip2*^{+/-} mice might reflect a pathological condition of the mice rather than augmentation of PFC functions. Furthermore, understanding any relationship between the enhanced ASSR and previously reported altered sensorimotor gating²⁰ of *Cyfip2*^{+/-} mice will be an interesting topic for future studies.

RNA-sequencing analysis showed reduced expression of potassium channel genes in the adult *Cyfip2*^{+/-} PFC. How decreased CYFIP2 caused transcriptional changes of potassium channels is not immediately clear. However, one possible candidate is β -catenin, a CYFIP2 interactor in the mouse PFC,⁵⁰ which can translocate from the synaptic compartment to the nucleus to induce changes in gene expression.⁵¹ Another potential mechanism underlying the hyperexcitability could be that the WRC can interact with diverse membrane proteins, including ion channels.⁵² Given that CYFIP forms an interaction surface of the WRC toward the membrane proteins, it is possible that interactions between WRC and some ion channels, and thus the properties of such ion channels, might be altered in the *Cyfip2*^{+/-} mPFC. Furthermore, increased F-actin levels in *Cyfip2*^{+/-} neurons may affect ion channel activities.⁵³

Molecular and functional changes in the mPFC and abnormal behaviors were observed in adult, but not juvenile, *Cyfip2*^{+/-} mice. Furthermore, in contrast to the increased seizure susceptibility of adult *Cyfip2*^{+/-} mice, juvenile *Cyfip2*^{+/-} mice unexpectedly showed reduced behavioral seizure susceptibility compared with that of WT mice, suggesting that CYFIP2 can have age-specific brain functions. Intriguingly, we noticed that some potassium channel genes were identified as upregulated DEGs in the juvenile *Cyfip2*^{+/-} PFC (Table S3). Whether the age-specific differences in the regulation of potassium channel genes in the PFC and possibly other brain regions are involved in the age-specific phenotypes of *Cyfip2*^{+/-} mice requires further investigation. More importantly, patients with *CYFIP2* missense variants show early-onset clinical symptoms.^{7–10} Therefore, our results for adult *Cyfip2*^{+/-} mice should be interpreted with caution and might not translate directly to human *CYFIP2*-associated brain disorders.

Lithium, as a mood stabilizer, is the US Food and Drug Administration-approved first-line treatment for bipolar disorder.⁵⁴ Additionally, owing to its effects on multiple cellular signals, the clinical potential of lithium as a treatment for various brain disorders has been considered.⁵⁵ For example, lithium treatment showed beneficial effects on FXS patients in an open-label clinical trial.⁵⁶ Mechanistically, direct inhibition of glycogen synthase kinase-3 (GSK3) by lithium mediates its effect on behaviors and cognitive functions of *Fmr1*-null mice that have basally hyperactive GSK3 compared with WT mice.³² Whether GSK3 inhibition is also involved in the response to lithium in *Cyfip2*^{+/-} mice should be investigated, especially considering the normal GSK3 activity in the *Cyfip2*^{+/-} mPFC (Fig 2H). In the *Cyfip2*^{+/-} mPFC, lithium treatment did not increase either mRNA or protein levels of the reduced potassium channel genes, which could be expected because of the short period of time (30 minutes) between lithium injection and the assays. Therefore, other acute mechanisms might be involved in the effects of lithium on *Cyfip2*^{+/-} mice. Lithium can modulate neuronal excitability by affecting ion channel functions at the protein level. Specifically, lithium passes through other cation channels and affects the channel ion influx.⁵⁷ Lithium also modulates phosphorylation of diverse ion channels and their functions by inhibiting GSK3.⁵⁸ In addition, lithium modulates G protein-gated potassium (GIRK) channel activity by acting on upstream G proteins.⁵⁹ These mechanisms might contribute collectively to lithium-mediated normalization of hyperexcitability in *Cyfip2*^{+/-} L5 neurons.

The effect of lithium on hyperactivity of EGFP-Cre-injected *Cyfip2*^{fl/fl} mice was relatively weaker than that

on *Cyfp2*^{+/-} mice. The homozygous (ie, flox/flox) and heterozygous (ie, *Cyfp2*^{+/-}) difference might contribute to the differential responses to lithium. In addition, CYFIP2 proteins were reduced for 2 weeks of the adult stage in EGFP-Cre-injected *Cyfp2*^{off} mice, whereas they were reduced for whole developmental stages in *Cyfp2*^{+/-} mice. Moreover, CYFIP2 reduction and functional changes in brain regions other than the mPFC might contribute to the behavioral phenotypes and response to lithium of *Cyfp2*^{+/-} mice. For example, we previously showed abnormal dendritic spines in L2/3 neurons of the somatosensory cortex of adult *Cyfp2*^{+/-} mice.²⁰ Spatiotemporally more comprehensive analysis on the expression and function of CYFIP2 is required to understand these important issues.

In conclusion, our study suggests that L5-specific prefrontal dysfunction, especially hyperexcitability, underlies both the pathophysiology and the lithium-mediated amelioration of neurobehavioral phenotypes in adult *Cyfp2*^{+/-} mice. Beyond *CYFIP2* haploinsufficiency, these results, if supported by follow-up studies, might also be implicated in ID and epilepsy of patients with *CYFIP2* missense variants,^{7–10} because both types of *CYFIP2* variants are expected to induce common molecular changes, such as aberrant actin polymerization.^{18,60} Prefrontal L5 neurons might be more vulnerable to these CYFIP2-dependent changes in the maintenance of their normal function. Moreover, whether lithium has any clinical potential for the treatment of *CYFIP2*-associated brain disorders is an important issue for further investigation.

Acknowledgments

This work was supported by National Research Foundation of Korea (NRF) grants funded by the Korea Government Ministry of Science and Information and Communications Technology (ICT) (NRF-2018R1C1B6001235 and NRF-2018M3C7A1024603, K.H.; NRF-2020R1A2C3011464, Se-Y.C.; NRF-2017M3C7A1048086, K.J.L.; and NRF-2017R1A2B3012659, B.K.), the Korea Brain Research Institute (KBRI) basic research program funded by Ministry of Science and ICT (18-BR-01-01 and 18-BR-04-01, K.J.L), and KBSI R&D program (Project No. D37700). Serial block-face EM images were acquired in the Advanced Neural Imaging Center in KBRI.

We thank C. H. Lee, N. Y. Do and N-Y. Seo for EM image segmentation and Dr N. Ohno (Jichi Medical University, Japan) for the kind gift of Ketjen black powder.

Author Contributions

K.J.L., Se-Y.C., and K.H. contributed to the conception and design of the study. S-H.L., Y.Z., J.P., B.K., Ya.K.,

S.H.L., G.H.K., Y.H.H., B.L., Yo.K., Ye.L., J.Y.K., Hyo.K., Su-Y.C., S.J., Ya.L., S.K., C.J., K.P., Eunje.K., Yo.L., Hyu.K., Eunjo.K., J.H.C., J.K., and K.J.L. contributed to data acquisition and analysis. S-H.L., Y.Z., J.P., B.K., Hyo.K., K.P., J.H.C., J.K., K.J.L., Se-Y.C., and K.H. wrote the paper and prepared the figures.

Potential Conflicts of Interest

Nothing to report.

References

1. Schenck A, Bardoni B, Moro A, et al. A highly conserved protein family interacting with the fragile X mental retardation protein (FMRP) and displaying selective interactions with FMRP-related proteins FXR1P and FXR2P. *Proc Natl Acad Sci U S A* 2001;98:8844–8849.
2. Abekhouk S, Bardoni B. CYFIP family proteins between autism and intellectual disability: links with fragile X syndrome. *Front Cell Neurosci* 2014;8:81.
3. Cox DM, Butler MG. The 15q11.2 BP1-BP2 microdeletion syndrome: a review. *Int J Mol Sci* 2015;16:4068–4082.
4. Oguro-Ando A, Rosensweig C, Herman E, et al. Increased CYFIP1 dosage alters cellular and dendritic morphology and dysregulates mTOR. *Mol Psychiatry* 2015;20:1069–1078.
5. Lee JH, Kim HJ, Yoon JM, et al. Interstitial deletion of 5q33.3q35.1 in a boy with severe mental retardation. *Korean J Pediatr* 2016;59:S19–S24.
6. Spranger S, Rommel B, Jauch A, et al. Interstitial deletion of 5q33.3q35.1 in a girl with mild mental retardation. *Am J Med Genet* 2000;93:107–109.
7. Zweier M, Begemann A, McWalter K, et al. Spatially clustering de novo variants in CYFIP2, encoding the cytoplasmic FMRP interacting protein 2, cause intellectual disability and seizures. *Eur J Hum Genet* 2019;27:747–759.
8. Peng J, Wang Y, He F, et al. Novel west syndrome candidate genes in a Chinese cohort. *CNS Neurosci Ther* 2018;24:1196–1206.
9. Nakashima M, Kato M, Aoto K, et al. De novo hotspot variants in CYFIP2 cause early-onset epileptic encephalopathy. *Ann Neurol* 2018;83:794–806.
10. Zhong M, Liao S, Li T, et al. Early diagnosis improving the outcome of an infant with epileptic encephalopathy with cytoplasmic FMRP interacting protein 2 mutation: case report and literature review. *Medicine* 2019;98:e17749.
11. Bagni C, Tassone F, Neri G, Hagerman R. Fragile X syndrome: causes, diagnosis, mechanisms, and therapeutics. *J Clin Invest* 2012;122:4314–4322.
12. Soderling SH, Guire ES, Kaech S, et al. A WAVE-1 and WRP signaling complex regulates spine density, synaptic plasticity, and memory. *J Neurosci* 2007;27:355–365.
13. Bozdagi O, Sakurai T, Dorr N, et al. Haploinsufficiency of *Cyfp1* produces fragile X-like phenotypes in mice. *PLoS One* 2012;7:e42422.
14. Davenport EC, Szulc BR, Drew J, et al. Autism and schizophrenia-associated CYFIP1 regulates the balance of synaptic excitation and inhibition. *Cell Rep* 2019;26:2037–2051.e6.
15. Dominguez-Iturza N, Lo AC, Shah D, et al. The autism- and schizophrenia-associated protein CYFIP1 regulates bilateral brain connectivity and behaviour. *Nat Commun* 2019;10:3454.
16. Abekhouk S, Sahin HB, Grossi M, et al. New insights into the regulatory function of CYFIP1 in the context of WAVE- and FMRP-containing complexes. *Dis Model Mech* 2017;10:463–474.
17. Cioni JM, Wong HH, Bressan D, et al. Axon-axon interactions regulate topographic optic tract sorting via CYFIP2-dependent WAVE complex function. *Neuron* 2018;97:1078–1093.e6.

18. Zhang Y, Lee Y, Han K. Neuronal function and dysfunction of CYFIP2: from Actin dynamics to early infantile epileptic encephalopathy. *BMB Rep* 2019;52:304–311.
19. Zhang Y, Kang HR, Han K. Differential cell-type-expression of CYFIP1 and CYFIP2 in the adult mouse hippocampus. *Anim Cells Syst* 2019; 23:380–383.
20. Han K, Chen H, Gennarino VA, et al. Fragile X-like behaviors and abnormal cortical dendritic spines in cytoplasmic FMR1-interacting protein 2-mutant mice. *Hum Mol Genet* 2015;24:1813–1823.
21. Ure K, Lu H, Wang W, et al. Restoration of Mecp2 expression in GABAergic neurons is sufficient to rescue multiple disease features in a mouse model of Rett syndrome. *Elife* 2016;5:e14198.
22. Han K, Holder JL Jr, Schaaf CP, et al. SHANK3 overexpression causes manic-like behaviour with unique pharmacogenetic properties. *Nature* 2013;503:72–77.
23. Lee Y, Kim SG, Lee B, et al. Striatal transcriptome and interactome analysis of Shank3-overexpressing mice reveals the connectivity between Shank3 and mTORC1 signaling. *Front Mol Neurosci* 2017;10:201.
24. Lee B, Zhang Y, Kim Y, et al. Age-dependent decrease of GAD65/67 mRNAs but normal densities of GABAergic interneurons in the brain regions of Shank3-overexpressing manic mouse model. *Neurosci Lett* 2017;649:48–54.
25. Walton J. Lead aspartate, an en bloc contrast stain particularly useful for ultrastructural enzymology. *J Histochem Cytochem* 1979;27:1337–1342.
26. Nguyen HB, Thai TQ, Saitoh S, et al. Conductive resins improve charging and resolution of acquired images in electron microscopic volume imaging. *Sci Rep* 2016;6:23721.
27. Fiala JC, Harris KM. Cylindrical diameters method for calibrating section thickness in serial electron microscopy. *J Microsc* 2001;202:468–472.
28. Cardona A, Saalfeld S, Schindelin J, et al. TrakEM2 software for neural circuit reconstruction. *PLoS One* 2012;7:e38011.
29. Kang MS, Choi TY, Ryu HG, et al. Autism-like behavior caused by deletion of vaccinia-related kinase 3 is improved by TrkB stimulation. *J Exp Med* 2017;214:2947–2966.
30. Seo J, Hong J, Lee SJ, Choi SY. C-Jun N-terminal phosphorylation is essential for hippocampal synaptic plasticity. *Neurosci Lett* 2012;531:14–19.
31. Shahriari Y, Krusienski D, Dadi YS, et al. Impaired auditory evoked potentials and oscillations in frontal and auditory cortex of a schizophrenia mouse model. *World J Biol Psychiatry* 2016;17:439–448.
32. Yuskaitis CJ, Mines MA, King MK, et al. Lithium ameliorates altered glycogen synthase kinase-3 and behavior in a mouse model of fragile X syndrome. *Biochem Pharmacol* 2010;79:632–646.
33. Riga D, Matos MR, Glas A, et al. Optogenetic dissection of medial prefrontal cortex circuitry. *Front Syst Neurosci* 2014;8:230.
34. Schubert D, Martens GJ, Kolk SM. Molecular underpinnings of prefrontal cortex development in rodents provide insights into the etiology of neurodevelopmental disorders. *Mol Psychiatry* 2015;20:795–809.
35. Focking M, Lopez LM, English JA, et al. Proteomic and genomic evidence implicates the postsynaptic density in schizophrenia. *Mol Psychiatry* 2015;20:424–432.
36. Zhao L, Wang D, Wang Q, et al. Drosophila cyfp regulates synaptic development and endocytosis by suppressing filamentous Actin assembly. *PLoS Genet* 2013;9:e1003450.
37. Bachmann SO, Sledziowska M, Cross E, et al. Behavioral training rescues motor deficits in Cyfp1 haploinsufficiency mouse model of autism spectrum disorders. *Transl Psychiatry* 2019;9:29.
38. van Aerde KI, Feldmeyer D. Morphological and physiological characterization of pyramidal neuron subtypes in rat medial prefrontal cortex. *Cereb Cortex* 2015;25:788–805.
39. Arellano JI, Benavides-Piccione R, Defelipe J, Yuste R. Ultrastructure of dendritic spines: correlation between synaptic and spine morphologies. *Front Neurosci* 2007;1:131–143.
40. Feng G, Mellor RH, Bernstein M, et al. Imaging neuronal subsets in transgenic mice expressing multiple spectral variants of GFP. *Neuron* 2000;28:41–51.
41. Risher WC, Patel S, Kim IH, et al. Astrocytes refine cortical connectivity at dendritic spines. *Elife* 2014;3:e04047.
42. Contractor A, Klyachko VA, Portera-Cailliau C. Altered neuronal and circuit excitability in fragile X syndrome. *Neuron* 2015;87:699–715.
43. O'Donnell BF, Vohs JL, Krishnan GP, et al. The auditory steady-state response (ASSR): a translational biomarker for schizophrenia. *Suppl Clin Neurophysiol* 2013;62:101–112.
44. Whittington MA, Traub RD, Kopell N, et al. Inhibition-based rhythms: experimental and mathematical observations on network dynamics. *Int J Psychophysiol* 2000;38:315–336.
45. Kim H, Ahrlund-Richter S, Wang X, et al. Prefrontal parvalbumin neurons in control of attention. *Cell* 2016;164:208–218.
46. Hong LE, Summerfelt A, McMahon R, et al. Evoked gamma band synchronization and the liability for schizophrenia. *Schizophr Res* 2004;70:293–302.
47. Mulert C, Kirsch V, Pascual-Marqui R, et al. Long-range synchrony of gamma oscillations and auditory hallucination symptoms in schizophrenia. *Int J Psychophysiol* 2011;79:55–63.
48. Hwang E, Brown RE, Kocsis B, et al. Optogenetic stimulation of basal forebrain parvalbumin neurons modulates the cortical topography of auditory steady-state responses. *Brain Struct Funct* 2019;224:1505–1518.
49. Kozono N, Honda S, Tada M, et al. Auditory steady state response; nature and utility as a translational science tool. *Sci Rep* 2019;9:8454.
50. Li J, Zhang W, Yang H, et al. Spatiotemporal profile of postsynaptic interactomes integrates components of complex brain disorders. *Nat Neurosci* 2017;20:1150–1161.
51. Qin L, Ma K, Wang ZJ, et al. Social deficits in Shank3-deficient mouse models of autism are rescued by histone deacetylase (HDAC) inhibition. *Nat Neurosci* 2018;21:564–575.
52. Chen B, Brinkmann K, Chen Z, et al. The WAVE regulatory complex links diverse receptors to the Actin cytoskeleton. *Cell* 2014;156:195–207.
53. Zhang Y, Zhang XF, Fleming MR, et al. Kv3.3 Channels bind Hax-1 and Arp2/3 to assemble a stable local Actin network that regulates channel gating. *Cell* 2016;165:434–448.
54. Alda M. Lithium in the treatment of bipolar disorder: pharmacology and pharmacogenetics. *Mol Psychiatry* 2015;20:661–670.
55. Chiu CT, Chuang DM. Molecular actions and therapeutic potential of lithium in preclinical and clinical studies of CNS disorders. *Pharmacol Ther* 2010;128:281–304.
56. Berry-Kravis E, Sumis A, Herve C, et al. Open-label treatment trial of lithium to target the underlying defect in fragile X syndrome. *J Dev Behav Pediatr* 2008;29:293–302.
57. Jakobsson E, Arguello-Miranda O, Chiu S-W, et al. Towards a unified understanding of lithium action in basic biology and its significance for applied biology. *J Membr Biol* 2017;250:587–604.
58. Wildburger NC, Laezza F. Control of neuronal ion channel function by glycogen synthase kinase-3: new prospective for an old kinase. *Front Mol Neurosci* 2012;5:80.
59. Farhy Tselnick I, Tsemakhovich V, Rishal I, et al. Dual regulation of G proteins and the G-protein-activated K⁺ channels by lithium. *Proc Natl Acad Sci U S A* 2014;111:5018–5023.
60. Lee Y, Zhang Y, Ryu JR, et al. Reduced CYFIP2 stability by Arg87 variants causing human neurological disorders. *Ann Neurol* 2019;86: 803–805.