Common Pathophysiology in Multiple Mouse Models of Pitt–Hopkins Syndrome

Courtney Thaxton,^{1,2*} Alexander D. Kloth,^{1,2*} Ellen P. Clark,^{1,2} Sheryl S. Moy,^{3,4} Raymond A. Chitwood,⁵ and Benjamin D. Philpot^{1,2,4}

¹Department of Cell Biology and Physiology, ²Neuroscience Center, ³Department of Psychiatry, ⁴Carolina Institute for Developmental Disabilities, the University of North Carolina, Chapel Hill, North Carolina 27599, and ⁵Center for Learning and Memory, Department of Neuroscience, University of Texas, Austin, Texas 78712

Mutations or deletions of the transcription factor *TCF4* are linked to Pitt–Hopkins syndrome (PTHS) and schizophrenia, suggesting that the precise pathogenic mutations dictate cellular, synaptic, and behavioral consequences. Here, we generated two novel mouse models of PTHS, one that mimics the most common pathogenic *TCF4* point mutation (human R580W, mouse R579W) and one that deletes three pathogenic arginines, and explored phenotypes of these lines alongside models of pan-cellular or CNS-specific heterozygous *Tcf4* disruption. We used mice of both sexes to show that impaired *Tcf4* function results in consistent microcephaly, hyperactivity, reduced anxiety, and deficient spatial learning. All four PTHS mouse models demonstrated exaggerated hippocampal long-term potentiation (LTP), consistent with deficits in hippocampus-mediated behaviors. We further examined R579W mutant mice and mice with pan-cellular *Tcf4* heterozygosity and found that they exhibited hippocampal NMDA receptor hyperfunction, which likely drives the enhanced LTP. Together, our data pinpoint convergent neurobiological features in PTHS mouse models and provide a foundation for preclinical studies and a rationale for testing whether NMDAR antagonists might be used to treat PTHS.

Key words: autism; mouse; NMDA; Pitt–Hopkins syndrome; schizophrenia; *TCF4*

Significance Statement

Pitt–Hopkins syndrome (PTHS) is a rare neurodevelopmental disorder associated with *TCF4* mutations/deletions. Despite this genetic insight, there is a need to identify the function of *TCF4* in the brain. Toward this goal, we developed two mouse lines, including one harboring the most prevalent pathogenic point mutation, and compared them with two existing models that conditionally delete *Tcf4*. Our data identify a set of overlapping phenotypes that may serve as outcome measures for preclinical studies of PTHS treatments. We also discovered penetrant enhanced synaptic plasticity across mouse models that may be linked to increased NMDA receptor function. These data reveal convergent neurobiological characteristics of PTHS mouse models and support the further investigation of NMDA receptor antagonists as a possible PTHS treatment.

Introduction

Pitt–Hopkins syndrome (PTHS) is a rare neurodevelopmental disorder caused by mutation or deletion of the transcription fac-

tor 4 gene *TCF4* located in chromosomal region 18q21.1 (Amiel et al., 2007; Zweier et al., 2007, 2008). PTHS is associated with intellectual disability, seizures, loss of speech, microcephaly, reductions in hippocampal volume, and other neuroanatomical abnormalities (Amiel et al., 2007; Brockschmidt et al., 2007; Zweier et al., 2008; Rosenfeld et al., 2009; de Pontual et al., 2009; Whalen et al., 2012; Marangi and Zollino, 2015). Although *de novo* mutations or deletions in one copy of *TCF4* cause PTHS, alterations in *TCF4* have also been linked to autism and schizophrenia (Stefansson et al., 2009; Blake et al., 2010; Hasi et al.,

Received May 10, 2017; revised Nov. 8, 2017; accepted Nov. 30, 2017.

Author contributions: C.T., A.D.K., S.S.M., R.A.C., and B.D.P. designed research; C.T., A.D.K., E.P.C., S.S.M., and R.A.C. performed research; C.T., A.D.K., S.S.M., and R.A.C. analyzed data; C.T., A.D.K., and B.D.P. wrote the paper.

This work was supported by the Pitt–Hopkins Research Foundation (B.D.P., C.T., and A.D.K.), University of Pennsylvania Orphan Disease Center Million Dollar Bike Ride (Grant MDBR-15-108-PH to B.D.P. and C.T.), the Brain and Behavior Research Foundation (NARSAD Young Investigator Grant 20653 to C.T.), and the National Institute of General Medical Sciences (Training, Workforce Development and Diversity Grant K12GM000678 to A.D.K.). Confocal imaging was supported by the National Institute of Neurological Disorders and Stroke (Grant P30NS045892) and the Eunice Kennedy Shriver National Institute of Child Health and Human Development (NICHD Grant U54HD079124). The mouse behavioral phenotyping core was supported by the NICHD (Grant U54HD079124 to S.S.M.). We thank Natalia Riddick and Viktoriya Nikolova for work on behavioral phenotyping, Dan Holmberg (Lund University, Sweden) for *Tcf4*^{fllox} mice, and Tonis Timmusk (Tallinn University, Estonia) for luciferase constructs.

The authors declare no competing financial interests.

*C.T. and A.D.K. contributed equally to this work.

Correspondence should be addressed to Dr. Benjamin D. Philpot, University of North Carolina School of Medicine, 115 Mason Farm Road, Chapel Hill, NC 27599-7545. E-mail: bphilpot@med.unc.edu.

DOI:10.1523/JNEUROSCI.1305-17.2017

Copyright © 2018 the authors 0270-6474/18/380918-19\$15.00/0

2011; Navarrete et al., 2013; Quednow et al., 2014; Stessman et al., 2017), suggesting a need to identify the role of *TCF4* in the pathophysiology of these disorders.

TCF4 (also known as *E2-2*, *ITF2*, and *SEF-2*) encodes a basic helix-loop-helix (bHLH) transcription factor that regulates gene transcription through homodimerization or heterodimerization with other bHLH transcription factors, including several neuron-specific bHLH transcription factors. *TCF4* is highly expressed during embryonic and early postnatal development (de Pontual et al., 2009) and has particularly high expression in the hippocampus (Brzózka et al., 2010; Sepp et al., 2011; Navarrete et al., 2013). Mice with reduced (Kennedy et al., 2016) or increased (Brzózka et al., 2010) *Tcf4* expression exhibit severe cognitive deficits. Together, these findings suggest that perturbation of *TCF4* may disrupt synaptic function in the hippocampus and other regions, resulting in impaired learning and memory.

Approximately 30% of PTHS individuals have loss-of-function mutations or deletions of *TCF4*; the remaining 70% of individuals with PTHS have missense mutations, nonsense mutations, or small insertion-deletions (indels) that can result in dominant-negative isoforms of *TCF4* protein (Whalen et al., 2012). Such mutant *TCF4* isoforms can perturb the function of wild-type *TCF4* as well as other bHLH transcription factors (Sepp et al., 2011, 2012; Tamberg et al., 2015), making it difficult to predict their functional impact. Therefore, it is critical to identify the common and distinct pathophysiological disruptions that arise across multiple genetic subtypes of PTHS because this will ultimately facilitate therapeutic strategies for PTHS. Toward this goal, we developed two novel mouse models, one that harbors the highly prevalent R580W (mouse R579W) point mutation and one that has a small in-frame deletion that eliminates three pathogenic arginines, and studied them in parallel with existing PTHS mouse models. We found that these PTHS model mice exhibited remarkably consistent behavioral and electrophysiological deficits, which can serve as a basis for preclinical studies. Our data indicate that hippocampal NMDA receptor (NMDAR) hyperfunction is a highly penetrant feature of PTHS and thus presents an important translational opportunity.

Materials and Methods

Animals

All mice were raised on a 12:12 light:dark cycle with *ad libitum* access to food and water. The *Tcf4*^{Flox/+} mice were previously generated in the laboratory of Dan Holmberg (Bergqvist et al., 2000) and were maintained on a C57BL/6 background. The β -Actin-Cre (JAX stock # 019099, RRID:IMSR_JAX:019099) and Nestin-Cre (JAX stock #003771, RRID:IMSR_JAX:003771) mice were purchased from The Jackson Laboratory and maintained on a C57BL/6 background. Homozygous β -Actin-Cre mice were bred with heterozygous *Tcf4*^{Flox/+} mice to produce the *Tcf4*^{Flox/+;Actin-Cre} line (cHet). Heterozygous Nestin-Cre mice were bred with homozygous *Tcf4*^{Flox/Flox} mice to produce the *Tcf4*^{Flox/+;Nestin-Cre} line (CNS-Het). Littermate controls consisted of the genotypes *Tcf4*^{+/+} and *Tcf4*^{Flox/+}. All *Tcf4* mutant mice used were heterozygous for the *Tcf4* mutation because homozygous mutations of *Tcf4* result in embryonic to postnatal day 1 (P1) lethality. Experiments were conducted in strict compliance with protocols approved by the Institutional Animal Care and Use Committee at the University of North Carolina–Chapel Hill (UNC).

Generation and confirmation of mouse models

The *Tcf4*^{R579W} (R579W) and *Tcf4* ^{Δ 574–579} (Δ 574–579) mouse models were generated using CRISPR/Cas9 technology by the Animals Models Core facility at UNC. The sequence of the guide RNA (gRNA) was 5'-AATGCCCGTGAGCGCTGAGGG-3' (the bold sequence is the PAM) and was targeted to bp 2253–2274 of mouse *Tcf4*. The sequence of DNA

containing the R579W codon change (R579W-T RNA) was 5'-AAGGCTGAGCGCGAGAAGGAACGGAGGATGGCCAATAATGCCCGTGAGCGCTGTTGGTCCGAGATATCAACGAGGCTTCAAGGAGCTTGCCCGTATGGTGCAGCTC-3'. A silent mutation oligo (5'-AAGGCTGAGCGCGAGAAGGAACGGAGGATGGCCAATAATGCCCGTGAGCGGCTCAGAGTCCGAGATATCAACGAGGCTTCAAGGAGCTTGCCCGTATGGTGCAGCTC-3') was also used to encourage insertion of the oligos on both alleles with no deleterious effects. C57BL/6J embryos were microinjected with a mixture of 10 ng/ μ l Cas9 mRNA (*in vitro* transcribed and polyadenylated RNA), 10 ng/ μ l *Tcf4* guide RNA (purified and *in vitro* transcribed RNA), and 100 ng/ μ l *TCF4* R579W-T oligo. Embryonic injections produced a unique founder with an indel containing an insertion of the silent mutation oligo and an 18 bp in-frame deletion of aa 574–579 of *Tcf4* was generated (Δ 574–579), as well as two founder mice heterozygous for the R579W mutation. Founders from each mutation were checked for off-target effects of the guide RNA. No changes to any other nontargeted gene(s) of interest were found. The R579W and Δ 574–579 mice were maintained on a C57BL/6 background. These mice were genotyped by Sanger sequencing using the following steps: (1) DNA from either a small toe, tail, or ear sample was extracted; (2) the PCR product of ~500 bp was amplified using a forward primer mTCF4 Intron 17-FP (5'-CCAGCTCATCCCTTCCATTG-3') and a reverse primer mTCF4 Intron 18-RP (5'-CAATCATGTCTGAGGATTGAG-3'); (3) the PCR product was purified using the ISOLATE II PCR and Gel Kit (Bioline) according to the manufacturer's instructions; and (4) the purified product was sequenced by Sanger sequencing (Eton Biosciences) using the nested primer Nest mTCF4 R579W Seq-FP (5'-CAATAACGATGATGAGGACC-3'). Either the A-to-T transition at bp 1135 for R579W mice or the presence of deletion beginning at bp 1119 for the Δ 574–579 was used to confirm heterozygosity of the mutant alleles.

Luciferase assays

Constructs. We used the following constructs described previously (Sepp et al., 2011, 2012): mouse *TCF4* (pcDNA3.1_mTCF4-B⁻), human Achaete-Scute homolog 1 [ASCL1; pcDNA3.1_hASCL1(m)], 12 μ E5 promoter containing the luciferase reporter *Photinus pyralis* (pGL4.29 [luc2P_12mE5_Hygro]), and a control minimal reporter that expresses *Renilla reniformis* (pGL4[hRLucP_min_Hygro]).

Mutagenesis. To generate mouse *Tcf4*^{R575Q} (R575Q), *Tcf4*^{R577H} (R577H), *Tcf4*^{R579W} (R579W), *Tcf4*^{R581P} (R581P), and *Tcf4* ^{Δ 574–579} (Δ 574–579) mutations, we mutagenized the pcDNA3.1_mTCF4-B⁻ (*Tcf4*) construct using the Velocity Mutagenesis kit (Bioline) according to the manufacturer's instructions. We used the following primers for mutagenesis: for R575Q, the forward primer is GGCCAATAATGCCCA GGAGCGCTGAGGGTCC and the reverse primer is GGACCTCA GGCGCTCCTGGGCATTATTGGCC; for R577H the forward primer is CCAATAATGCCCGTGAGCACCTGAGGGTCCGAG and the reverse primer is CTCGGACCCTCAGGTGCTCACGGGCATTATTGG; for R579W the forward primer is GCGCGTGAGCGCCTGTGGGTCCGA GATATC and the reverse primer is GATATCTCGACCCACAGGCG CTCACGGGC; for R581P the forward primer is GCGCCTGAGGGTCCC AGATATCAACGAGGC and the reverse primer is GCCTCGTTGATA TCTGGGACCCTCAGGCGC; for Δ 574–579 the forward primer is GGA GGATGGCCAATAAT-GTCCGAGATATCAACGAGGC and the reverse primer is GCCTCGTTGATATCTCGGAC-ATTATTGGCCATCCTCC. We used the following cycling parameters: 98°C for 2 min; 18 cycles of 98°C (30 s), 57°C (30 s), 68°C (5.5 min); and 68°C (5 min). The mutagenized PCR products were digested with DpnI for 30 min at 37°C. Beta-mercaptoethanol (2 μ l) was added to the DpnI-digested products and transformed into BioBlue (Bioline) chemically competent cells by heat shock for 30 s at 42°C, followed by incubation on ice for 2 min. Prewarmed SOC/LB medium (500 μ l) was added and the transformed cells were incubated at 37°C with shaking (170 rpm) for at least 1 h before plating on LB agar plates supplemented with ampicillin (100 μ g/ml) and incubated overnight at 37°C. Clones were picked from the plates and grown in LB medium (5 ml) supplemented with ampicillin (100 μ g/ml) overnight at 37°C. An aliquot of the bacterial prep was mixed with 50% glycerol and stored at –80°C. The remaining prep was extracted for the

plasmid DNA using the ISOLATE II Plasmid mini kit (Bioline) according to the manufacturer's instructions. The plasmid DNA was sequenced by Sanger sequencing using the mTCF4 Intron 17-FP primer (described above) to identify positive clones.

Cell culture. HEK293T cells were grown in D10 medium supplemented with 10% fetal bovine serum (FBS) and penicillin–streptomycin (Pen-Strep; 10 U/ml; Thermo Fisher Scientific) at 37°C in 50% CO₂. Cells were maintained in the 10% FBS/Pen-Strep/D10 medium and passaged every 2–3 d. For the luciferase assays, the HEK293T cells were passaged and plated into 48-well plates and maintained in 10% FBS/Pen-Strep/D10 medium before transfection.

Transfections. HEK293T cells were transfected with cDNA constructs for luciferase assays using the Lipofectamine 2000 reagents as described previously (Sepp et al., 2011, 2012). For each luciferase assay, the 12 μ E5 promoter construct and the minimal reporter *R. reniformis* construct were cotransfected with either empty vector (pCDNA3.1 alone), mTCF4 (Tcf4), R575Q, R577H, R579W, R581P, or Δ 574–579 constructs. A total of 150 ng of cDNA was transfected per well, with equal amounts of cDNA from the 12 μ E5 promoter construct and the minimal reporter *R. reniformis* constructs. The HEK293T cells were incubated with transfection medium containing all of the cDNAs for 12–18 h at 37°C in 50% CO₂. After transfection, the medium was replaced with fresh D10/FBS/Pen-Strep medium. The cells were used for luciferase assays 24–48 h after transfection.

Luciferase assays. The Dual-Glo Luciferase Assay System (Promega) was used according to the manufacturer's instructions. The transfected cells were extracted in 50 μ l of passive lysis buffer using the above kit and assayed on a luminometer. As a background control, untransfected cell lysate was used to assess any inherent luminescence in the lysates. The background level was subtracted from all values obtained from transfections containing the 12 μ E5 promoter construct and these values were further normalized to the minimal reporter *R. reniformis* signal.

Behavioral testing

All behavioral testing was performed through the UNC Mouse Phenotyping Core. Testing began when animals were between 6 and 8 weeks of age and ended when they were between 16 and 21 weeks of age.

Elevated plus maze. The elevated plus maze is constructed to have two open arms and two closed arms, all 20 cm in length; the height in the closed arms is 30 cm. The entire maze is elevated 50 cm above the floor. Mice were given 15 min trial on the elevated plus maze. For testing, the mice were placed on the center section (8 $\times$ 8 cm) and allowed to explore the maze freely. Time and the number of entries into the open and closed arms were recorded. For the R579W mice, only data from the male mice from the first cohort are presented due to the high variability in the results from the females (two controls, three R579W). The second cohort of R579W did not undergo testing on the elevated plus maze.

Open field test. The mice were given a 60 min trial in an open-field chamber (41 $\times$ 41 $\times$ 30 cm) that was crossed by a grid of photobeams (VersaMax system; AccuScan Instruments). Counts were taken of the number of photobeams broken during the 60 min trial in 5 min intervals. Measures included locomotor activity (total distance traveled) and time spent in the center region.

Rotarod. The mice were tested for motor coordination and learning on an accelerating rotarod (Ugo Basile SRL). For initial testing, the mice were given three trials with 45 s between each trial. Two additional trials were given 48 h later. The initial rotation speed was set at 3 rpm and progressed to a maximum of 30 rpm across a 5 min maximum trial duration. The latency to fall from the top of the rotating barrel was recorded.

Three-chamber social approach task. The social testing apparatus was a rectangular, three-chambered box fabricated from clear Plexiglas. The dividing walls had doorways allowing access into each chamber. An automated image tracking system (Ethovision, RRID:SCR_000441; Noldus) provided measures of time spent in each chamber and the number of entries into each chamber. Each subject was allowed an initial habituation period when they could explore the fully open chamber freely for 10 min. After habituation, the test mouse was enclosed in the center compartment of the social test box. The first novel mouse (Novel 1) was

placed in one of the side chambers and was enclosed in a small Plexiglas cage drilled with holes, allowing for nose contact. An identical empty Plexiglas cage was placed in the opposite side of the chamber. After placement of the Novel 1 mouse and the empty cage, the doors were reopened and the subject was allowed to explore the social test box for 10 min. At the end of the sociability phase, another novel mouse (Novel 2) was placed in the empty Plexiglas container. The test mouse was again enclosed within the center region and then allowed to explore the social test box and chambers containing the Novel 1 mouse or the Novel 2 mouse for 10 min. Each novel mouse used was sex and strain matched to the test mouse. For the R579W cohort, only the male data are presented due to the low number and high variability in the female cohort.

Acoustic startle. Mice were tested twice: the first test was between 7 and 12 weeks of age and the second test at 16–21 weeks of age. For each test, mice were placed into individual small Plexiglas cylinders within larger, sound-attenuating chambers (San Diego Instruments SR-Lab system). Each cylinder was seated upon a piezoelectric transducer that quantified the vibrations. The chambers include a ceiling light, a fan, and a loudspeaker for the acoustic stimuli. Background sound levels (70 dB) and calibration of the acoustic stimuli were confirmed with a digital sound level meter (San Diego Instruments). Each session began with a 5 min habituation period, followed by 42 trials (seven of each type): no-stimulus trials, acoustic startle stimulus alone (120 dB for 40 ms) trials, and prepulse stimulus (20 ms; 74, 78, 82, 86, or 90 dB) trials that occurred 100 ms before the onset of the acoustic startle stimulus. Measures were taken of the startle amplitude for each trial across a 65 ms sampling window. An overall analysis was performed for each subject's data for levels of prepulse inhibition at each prepulse sound level, calculated as $100 - [(response\ amplitude\ for\ prepulse\ stimulus\ and\ startle\ stimulus\ together / response\ amplitude\ for\ startle\ stimulus\ alone) \times 100]$. For the R579W cohort, combined data from both cohorts are presented for the first test (7–11 weeks) for both acoustic startle and pre-pulse inhibition. For the second test of acoustic startle, only the first cohort of R579W mice is presented.

Morris water maze. The water maze consisted of a large circular pool (diameter, 122 cm) partially filled with water (45 cm deep, 24–26°C) located in a room with numerous visual cues. The procedure involved three different phases: (1) a visible platform test, (2) acquisition in the hidden platform task, and (3) a test for reversal learning. For the visible platform test, each mouse was given 4 trials/d for 2 d to swim to an escape platform cued by a patterned cylinder extending above the surface of the water. For each trial, the mouse was placed in the pool at one of four possible locations (randomly ordered) and then given 60 s to find the visible platform. If the mouse found the platform, the trial ended and the animal was allowed to remain for 10 s on the platform before the next trial began. If the platform was not found, the mouse was placed on the platform for 10 s and then given the next trial. Measures were taken of latency to find the platform and swimming speed via an automated tracking system (Ethovision; Noldus). After the visible platform task, mice were tested for their ability to find a submerged, hidden escape platform (diameter, 12 cm). Each animal was given 4 trials per day with 1 min per trial to swim to the hidden platform. The criterion for learning was an average group latency of 15 s or less to locate the platform. Mice were tested until the group reached criterion, with a maximum of 9 d of testing. After the acquisition phase, mice were tested for reversal learning using the same procedure described above. In this phase, the hidden platform was relocated to the opposite quadrant in the pool. Measures were taken of latency to find the platform. For the initial cohort of R579W mice, the acquisition and hidden tasks were only performed for 3 d, whereas the second cohort was tested for 7 d for the hidden task (only the first 5 d are shown in Fig. 6). Similarly, the first R579W cohort was only tested for 3 d for the reversal versus 8 d for the second cohort.

Histology and microscopy

A set of adult mice was perfused for brain collection and histology was performed. Mice were deeply anesthetized with sodium pentobarbital (60 mg/kg, i.p.) before being transcardially perfused with room temperature paraformaldehyde in PBS. Brains were removed from the skull and placed in fixative overnight at 4°C; the brains were then cryoprotected in

20% sucrose in PBS for 12 h and 30% sucrose in PBS thereafter. Coronal brain slices were collected using a freezing sliding microtome (Thermo Fisher Scientific) at a thickness of 30 μ m after the brains were frozen on dry ice. Sections were cryopreserved at -20°C in a solution of 45% PBS, 30% ethylene glycol, and 25% glycerol by volume.

In preparation for imaging, tissue sections were washed at least two times in PBS for 20 min, stored overnight, then washed at least three times in PBST for 30 min and again in PBS for 20 min. The sections were then incubated for 60–120 min in NeuroTrace 640/660 Deep-Red Fluorescent Nissl Stain (1:100 in PBS; Thermo Fisher Scientific) and/or DAPI (1:3000 in PBS) and then washed in PBS and mounted on gelatinized slides. Comparative brain sections within figures were stained within the same experiment under identical conditions.

Mounted tissue sections from the forebrain were imaged using a Zeiss LSM 710 confocal microscope. Single-plane optical slices were acquired with a 10 $\times$ objective (10 $\times$ /0.3 Neofluar) with 1 $\times$ averaging. Images were obtained by tiling micrographs of 512 pixels $\times$ 512 pixels (1.66 μ m pixel size, 848.5 μ m $\times$ 848.5 μ m image size) that were stitched together using Zen imaging software (correlation threshold, 9.0). Images were then adjusted for angle, brightness, and contrast and then merged using ImageJ software (RRID:SCR_003070). Images compared within figures were acquired using identical acquisition parameters. All images to be compared underwent identical manipulations for brightness and contrast.

Electrophysiology

For electrophysiological recordings, P60–P120 sex-matched, age-matched mutant mice and control littermates were used. All reagents were purchased from Sigma-Aldrich unless otherwise specified. All drugs were prepared as stock aliquots in water and stored at -20°C .

Hippocampal slice preparation. Mice were anesthetized with an overdose of pentobarbital (Euthasol; Virbac) diluted in distilled water. The brain was quickly dissected and blocked. For extracellular field recordings, mice were decapitated rapidly after the cessation of the toe-pinch reflex and the brain was quickly dissected and blocked. Then, hippocampal sections were cut transversely at a thickness of 350 μ m in ice-cold oxygenated cutting solution containing the following (in mM): 87 NaCl, 2.5 KCl, 1.25 sodium monobasic monophosphate, 25 NaHCO₃, 10 dextrose, 75 sucrose, 1.3 sodium ascorbate, 0.5 CaCl₂, and 7 MgCl₂ bubbled with 95% O₂/5% CO₂ on a Leica 1000S vibrating microtome. During slicing, hippocampal area CA3 was removed. Slices recovered for >60 min at 32 $^{\circ}\text{C}$ in oxygenated artificial CSF (ACSF) containing the following (in mM): 124 NaCl, 3 KCl, 1.25 sodium monobasic monophosphate, 26 NaHCO₃, 20 dextrose, 2 CaCl₂, and 1 MgCl₂ bubbled with 95% O₂/5% CO₂ before recordings. For whole-cell recordings, mice were transcardially perfused after the cessation of the toe-pinch reflex with ice-cold cutting solution containing the following (in mM): 2.5 KCl, 1.25 sodium monobasic monophosphate, 25 NaHCO₃, 7 dextrose, 1.3 sodium ascorbate, 3 sodium pyruvate, 210 sucrose, 7 MgCl₂, and 0.5 CaCl₂ and the brain was quickly dissected. Then, the hippocampus was blocked to expose the transverse surface (Bischofberger et al., 2006) and cut at a thickness of 300–350 μ m in ice-cold cutting solution. Slices recovered for 20 min at 35 $^{\circ}\text{C}$ followed by >40 min at room temperature in ACSF (same as above, with the addition of 10 mM dextrose, 3 mM sodium pyruvate, and 1.3 mM sodium ascorbate added to the recovery chamber) before recordings.

Field potential recordings. Slices were placed in a submersion recording chamber (Warner Instruments) perfused with oxygenated ACSF at a rate of 1.5–1.75 ml/min and maintained at a temperature of 30–32 $^{\circ}\text{C}$. Extracellular recording micropipettes were pulled (P-97; Sutter Instruments) from thin borosilicate glass (fire-polished with filament; outer diameter, 1.0 mm; inner diameter, 0.78 mm; length, 10 cm; Sutter Instruments) and filled with ACSF. Field EPSPs (fEPSPs) were recorded from micropipettes placed in the stratum radiatum of hippocampal area CA1 in response to stimulation of Schaffer collaterals by a 2-contact platinum/iridium cluster electrode (2 $\times$ 25 μ m; FHC) placed 300–500 μ m from the recording electrode. Signals were filtered between 0.1 Hz and 3 kHz, amplified, and sampled at a rate of 10 kHz using pCLAMP 9.2 software (RRID:SCR_011323; Molecular Devices). To set the stimulation intensity, an input/output (I/O) characteristic for the slice was first generated by monitoring the postsynaptic response (measured as the slope of the

fEPSP during 0.7 ms following the fiber volley) evoked by brief pulses (200 μ s; 2–60 mA) delivered every 15 s and then selecting the current that elicited 40–60% of the maximum response within the stimulation range. For analysis of I/O characteristics, we omitted trials in which the presence of a population spike occluded our ability to measure fEPSP slope accurately. For analysis of fiber volley, we included only recordings in which the evoked fiber volley was discernible. In a subset of experiments, the paired-pulse ratio was tested before the induction of LTP using two iterations of a family of interpulse intervals (20–100 ms, 20 ms steps, followed by 50–300 ms, 50 ms steps). Evoked responses were then monitored every 30 s (0.03 Hz) for $\geq$ 20 min to record a stable baseline (<5% increase over the last 15 min of monitoring). After a stable baseline was achieved, slices were stimulated with 1 of 4 stimulation protocols: 3 1 s 100 Hz bursts separated by 20 s; 3 1 s 40 Hz bursts separated by 20 s; 3 1 s 20 Hz bursts separated by 20 s; or 900 pulses at 1 Hz (15 min). Monitoring of synaptic responses resumed shortly after the cessation of stimulation and fEPSP slopes were recorded every 30 s for 45 min. Experiments were aligned to the time of stimulation and changes in synaptic strength were measured by comparing the average fEPSP slopes to 30–45 min after conditioning with the baseline response 0–15 min before stimulation. Some experiments were performed in the presence of 100 μ M D,L-2-amino-5-phosphonopentanoic acid (D,L-APV; Tocris Bioscience).

Whole-cell recordings. Slices were placed in a submersion recording chamber (Harvard Apparatus), perfused with oxygenated ACSF at a rate of 2 ml/min, and maintained at a temperature of 30–32 $^{\circ}\text{C}$. Hippocampal area CA1 pyramidal cells were visualized using a Zeiss Examiner microscope outfitted with infrared differential interference contrast optics. Patch pipettes were pulled (P-97; Sutter Instruments) from thick-walled borosilicate glass (fire-polished with filament; outer diameter, 1.5 mm; inner diameter, 0.86 mm; length, 10 cm; Sutter Instruments). Open-tip resistances were 2–5 M Ω . For voltage-clamp experiments, the patch pipettes were backfilled with a cesium-based internal solution containing the following (in mM): 100 CsCHSO₃, 15 CsCl, 2.5 MgCl₂, 10 HEPES, 5 QX-314, 5 BAPTA, 4 Mg-ATP, and 0.3 Mg-GTP, pH adjusted to 7.25 with 1 M CsOH; osmolarity adjusted to $\sim$ 300 mOsm with sucrose (Berrios et al., 2016). For current-clamp experiments, the patch pipettes were backfilled with a potassium-based internal solution containing the following (in mM): 100 potassium gluconate, 20 KCl, 10 HEPES, 0.2 EGTA, 4 Mg-ATP, 0.3 Na-GTP, 10 Na₂ phosphocreatine, pH adjusted to 7.2–7.3 with 1 M KOH; osmolarity adjusted to 290–300 mOsm with sucrose (Wallace et al., 2012). In some experiments, the internal solution contained 0.025 mM Alexa Fluor 594 hydrazide (Thermo Fisher Scientific). Recordings were performed in the whole-cell configuration using a patch-clamp amplifier (Multiclamp 700B; Molecular Devices) and a data acquisition driven by PClamp 10 software (Molecular Devices). To obtain recordings in voltage-clamp mode, initial pipette seal resistances exceeded 1 G Ω , fast and slow pipette capacitive transients were compensated for before break-in, and cells were held at a holding potential of -70 mV. For current-clamp recordings, the amplifier switched into current-clamp mode after 3–5 min wash-in of the internal, the resting membrane potential (RMP) was recorded at a holding current of $I_{inj} = 0$ pA, the bridge was balanced, and the pipette capacitance was compensated. Changes in series and input resistances were monitored every 30 s throughout the experiments. Data were excluded if series resistances exceeded 30 M Ω . For current-clamp experiments, the bridge was balanced before each recording protocol.

All voltage-clamp recordings were performed in the presence of 2 μ M gabazine (Abcam) to isolate EPSPs. For evoked-current experiments, a 2-contact platinum/iridium cluster stimulating electrode (2 $\times$ 25 μ m; FHC) was placed in the stratum radiatum $\sim$ 250 μ m orthogonal to the recording site (50% layer depth). Once whole-cell voltage-clamp recordings were established at a holding potential of -70 mV, the intensity of the stimulus (200 μ s, 0.05 Hz) was adjusted to elicit AMPA receptor (AMPA)-elicited EPSCs with an amplitude of 250–350 pA that remained stable for 2 min. AMPAR majority currents were then recorded for 15 sweeps. Mixed NMDA receptor (NMDAR)-mediated EPSCs were recorded at a holding potential of +40 mV for 15 sweeps. NMDAR-to-AMPA analysis (NMDA/AMPA) for each cell were computed by mea-

suring the average amplitude 50 ms after stimulus onset of mixed EPSCs collected at +40 mV (the NMDAR-specific component) and the average peak amplitude of EPSCs collected at –70 mV (the AMPAR-specific component) dividing the AMPAR-specific component by the NMDAR-specific component (Etherton et al., 2011). In addition, the decay kinetics of NMDAR-mediated EPSCs were analyzed by computing the decay time constant.

NMDAR-mediated EPSCs were well fit in Clampfit (Molecular Devices) by a double exponential using the equation $I(t) = I_f \exp(-t/\tau_f) + I_s \exp(-t/\tau_s)$, where I is the current amplitude, t is time, I_f and I_s are the peak amplitudes of the slow and fast components of the current, respectively, and τ_f and τ_s are the decay time constants, respectively; the overall weighted decay constant, τ_w , was quantified as $\tau_w = \tau_f * [I_f/(I_f + I_s)] + \tau_s * [I_s/(I_f + I_s)]$ (Philpot et al., 2001; de Marchena et al., 2008). NMDAR-mediated EPSCs were confirmed from recordings performed at a holding potential of +40 mV after ACSF supplemented with 20 μ M DNQX (Abcam) was washed onto the slice; effectiveness of DNQX wash-on was confirmed by the loss of AMPAR-mediated EPSCs at a holding potential of –70 mV.

In some experiments, 5 μ M 4-[2-(4-benzylpiperidin-1-yl)-1-hydroxypropyl]phenol (ifenprodil (+)-tartrate; Abcam) + 20 μ M DNQX was washed onto the slice for 20 min while the cell was maintained at a holding potential of –70 mV. After wash-on, the effect of ifenprodil on NMDAR-mediated EPSC amplitude and decay time constant τ_w by ifenprodil was computed as the ratio of the postdrug to predrug values.

All current-clamp recordings were performed in the presence of 2 μ M gabazine (Abcam), 100 μ M D,L-APV (Tocris Bioscience), and 20 μ M DNQX (Abcam) to block inhibitory, NMDAR-mediated excitatory, and AMPAR-mediated excitatory currents, respectively. Passive membrane properties were measured from the response to a family of hyperpolarizing and depolarizing current steps (500 ms, –150 pA to 50 pA in 20 pA steps, 1 s intertrial interval) (Routh et al., 2009; Dembrow et al., 2010). Membrane time constant was determined from the responses to 100 alternating hyperpolarizing and depolarizing pulses (2 ms, –400 pA and +400 pA, delivered with the minimum possible intertrial interval) (Routh et al., 2009; Dembrow et al., 2010). The relationship between firing frequency and current magnitude was determined from a family of depolarizing current steps (1000 ms, 50 pA to 400 pA, 15 s intertrial interval) (Routh et al., 2009; Dembrow et al., 2010).

Data from current-clamp experiments were analyzed in Clampfit (Molecular Devices). Input resistance was computed as the slope of the best-fit linear line of the voltage–current plot generated from the family of hyperpolarizing and depolarizing current steps. The sag ratio was computed as the proportional increase of the maximum negative-going voltage transient relative to the steady-state voltage transient in response to hyperpolarizing current steps. The rebound was computed as the slope of the best-fit linear line of the plot showing the relationship between the steady-state voltage transient and the maximum positive-going voltage transient at the cessation of the current step relative to the holding potential. The membrane time constant (τ_m) was computed as the slow component of a double-exponential fit ($V_m(t) = A_{\text{slow}} \exp(-t/\tau_{\text{slow}}) + A_{\text{fast}} \exp(-t/\tau_{\text{fast}}) + C$) of the average response to hyperpolarizing and depolarizing current steps. Single action potentials were analyzed to yield spiking threshold (where the upward voltage inflection first exceeds $dV_m/dt = 20$), maximum dV_m/dt , spike amplitude, and spike half-maximum width. Firing rate was determined using the Clampfit threshold-crossing algorithm across the 1 s current injection.

Experimental design and statistical analysis

All experiments were conducted blinded to genotype. Minimum sample sizes were estimated from previously published datasets with similar experimental parameters. For behavior, electrophysiology, and brain and body weight experiments, measurements were drawn from mutant animals and their age-matched, sex-matched littermates and we attempted to balance the number of animals drawn from each sex. For behavior experiments, the sample sizes were as follows: 11 cHet mutant mice (5 males, 6 females) and 10 controls (5 males, 5 females); a cohort of 12 R579W mutant mice (10 males, 2 females) and 12 controls (9 males, 3 females), and second cohort of 9 R579W mutant mice (5 males, 4 fe-

males) and 9 controls (5 males, 4 female), where the R579W cohorts were combined for all tests (21 pairs total, sexes combined) except for the 3-chamber assay test, in which only the male data from the first cohort were used. For extracellular field recording measurements, we used ≥ 6 slices for each set with no more than 2 slices per animal. For whole-cell recordings, we used ≥ 7 cells with at least 4 mice per genotype/experiment. Data were analyzed using GraphPad Prism 6 software (RRID: SCR_002798). Statistical outliers were identified by the Prism ROUT routine and removed from further analysis. For hypothesis testing, we used unpaired two-sample t tests and one-way and two-way repeated-measures ANOVA, as described in the Results and figure legends. For behavioral data, “protected” Fisher’s least significant difference (LSD) *post hoc* tests were conducted if a significant F value was detected by two-way ANOVA. All other *post hoc* tests were unpaired two-sample t tests corrected for multiple comparisons using the Bonferroni correction. Data are depicted as mean $\pm$ SEM unless otherwise indicated.

Results

Generation and validation of PTHS mouse models

The bHLH region in exon 18 of *TCF4* is a hotspot for missense mutations associated with PTHS (Whalen et al., 2012), including highly penetrant mutations at arginine residues R576Q, R578H, R580W, and R582P (mouse R575Q, R577H, R579W, and R581P, respectively). The most common PTHS mutation to date is the R580W missense mutation, which disrupts the ability of TCF4 to bind DNA (Zhuang et al., 1996; Whalen et al., 2012; Hsu et al., 2014; Rannals et al., 2016b). To target these common mutation sites, we used the CRISPR/Cas9 gene editing technology to produce a PTHS mouse model that specifically modeled the R580W (mouse R579W) point mutation. During this process, we also serendipitously generated a mouse with an in-frame deletion of six amino acids (aa 574–579) on one allele and the insertion of a silent mutation on the other allele (Δ 574–579 mice) affecting three of five known pathogenic arginines and potentially having a more severe phenotypic presentation (Fig. 1A,B). Homozygosity in these mice was embryonically lethal, similar to mice with homozygous loss of *Tcf4* (Zhuang et al., 1996; Rannals et al., 2016b).

To test the transcriptional activity of mutant TCF4, we performed luciferase assays by transfecting HEK293 cells with wild-type or mutant TCF4, a control plasmid carrying *Renilla* luciferase (*hRlucP*), and the firefly luciferase gene (*luc2P*) under the control of 12 μ E5 (CACCTG) E-boxes (Sepp et al., 2011, 2012). All four causative point mutations perturbed significantly the ability of TCF4 to activate the 12 μ E5 promoter (Fig. 1C; one-way ANOVA, $F_{(5,12)} = 54.69$, $p < 0.0001$; *post hoc* unpaired t tests with Bonferroni corrections – R575Q, $t_{(12)} = 12.82$, $p < 0.0001$; R577H, $t_{(12)} = 12.82$, $p < 0.0001$; R579W, $t_{(12)} = 12.78$, $p < 0.0001$; R581P, $t_{(12)} = 12.82$, $p < 0.0001$). Comparatively, the Δ 574–579 deletion was significantly less transcriptionally active than wild-type TCF4 (Fig. 1D; one-way ANOVA, $F_{(3,8)} = 23.28$, $p = 0.0003$; *post hoc* unpaired t test with Bonferroni correction for Δ 574–579, $t_{(8)} = 6.823$, $p = 0.0007$). These studies demonstrate that perturbation of the bHLH domain severely impairs TCF4 transcriptional activity and emphasizes the pathogenicity of these mutations in PTHS.

E-proteins such as TCF4 can promote transcription either independently or through homodimerization or heterodimerization to other E-proteins, including ASCL1 (Achaete-Scute homolog 1) (Sepp et al., 2012). To test whether the R579W and Δ 574–579 mutant constructs altered ASCL1 enhanced transcription, we transfected HEK293 cells with TCF4, ASCL1, or ASCL1 combined with either wild-type TCF4, the R579W mutant, or the Δ 574–579 mutant (Fig. 1E). Consistent with previous results (Sepp et al., 2012), we found that TCF4 and ASCL1 indepen-

Figure 1. Generation and validation of PTHS model mice that express a dominant-negative form of TCF4. **A**, Several mutation sites (pink text) within the bHLH region (exon 18, yellow) are associated with PTHS. We generated mice with a pathogenic point mutation (*Tcf4*^{R579W}, R579W) or a unique in-frame deletion that removes three of five pathogenic arginines associated with PTHS (*Tcf4*^{Δ574–579}, Δ574–579). The table lists mouse models and abbreviations used in this study. **B**, Electropherograms showing heterozygosity of the mutations: a c.1735A>T transition in the R579W mice and a c.1120_1137del frameshift mutation in the Δ574–579 mice. **C**, Transcriptional activity measured by luciferase assays of wild-type TCF4 and pathogenic point mutations. **D**, Transcriptional activity of wild-type TCF4 compared with the R579W and Δ574–579 variants. **E**, Transcriptional activity of TCF4, the transcriptional activator ASCL1, R579W, or Δ574–579 constructs (orange hash) either alone or in combination as indicated. Data represent mean ± SEM. *Post hoc* unpaired *t* tests with Bonferroni correction: ****p* ≤ 0.001, *****p* ≤ 0.0001.

dently supported transcriptional activity and, when combined, their transcriptional activities were enhanced (one-way ANOVA, $F_{(5,12)} = 3.043$, $p < 0.0001$; *post hoc* unpaired *t* tests: TCF4 vs TCF4+ASCL1, $t_{(12)} = 6.314$, $p = 0.0005$; ASCL1 vs TCF4+ASCL1, $t_{(12)} = 7.33$, $p = 0.0001$; control vs TCF4+ASCL1, $t_{(12)} = 7.669$, $p < 0.0001$). However, the R579W and Δ574–579 mutant constructs reduced transcriptional activity with ASCL1 significantly compared with wild-type TCF4 (*post hoc* unpaired *t* tests with Bonferroni corrections; TCF4+ASCL1 vs R579W+ASCL1, $t_{(12)} = 7.638$, $p < 0.0001$; TCF4+ASCL1 vs Δ574–579+ASCL1, $t_{(12)} = 7.638$, $p < 0.0001$). These findings further suggest that the R579W mutation produces a TCF4 variant that, at least partially, acts in a dominant-negative fashion (Sepp et al., 2011, 2012; Tamberg et al., 2015) and indicate that the unique Δ574–579 indel produces a similar dominant-negative consequence.

We next characterized these two new PTHS mouse models alongside an existing conditional deletion mouse model (*Tcf4*^{Flox/+}) that we used to produce either pan-cellular or CNS-specific *Tcf4* deletion. Exons 18–20 are flanked by loxP sites in the *Tcf4*^{Flox/+} mice (Bergqvist et al., 2000) such that *Cre* recombinase (*Cre*)-mediated

excision of the loxP sites heterozygously removes the bHLH region of *Tcf4* similar to that observed in *Tcf4*^{+/-} mice (Zhuang et al., 1996; Rannals et al., 2016a, b). We generated two conditional deletion mouse models: (1) a ubiquitous deletion by crossing *Tcf4*^{Flox/+} mice with β -Actin-*Cre* transgenic mice (cHet), which allowed us to mimic pan-cellular heterozygous disruption of *Tcf4*, and (2) a CNS-specific deletion by crossing *Tcf4*^{Flox/+} mice with *Nestin-Cre* mice (CNS-cHet), which allowed us to eliminate consequences of peripheral *Tcf4* heterozygosity (Grubišić et al., 2015) that could affect growth trajectories.

As an initial measure of the consequences of *Tcf4* disruption, we measured body weight and brain weight of PTHS model mice and their sex- and age-matched littermate controls (Fig. 2). We observed significant effects or trends for smaller body and brain weights in all four PTHS mouse models: R579W and CNS-cHet mice had significantly reduced body and brain weights compared with controls (Fig. 2A, C; unpaired two-sample *t* test; $n = 17$ R579W and 19 control; body weight, $t_{(34)} = 2.186$, $p = 0.0358$; brain weight, $t_{(34)} = 2.858$, $p = 0.0072$; $n = 9$ CNS-cHet and 10 control; body weight, $t_{(17)} = 7.522$, $p < 0.0001$; brain weight, $t_{(17)} =$

Figure 2. PTHS model mice have reduced body and brain weight. **A–D**, Body and brain weights of adult (P70–P90) control littermates compared with R579W mice (**A**), cHet mice (**B**), CNS-cHet mice (**C**), and $\Delta 574$ –579 mice (**D**). Data represent individual data points and mean $\pm$ SEM. * $p \leq 0.05$, ** $p \leq 0.01$, **** $p \leq 0.0001$. **E**, Representative NeuroTrace (Nissl), DAPI, and merged stained coronal sections of adult control littermates compared with R579W mice, cHet, and $\Delta 574$ –579 mice. Scale bar, 2 mm.

2.208, $p = 0.0413$). Similarly, $\Delta 574$ –579 mice exhibited significant reductions in brain weight, whereas cHet mice exhibited significant reductions in body weight (Fig. 2*B,D*; unpaired two-sample t tests; $n = 11$ $\Delta 574$ –579 and 13 control; body weight, $t_{(22)} = 1.287$, $p = 0.2115$; brain weight, $t_{(22)} = 2.914$, $p = 0.008$; $n = 14$ cHet and 15 control, $t_{(27)} = 2.241$, $p = 0.0335$; brain weight, $t_{(27)} = 1.042$, $p = 0.3069$). The reduction in brain weight appeared, at least qualitatively, to be a consequence of uniform reductions in brain volume, as demonstrated by Nissl- and DAPI-stained coronal series from R579W, cHet, and $\Delta 574$ –579 mice compared with controls (Fig. 2*E*). The gross reduction in brain size in PTHS model mice suggests that they have a relative microcephaly.

Increased activity and reduced anxiety in PTHS model mice

To begin examining convergent and divergent behavioral phenotypes across mouse models, we tested R579W and cHet mouse models and their sex-matched littermate controls in a battery of behavioral tasks. In the open-field task, both the R579W and cHet PTHS model mice showed increased activity (Fig. 3*A*; two-way repeated-measures ANOVA; $n = 21$ R579W and 21 control; genotype effect, $F_{(1,40)} = 18.31$, $p = 0.0001$; time effect, $F_{(11,440)} = 49.97$, $p < 0.0001$; genotype $\times$ time interaction, $F_{(11,440)} = 0.8241$, $p = 0.6160$; $n = 11$ cHet and 10 control; genotype effect, $F_{(1,40)} = 8.445$, $p = 0.0091$; time effect, $F_{(11,440)} = 11.68$, $p < 0.0001$; genotype $\times$ time interaction, $F_{(11,440)} = 0.694$, $p =$

Figure 3. TCF4 mutant mice are hyperactive and show reduced anxiety. Behavioral data from the open-field (A–C) and elevated plus maze (D–F) tasks. **A**, Distance traveled in an open field plotted in 5 min intervals. **B**, Total distance traveled in the open field over a 1 h testing period. **C**, Center time in an open field plotted in 5 min intervals. **D**, Time spent in either the closed or open arms of an elevated plus maze. **E**, Percentage of time spent in the open arms. **F**, Total number of entries made into either the closed or open arms. Data represent mean $\pm$ SEM and individual data points are plotted when feasible. Protected Fisher's LSD tests following significant ANOVA effects (A, C, D, F) or unpaired two-sample *t* tests (B, E); * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$, **** $p \leq 0.0001$.

0.7436) and total distance traveled (Fig. 3B; unpaired two-sample *t* tests; $n = 21$ R579W and 21 control; $t_{(40)} = 4.279$, $p < 0.0001$; $n = 10$ cHet and 11 control; $t_{(19)} = 1.899$, $p = 0.0728$), consistent with previous findings in *Tcf4*^{+/-} model mice (Kennedy et al., 2016; Rannals et al., 2016a, b). These results also highlight a possible deficit in habituation to novel environments in the cHet mice; both cHet mice and controls show a similar level of activity at the outset of the task, but the level of activity appears to wane more slowly for the cHet mice (Fig. 3A). We assessed time spent in the center region of the novel open field as a measure of anxiety and found that both the R579W and cHet mice spent significantly more time in the center region during the last 20 min of the trial (Fig. 3C; two-way repeated-measures ANOVA; $n = 21$ R579W and 21 control; genotype effect, $F_{(1,40)} = 0.0079$, $p = 0.0079$; time effect, $F_{(11,440)} = 0.8317$, $p = 0.6083$; genotype $\times$ time interaction, $F_{(11,440)} = 3.415$, $p = 0.0001$; $n = 11$ cHet and 10 control; genotype effect, $F_{(1,19)} = 5.657$, $p < 0.0001$; time effect, $F_{(11,209)} = 1.03$, $p = 0.4207$; genotype $\times$ time interaction, $F_{(11,209)} = 1.409$, $p = 0.1704$). These data suggest that these mice experienced reduced anxiety. To further assess anxiety, we evaluated mice in the elevated plus maze task (Fig. 3D–F) and found that both the R579W and cHet mice spent significantly more total time (two-way repeated-measures ANOVA with protected Fisher's LSD tests; $n = 12$ R579W and 12 control; genotype effect, $F_{(1,22)} = 3.415$, $p = 0.0781$; arm effect, $F_{(1,22)} = 1.372$, $p = 0.2539$; genotype $\times$ arm interaction, $F_{(1,22)} = 7.013$, $p = 0.0147$; R579W vs

control in open arms, $t_{(44)} = 2.945$, $p = 0.0041$; $n = 11$ cHet and 10 control; genotype effect, $F_{(1,19)} = 1.131$, $p = 0.3010$; arm effect, $F_{(1,19)} = 27.58$, $p < 0.0001$; genotype $\times$ arm interaction, $F_{(1,19)} = 11.13$, $p = 0.0035$; cHet vs control in open arms, $t_{(38)} = 3.082$, $p = 0.0038$) and a higher percentage of time (unpaired two-sample *t* tests; $n = 12$ R579W and 12 control; $t_{(22)} = 2.846$, $p = 0.0094$; $n = 11$ cHet and 10 control; $t_{(19)} = 3.25$, $p = 0.0042$) in the open arms (Fig. 3D,E). We also found that, in both models, the mutants made significantly more entries into the open arm than the closed arm compared with controls (Fig. 3F; two-way repeated-measures ANOVA with protected Fisher's LSD tests; $n = 12$ R579W and 12 control; genotype effect, $F_{(1,22)} = 0.03982$, $p = 0.8437$; entries effect, $F_{(1,22)} = 0.6828$, $p = 0.4175$; genotype $\times$ entries interaction, $F_{(1,22)} = 12.82$, $p = 0.0017$; open arm R579W vs control, $t_{(44)} = 2.551$, $p = 0.0143$; $n = 11$ cHet and 10 control; genotype effect, $F_{(1,19)} = 0.04737$, $p = 0.8301$; entries effect, $F_{(1,19)} = 36.98$, $p < 0.0001$; genotype $\times$ entries interaction, $F_{(1,19)} = 22.37$, $p = 0.0001$; open arm cHet vs control, $t_{(38)} = 2.075$, $p = 0.0448$). The increased time and entries made into the open arms by R579W and cHet mice were not a result of hyperactivity because the R579W and cHet mice made a similar number of arm entries as their respective littermate controls (R579W, 26 ± 3 ; R579W controls, 25 ± 1 ; cHet, 22 ± 3 ; cHet controls, 21 ± 2). Collectively, these data suggest that both the R579W and cHet mice are hyperactive and have reduced anxiety and/or a propensity for risky behavior.

Figure 4. TCF4 mutant mice have an impaired startle response. Mice were tested for auditory function, reactivity to environmental stimuli, and sensorimotor gating by the acoustic startle test at 7–12 weeks of age (**A, B**) and 16–19 weeks of age (**C, D**). **A**, Response to acoustic startle (AS: 120 dB) alone or in combination with a prepulse sound level (74, 78, 82, 86, or 90 dB). **B**, Percentage of prepulse inhibition (PPI). **C, D**, Startle amplitude (**C**) and %PPI (**D**) upon retest at 16–19 weeks of age. Note that only one cohort of data from R579W mice and controls is shown for the second acoustic startle test at 16 weeks. Data represent individual data points and mean $\pm$ SEM. Protected Fisher's LSD tests following significant ANOVA effects: * $p \leq 0.05$, ** $p \leq 0.01$, **** $p \leq 0.0001$.

Reduced startle magnitude but enhanced sensorimotor gating in PTHS model mice

Sensorimotor gating is often impaired in neuropsychiatric and neurodevelopmental disorders and can be assessed in mice by the auditory prepulse inhibition task. Accordingly, we measured auditory function, reactivity to environmental stimuli, and sensorimotor gating in an acoustic startle task. The R579W displayed highly significant decreases in the startle amplitude across a range of sound levels at 7–11 weeks of age (two-way repeated-measures ANOVA; $n = 21$ R579W and 21 control; genotype effect, $F_{(1,40)} = 19.72$, $p < 0.0001$; amplitude effect, $F_{(6,240)} = 92.57$, $p < 0.0001$; genotype $\times$ amplitude interaction, $F_{(6,240)} = 10.97$, $p < 0.0001$), but only mild deficits to the acoustic startle stimulus alone by 16 weeks of age (two-way repeated-measures ANOVA; $n = 10$ R579W and 9 control; genotype effect, $F_{(1,17)} = 1.773$, $p = 0.2006$, amplitude effect, $F_{(6,102)} = 21.27$, $p < 0.0001$; genotype $\times$ amplitude interaction, $F_{(6,102)} = 1.316$, $p = 0.2566$), indicating a dampened response with age in this model (Fig. 4A,C). Reduced startle to a 74 dB stimulus was observed in cHet mice at both 12 and 19 weeks of age (Fig. 4A,C; two-way repeated-measures ANOVA; 12 weeks of age: $n = 11$ R579W and 10 control; genotype effect, $F_{(1,19)} = 5.461$, $p = 0.0306$; amplitude effect, $F_{(6,114)} = 27.75$, $p < 0.0001$; genotype $\times$ amplitude interaction, $F_{(6,114)} = 3.169$, $p = 0.0065$; 19 weeks of age: $n = 11$ R579W and 10 control; genotype effect, $F_{(1,19)} = 6.259$, $p = 0.0217$; amplitude effect, $F_{(6,114)} = 26.59$, $p < 0.0001$; genotype $\times$ amplitude interaction, $F_{(6,114)} = 4.985$, $p = 0.0001$). The percentage prepulse inhibition was significantly enhanced in 7- to 11-week-old but not 16-week-old R579W mice (Fig. 4B,D; two-way repeated-measures ANOVA; 7–11 weeks of age: $n = 21$ R579W and 21 control; genotype effect, $F_{(1,39)} = 4.837$, $p = 0.0339$; amplitude effect, $F_{(4,156)} = 144.5$, $p < 0.0001$; genotype $\times$ amplitude interaction, $F_{(4,156)} = 3.238$, $p = 0.0139$; 16 weeks of age: $n = 10$ R579W and 9 control; genotype effect, $F_{(1,17)} = 0.03478$, $p = 0.8543$; amplitude effect, $F_{(4,68)} = 66.92$, $p < 0.0001$, genotype $\times$ amplitude interaction, $F_{(4,76)} = 0.7356$, $p < 0.0001$). This same measure was decreased in 19-week-old but not 12-week-old cHet mice (Fig.

4B,D; two-way repeated-measures ANOVA; 12 weeks of age: $n = 11$ cHet and 10 control; genotype effect, $F_{(1,19)} = 1.64$, $p = 0.2157$; amplitude effect, $F_{(4,76)} = 51.38$, $p < 0.0001$; genotype $\times$ amplitude interaction, $F_{(4,76)} = 0.5385$, $p = 0.7078$; 19 weeks of age: $n = 11$ mutant and 10 control; genotype effect, $F_{(1,19)} = 5.498$, $p = 0.0301$; amplitude effect, $F_{(4,76)} = 39.21$, $p < 0.0001$; genotype $\times$ amplitude interaction, $F_{(4,76)} = 0.5475$, $p = 0.7013$). Overall, these results indicate that R579W and cHet mice have modestly impaired startle responses and divergent sensorimotor gating phenotypes that each manifest in an age-dependent manner.

Motor coordination and sociability appear normal in PTHS model mice

PTHS individuals often exhibit motor impairments (de Pontual et al., 2009; de Winter et al., 2016) that can potentially affect other behaviors. These behaviors may include sociability, dysfunction of which is common in individuals with autism spectrum disorder but documented to be normal in PTHS individuals (Marangi and Zollino, 2015). In the accelerating rotarod test of motor function, the R579W and cHet mice had similar motor performance to their littermate controls (Fig. 5A; two-way repeated-measures ANOVA; $n = 12$ R579W and 12 control; genotype effect, $F_{(1,22)} = 1.298$, $p = 0.2668$; time effect, $F_{(4,88)} = 15.98$, $p < 0.0001$; genotype $\times$ time interaction, $F_{(4,88)} = 0.607$, $p = 0.6586$; $n = 11$ cHet and 10 control; genotype effect, $F_{(1,19)} = 0.1183$, $p = 0.7347$, time effect, $F_{(4,76)} = 13.99$, $p < 0.0001$; genotype $\times$ time interaction, $F_{(4,76)} = 1.087$, $p = 0.3691$). This suggests that the mice exhibit normal motor performance and are thus physically capable of normal social interactions. To test for sociability, we measured mice in a three-chamber task. Both R579W and cHet mice and their littermate controls preferred the novel mouse chamber over the empty cage and, on average, spent significantly more time in the novel chamber (Fig. 5B; two-way repeated-measures ANOVA with protected Fisher's LSD tests; $n = 10$ R579W and 9 control; genotype effect, $F_{(1,17)} = 3.767$, $p = 0.69$, object effect; $F_{(1,17)} = 47.68$, $p < 0.0001$; genotype $\times$ object interaction, $F_{(1,17)} = 1.578$,

Figure 5. TCF4 mutant mice have typical motor coordination and social behavior. **A**, Latency to fall on an accelerating rotarod. **B**, Amount of time the mice spent in either the empty chamber or the chamber housing a novel mouse (Novel 1) in a three-chambered assay. **C**, Number of entries made into the empty versus occupied (Novel 1) chamber in the three-chambered assay. **D**, Time spent with either a now familiar (Novel 1) or a new mouse (Novel 2) in a three-chambered assay. **E**, Number of entries that each mouse made into the chamber housing either a now familiar mouse (Novel 1) or a novel mouse (Novel 2) in a three-chambered assay. Only data from the male R579W mice and control are presented for the three-chambered task due to high variability in the female mice. Data represent individual data points and mean ± SEM. Protected Fisher's LSD tests following significant ANOVA effects: * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$, **** $p \leq 0.0001$.

$p = 0.2260$; R579W empty vs novel, $t_{(17)} = 3.893$, $p = 0.0012$; control empty vs novel, $t_{(17)} = 5.629$, $p < 0.0001$; $n = 11$ cHet and 10 control; genotype effect, $F_{(1,19)} = 0.5956$; $p = 0.4497$, object effect; $F_{(1,19)} = 15.97$, $p = 0.0008$; genotype × object interaction, $F_{(1,19)} = 0.5956$, $p = 0.4497$; cHet empty vs novel, $t_{(19)} = 2.227$; control empty vs novel, $t_{(19)} = 3.454$, $p = 0.0027$). Both R579W and cHet mice and their littermate controls made significantly more entries into the novel chamber than the empty chamber (Fig. 5C; two-way repeated-measures ANOVA with protected Fisher's LSD tests; $n = 10$ R579W and 9 control; genotype effect, $F_{(1,17)} = 7.026$, $p = 0.0168$; object effect, $F_{(1,17)} = 2.765$, $p = 0.1147$; genotype × object interaction, $F_{(1,17)} = 0.2663$, $p = 0.6124$; R579W empty vs novel, $t_{(34)} = 2.492$, $p = 0.0177$; control empty vs novel, $t_{(34)} = 1.921$, $p = 0.0632$; $n = 11$ cHet and 11 control; genotype effect, $F_{(1,19)} = 3.838$, $p = 0.0649$, object effect, $F_{(1,19)} = 16.37$, $p = 0.0007$; genotype × object interaction, $F_{(1,19)} = 2.61$, $p = 0.1227$, cHet empty vs novel $t_{(19)} = 1.679$, $p = 0.0006$;

control empty vs novel, $t_{(19)} = 4.102$, $p = 0.1096$). During the next phase, the mice were given the choice between the now-familiar mouse (Novel 1) and a novel mouse (Novel 2) (Fig. 5D,E). We found that, similar to their respective controls, R579W and cHet mice showed no strong preference for either novel test mouse (two-way repeated-measures ANOVA; time spent: $n = 10$ R579W and 9 control; genotype effect, $F_{(1,17)} = 0.03902$, $p = 0.8457$; object effect, $F_{(1,17)} = 21.84$, $p = 0.0002$; genotype × object interaction, $F_{(1,17)} = 0.3852$, $p = 0.5430$; time spent: $n = 11$ cHet and 10 control; genotype effect, $F_{(1,19)} = 1.322$, $p = 0.2645$; object effect, $F_{(1,19)} = 4.763$, $p = 0.0418$; genotype × object interaction, $F_{(1,19)} = 0.6869$, $p = 0.4175$; entries: $n = 10$ R579W and 9 control; genotype effect, $F_{(1,17)} = 0.4395$, object effect, $F_{(1,17)} = 1.635$, genotype × object interaction, $F_{(1,17)} = 1.635$, $p = 0.2181$; $n = 11$ cHet and 10 control; genotype effect, $F_{(1,19)} = 1.666$, $p = 0.2122$; object effect, $F_{(1,19)} = 0.3849$, $p = 0.5423$; genotype × object interaction, $F_{(1,19)} = 0.3849$, $p = 0.5423$). Given the hyperactive nature of the R579W and cHet mice, we assessed the number of entries into the chambers and found small, albeit significant, decreases in entry numbers compared with controls, but only after the first phase (Fig. 5C,E). Overall, we found that the R579W and cHet mice display normal motor coordination and typical sociability.

Spatial learning and memory are impaired in PTHS model mice

We measured performance in the Morris water maze task to assess hippocampus-dependent spatial learning and memory. The R579W and cHet mice were able to locate the visible platform and largely had similar swim speeds during all phases of testing to their respective littermate controls (Table 1), suggesting that they had sufficient visual and motor skills to perform the task. However, both the R579W and cHet mice took a significantly longer time to locate the hidden platform than controls [Fig. 6A: two-way repeated-measures ANOVA; $n = 21$ R579W and 21 control (cohort 1 + 2 combined); genotype effect, $F_{(1,40)} = 14.3$, $p = 0.0005$, time effect, $F_{(2,80)} = 6.373$, $p = 0.0027$, genotype × time interaction, $F_{(2,80)} = 0.9883$, $p = 0.3767$; $n = 9$ R579W and 9 control (cohort 2 only); genotype effect, $F_{(1,16)} = 3.348$, $p = 0.0860$, time effect, $F_{(4,64)} = 3.706$, $p = 0.0089$, genotype × time interaction: $F_{(4,64)} = 6.373$, $p = 0.0699$; $n = 11$ cHet and 10 control; genotype effect, $F_{(1,19)} = 7.667$, $p < 0.0001$, time effect, $F_{(4,76)} = 9.794$, $p < 0.0001$, genotype × time interaction, $F_{(4,76)} = 1.512$, $p = 0.0122$]. Consistent with the finding of hyperactivity in the open field (Fig. 3), cHet and R579W mutant mice traveled farther during the acquisition phase than controls [Figure 6B: two-way repeated-measures ANOVA; $n = 21$ R579W and 21 control (cohort 1 + 2 combined); genotype effect, $F_{(1,40)} = 19.69$, $p < 0.0001$, time effect, $F_{(2,80)} = 8.406$, $p = 0.0005$, genotype × time interaction, $F_{(2,80)} = 0.8411$, $p = 0.4350$; $n = 9$ R579W and 9 control (cohort 2 only); genotype effect, $F_{(1,16)} = 4.755$, $p = 0.0455$, time effect, $F_{(4,64)} = 4.639$, $p = 0.0024$, genotype × time interaction, $F_{(4,64)} = 2.22$, $p = 0.0766$; $n = 11$ cHet and 10 control; genotype effect, $F_{(1,19)} = 8.194$, $p = 0.01$, time effect, $F_{(4,76)} = 9.669$, $p < 0.0001$, genotype × time interaction, $F_{(4,76)} = 1.787$, $p = 0.1401$]. Interestingly, during the reversal phase of the hidden platform test, the R579W performed comparably to controls, yet the cHet mice never met criterion (15 s) for learning across the entire 8 d regimen [Figure 6C; two-way repeated-measures ANOVA; $n = 21$ R579W and 21 control (cohort 1 + 2 combined); genotype effect, $F_{(1,40)} = 0.8178$, time effect, $F_{(2,80)} = 1.468$, $p = 0.2366$, genotype × time interaction, $F_{(2,80)} = 1.468$, $p = 0.2366$; $n = 9$ R579W and 9 control (cohort 2 only); genotype

Figure 6. PTHS model mice exhibit hippocampus-dependent spatial learning and memory deficits. **A**, Latency to find a hidden platform during training in the Morris water maze task. **B**, Distance traveled during hidden platform training. **C**, Latency to find a hidden platform in a reversal learning task in which the platform is placed in a new location. **D**, Distance traveled during reversal learning task. Insets in **A–D** represent the combined data for the first 3 d from 2 cohorts of R579W and control mice. Data represent mean $\pm$ SEM. Protected Fisher's LSD tests following significant ANOVA effects: * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$, **** $p \leq 0.0001$.

Table 1. Swimming distance and velocity in the Morris water maze

	Cohorts 1 + 2			Cohort 2 only			cHet		
	R579W (<i>n</i> = 21)	Control (<i>n</i> = 21)		R579W (<i>n</i> = 12)	Control (<i>n</i> = 12)		cHet (<i>n</i> = 10)	Control (<i>n</i> = 11)	
Visible platform, distance (cm)									
Day 1	349 $\pm$ 27	347 $\pm$ 25	$t_{(40)} = 0.0544; p = 0.9569$	311 $\pm$ 33	334 $\pm$ 33	$t_{(22)} = 0.4928; p = 0.6270$	334 $\pm$ 54	414 $\pm$ 35	$t_{(19)} = 1.2664; p = 0.2207$
Day 2	177 $\pm$ 35	229 $\pm$ 30	$t_{(40)} = 1.1280; p = 0.2660$	270 $\pm$ 49	194 $\pm$ 57	$t_{(22)} = 1.011; p = 0.3230$	135 $\pm$ 20	198 $\pm$ 45	$t_{(19)} = 1.2356; p = 0.2317$
Swim speed (cm/s)									
Day 1: Visible platform	15 $\pm$ 0.7	17 $\pm$ 0.5	$t_{(40)} = 2.3250; p = 0.0252^*$	17 $\pm$ 0.8	17 $\pm$ 0.7	$t_{(22)} < 0.001; p > 0.999$	17 $\pm$ 0.5	18 $\pm$ 0.4	$t_{(19)} = 1.5754; p = 0.1317$
Day 1: Acquisition	21 $\pm$ 0.5	20 $\pm$ 0.6	$t_{(40)} = 1.2804; p = 0.2078$	22 $\pm$ 0.6	20 $\pm$ 0.5	$t_{(22)} = 2.5607; p = 0.0178^*$	20 $\pm$ 0.5	20 $\pm$ 0.4	$t_{(19)} < 0.0001; p > 0.9999$
Day 1: Reversal	20 $\pm$ 0.6	19 $\pm$ 0.7	$t_{(40)} = 1.0847; p = 0.2846$	20 $\pm$ 0.8	20 $\pm$ 0.5	$t_{(22)} < 0.001; p > 0.999$	21 $\pm$ 0.9	19 $\pm$ 0.7	$t_{(19)} = 1.7719; p = 0.0925$

Data are shown as means $\pm$ SEM of four trials per day. Distance measures are shown for the 2 d visible platform test. Swim speeds are given for day 1 of each phase of testing. * $p < 0.05$.

effect, $F_{(1,16)} = 0.4242$, $p = 0.5241$; time effect, $F_{(7,112)} = 6.374$, $p < 0.0001$; genotype $\times$ time interaction, $F_{(7,112)} = 0.2958$, $p = 0.9543$ $n = 11$ cHet and 10 control; genotype effect, $F_{(1,19)} = 24.42$, $p < 0.0001$; time effect, $F_{(7,133)} = p < 0.0001$; genotype $\times$ time interaction, $F_{(7,133)} = 1.032$, $p = 0.4122$. In terms of distance traveled during the reversal phase, cHet but not R579W mutant mice traveled farther than controls [Figure 6D: two-way repeated-measures ANOVA; $n = 21$ R579W and 21 control (cohort 1 + 2 combined); genotype effect, $F_{(1,40)} = 0.4165$, $p = 0.5224$, time effect, $F_{(2,80)} = 3.666$, $p = 0.0300$, genotype $\times$ time interaction, $F_{(2,80)} = 5.364$, $p = 0.0065$; $n = 9$ R579W and 9 control (cohort 2 only); genotype effect, $F_{(1,16)} = 1.588$, $p = 0.2256$, time effect, $F_{(4,64)} = 5.039$, $p < 0.0001$, genotype $\times$ time interaction, $F_{(4,64)} = 1.156$, $p = 0.3341$; $n = 11$ cHet and 10 control; genotype effect, $F_{(1,19)} = 24.84$, $p < 0.0001$, time effect, $F_{(4,76)} = 5.649$, $p < 0.0001$, genotype $\times$ time interaction, $F_{(4,76)} = 1.094$, $p = 0.3707$]. Together, these data show that, although there may be subtle genotype–phenotype differences, both the

R579W and cHet mice have deficits in spatial learning and memory.

Hippocampal long-term potentiation is enhanced in four mouse models of PTHS

Given that both the R579W and cHet mice show learning deficits in the Morris water maze (Fig. 6), we determined whether hippocampal synaptic function was disrupted in the PTHS mouse models. We first measured basic properties of synaptic transmission by recording CA1 field EPSPs (fEPSPs) evoked by Schaffer collateral stimulation. The resulting I/O curves showed that AMPA receptor (AMPA)-mediated synaptic transmission was normal in all four PTHS mouse models relative to their respective control littermates (Fig. 7A1–A4; two-way repeated-measures ANOVA; $n = 31$ R579W and 32 control; genotype effect, $F_{(1,61)} = 0.009042$, $p = 0.9246$; current effect, $F_{(12,732)} = 116.0$, $p < 0.0001$; genotype $\times$ current interaction, $F_{(12,732)} = 1.087$, $p = 0.3679$; $n = 28$ cHet and 26 control; genotype effect, $F_{(1,52)} = 0.01123$, $p =$

Figure 7. Synaptic transmission and short-term plasticity are normal in PTHS mouse models. Extracellular field recordings from hippocampal area CA1 and evoked by Schaffer collateral stimulation. (**A**) I/O curves for R579W (**A1**), cHet (**A2**), CNS-cHet (**A3**), and $\Delta 574-579$ (**A4**) mice relative to control littermates. Insets in **A** show representative responses during generation of I/O characteristics. (**B**) Fiber volley responses for R579W (**B1**), cHet (**B2**), CNS-cHet (**B3**), and $\Delta 574-579$ (**B4**) mice relative to control littermates. (**C**) Paired-pulse ratio (PP2/PP1) for R579W (**C1**), cHet (**C2**), CNS-cHet (**C3**), and $\Delta 574-579$ (**C4**) mice relative to control littermates. Insets in **C** show representative paired-pulse responses with a 60 ms interpulse interval. Scale bar, 5 ms 0.25 mV.

0.9160; current effect, $F_{(12,624)} = 126.3$, $p < 0.0001$; genotype $\times$ current effect, $F_{(12,624)} = 0.3997$, $p = 0.9638$; $n = 35$ CNS-cHet and 30 control; genotype effect, $F_{(1,63)} = 0.7331$, $p = 0.3951$; current effect, $F_{(12,756)} = 224.9$, $p < 0.0001$; genotype $\times$ current interaction, $F_{(12,756)} = 0.6985$, $p = 0.7539$; $n = 17$ $\Delta 574-579$ and 15 control; genotype effect, $F_{(1,30)} = 1.336$, $p = 0.2569$; current effect, $F_{(12,360)} = 64.56$, $p < 0.0001$; $F_{(12,360)} = 0.5086$, $p = 0.9091$). Presynaptic function also appeared normal in PTHS model mouse in terms of the presynaptic fiber volley (Fig. 7B1–B4; two-way repeated-measures ANOVA; $n = 29$ R579W and 26 control; genotype effect, $F_{(1,53)} = 0.02714$, $p = 0.8698$; current effect, $F_{(12,636)} = 223.1$, $p < 0.0001$; genotype $\times$ current interaction, $F_{(12,636)} = 0.5708$, $p = 0.8633$; $n = 23$ cHet and 20 control; genotype effect, $F_{(1,41)} = 0.02937$, $p = 0.8777$; current effect, $F_{(12,492)} = 105.8$, $p < 0.0001$; genotype $\times$ current interaction, $F_{(12,492)} = 0.9958$, $p = 0.4516$; $n = 27$ CNS-cHet and 21 control; genotype effect, $F_{(1,46)} = 0.9902$, $p = 0.3249$; current effect, $F_{(12,552)} = 219.2$, $p < 0.0001$; genotype $\times$ current interaction, $F_{(12,552)} = 0.9564$, $p = 0.4899$; $n = 14$ $\Delta 574-579$ and 14 control; genotype effect, $F_{(1,26)} = 1.295$, $p = 0.2656$; current effect, $F_{(12,312)} = 135.2$, $p < 0.0001$; genotype $\times$ current interaction, $F_{(12,312)} = 1.053$, $p = 0.4000$). In addition, short-term plasticity appeared normal in PTHS model mice in terms of the paired-pulse ratio (Fig. 7C1–C4; two-way repeated-measures ANOVA; $n = 24$ R579W and 31 control; genotype effect, $F_{(1,53)} = 0.2462$, $p = 0.6218$; interval effect, $F_{(8,424)} = 62.25$, $p < 0.0001$; genotype $\times$ interval interaction, $F_{(8,424)} = 0.7348$, $p = 0.6608$; $n = 23$ cHet and 29 control; genotype effect, $F_{(1,50)} = 0.008154$, $p = 0.9773$; interval effect, $F_{(8,400)} = 73.82$, $p < 0.0001$; genotype $\times$

interval interaction, $F_{(8,400)} = 0.5870$, $p = 0.7887$; $n = 34$ CNS-cHet and 28 control; genotype effect, $F_{(1,60)} = 0.0127$, $p = 0.9106$; interval effect, $F_{(8,480)} = 100.9$, $p < 0.0001$; genotype $\times$ interval interaction, $F_{(8,480)} = 0.832$, $p = 0.5747$; $n = 13$ $\Delta 574-579$ and 13 control; genotype effect, $F_{(1,24)} = 0.02017$, $p = 0.8883$; interval effect, $F_{(8,192)} = 64.96$, $p < 0.0001$; genotype $\times$ interval effect, $F_{(8,192)} = 0.4969$, $p = 0.8575$). Together, these findings indicate that presynaptic function and AMPAR-mediated synaptic transmission appear grossly normal in hippocampal area CA1 in four PTHS mouse models.

To determine whether *Tcf4* mutations alter hippocampal synaptic plasticity, we measured the long-term changes in synaptic strength in response to stimulation patterns known to induce either LTP or LTD. We found significant enhancement of LTP after 3 s bursts of 100 Hz stimulation (separated by 20 s) in each of the mutant models compared with littermate controls (Fig. 8A1–A4; unpaired two-sample *t* tests; $n = 10$ R579W and 10 control; $t_{(18)} = 2.3720$, $p = 0.0290$; $n = 10$ cHet and 8 control; $t_{(16)} = 2.2434$, $p = 0.0394$; $n = 12$ CNS-cHet and 11 control; $t_{(21)} = 2.0817$, $p = 0.0498$; $n = 11$ $\Delta 574-579$ and 10 control; $t_{(19)} = 2.6588$, $p = 0.0155$). However, no significant differences in LTD after 15 min of 1 Hz stimulation were established among any of the PTHS mouse models compared with controls (Fig. 8B1–B4; unpaired two-sample *t* tests; $n = 6$ R579W and 7 control; $t_{(11)} = 0.1949$, $p = 0.8491$; $n = 7$ cHet and 6 control; $t_{(11)} = 0.8912$, $p = 0.3919$; $n = 6$ CNS-cHet and 8 control; $t_{(12)} = 0.5434$, $p = 0.5968$; $n = 6$ $\Delta 574-579$ and 6 control; $t_{(10)} = 0.7688$, $p = 0.4598$). The enhanced LTP was not limited to strong induction protocols because we found that LTP was consistently enhanced in all PTHS

Figure 8. Consistent enhancement of hippocampal LTP in PTHS model mice. (**A**) LTP of fEPSP slope induced by 3, 1 s bursts at 100 Hz separated by 20 Hz for R579W (**A1**), cHet (**A2**), CNS-cHet (**A3**), and $\Delta 574-579$ (**A4**) mice relative to control littermates. Top, Representative traces scale bars, 5 ms 0.25 mV. (**B**) LTD of fEPSP slope induced by 1 Hz stimulation for 15 min for R579W (**B1**), cHet (**B2**), CNS-cHet (**B3**), and $\Delta 574-579$ (**B4**) mice relative to control littermates. Representative trace scale bars: 5 ms 0.25 mV. (**C**) Frequency-response curves generated across a range of stimulation frequencies for R579W (**C1**), cHet (**C2**), CNS-cHet (**C3**), and $\Delta 574-579$ (**C4**) mice relative to control littermates. Data show mean $\pm$ SEM. * $p < 0.05$.

mouse models over a range of stimulation frequencies (Fig. 8C1–C4; unpaired two-sample t tests; R579W: 20 Hz, $t_{(13)} = 3.2841$, $p = 0.0059$, 40 Hz, $t_{(14)} = 2.4801$, $p = 0.0265$; cHet: 20 Hz, $t_{(12)} = 0.0137$, $p = 0.9893$, 40 Hz, $t_{(15)} = 3.4494$, $p = 0.0036$; CNS-cHet, 20 Hz, $t_{(12)} = 2.2721$, $p = 0.0423$, 40 Hz, $t_{(16)} = 2.1246$, $p = 0.0491$). Collectively, these data show that enhanced LTP is a highly penetrant feature of PTHS mouse models.

NMDA receptor hyperfunction and intrinsic firing properties in R579W and cHet mice

The enhanced LTP in all four PTHS mouse models was striking and warranted a deeper look at underlying intrinsic membrane properties and receptor-mediated transmission to gain mechanistic insights. Given the consistent hippocampus-dependent behavioral phenotypes and the intensive nature of whole-cell recordings, we focused on the R579W and cHet mouse models for further analysis of CA1 pyramidal neurons.

We found that CA1 pyramidal neurons from R579W mice and control littermates had similar RMPs (Fig. 9A; unpaired two-sample t test; $n = 18$ R579W and 14 control; $t_{(30)} = 0.1775$, $p = 0.8603$). Furthermore, at a series of holding potentials (-75 mV, -70 mV, and -65 mV), there were no statistically significant differences be-

tween neurons from R579W mutants and controls in input resistance (Fig. 9B; two-way repeated-measures ANOVA; $n = 14$ R579W and 13 control; genotype effect, $F_{(1,25)} = 1.662$, $p = 0.2013$; potential effect, $F_{(2,75)} = 14.31$, $p < 0.0001$; genotype $\times$ potential interaction, $F_{(2,75)} = 1.001$, $p = 0.3724$), nor were there differences in subthreshold responses to the application of a hyperpolarizing current injection (Fig. 9C; two-way repeated-measures ANOVA; $n = 14$ R579W and 13 control; genotype effect, $F_{(1,25)} = 3.185$; $p = 0.0783$, potential effect, $F_{(2,75)} = 1.722$, $p = 0.1858$; genotype $\times$ potential interaction, $F_{(2,75)} = 0.01825$, $p = 0.9819$) and cessation of a hyperpolarizing current injection (rebound, Fig. 9D; two-way repeated-measures ANOVA; $n = 14$ R579W and 13 control; genotype effect, $F_{(1,25)} = 1.546$, $p = 0.2177$; potential effect, $F_{(2,75)} = 0.6843$, $p = 0.5076$, genotype $\times$ potential interaction, $F_{(2,75)} = 0.5827$, $p = 0.4192$). In addition, we found no statistical differences in the membrane time constant between groups at a holding potential of -70 mV (τ_m , Fig. 9E; unpaired two-sample t tests; $n = 14$ R579W and 13 control; -400 pA: $t_{(21)} = 1.576$, $p = 0.1299$; $+400$ pA: $t_{(21)} = 0.9253$). Therefore, passive membrane properties of CA1 pyramidal neurons were largely unaffected by the R579W mutation.

When we investigated whether there were changes in intrinsic firing properties of CA1 pyramidal neurons, we found unexpect-

Figure 9. The R579W mutation alters active membrane properties in CA1 pyramidal neurons. Current-clamp measurements from CA1 pyramidal cells from R579W mice and control littermates of passive properties (**A–D**) and intrinsic excitability and action potential features (**E–J**). **A**, RMP. **B**, Averaged input resistance. Sample trace scale bars: 500 ms $\times$ 10 mV. **C**, Sag ratio. Sample trace (150 pA injection) scale bars, 250 ms $\times$ 10 mV. **D**, Rebound slope. Left, Representative rebound slope examples based on rebound/steady-state voltage relationship. **E**, Membrane time constant τ_m measured from hyperpolarizing (–400 pA) or depolarizing (+400 pA) current steps. **F**, Action potential firing rate to a depolarizing (200 pA) current step. Sample trace scale bar, 250 ms $\times$ 25 mV. **G**, Firing threshold. Left, Example action potentials. Scale bar, 20 ms $\times$ 25 mV. Right, Membrane voltage at which action potentials are initiated ($dV_m/dt = 20$). **H–J**, Maximum action potential dV_m/dt (**H**), height (**I**), and half-width (**J**). Error bars: mean $\pm$ SEM. $p \leq 0.05$. *Post hoc* unpaired two-sample *t* tests with Bonferroni correction (**B–D**, **F**) or unpaired two-sample *t* tests (**A**, **E**, **G**, **H–J**): * $p \leq 0.05$. ** $p \leq 0.005$. *** $p \leq 0.005$.

edly that neurons from R579W mice produced statistically fewer action potentials across a series of depolarizing current steps (Fig. 9F; two-way repeated-measures ANOVA; $n = 14$ R579W and 13 control; genotype effect, $F_{(1,25)} = 16.8$, $p = 0.0004$; current effect, $F_{(7,125)} = 338.8$, $p < 0.0001$; genotype $\times$ current interaction, $F_{(7,125)} = 5.12$, $p < 0.0001$; firing rate at +400 pA, *post hoc* unpaired *t* test with Bonferroni correction, $t_{(200)} = 1.682$, $p = 0.016$). Given the reduced firing rates and the statistically equivalent membrane resistances at –70 mV (Fig. 9B), we predicted that the threshold for firing an action potential would be more hyperpolarized in neurons from R579W mice. Indeed, action potentials in CA1 pyramidal neurons from R579W mice were initiated at a more depolarized membrane potential relative to controls (Fig. 9G; unpaired two-sample *t* test; $n = 14$ R579W and 13 control; $t_{(25)} = 4.203$, $p = 0.0003$). These neurons were also distinct in other firing properties, having a slower rate of action potential rise (Fig. 9H; unpaired two-sample *t* test; $n = 14$ R579W and 13 control; $t_{(25)} = 2.289$, $p = 0.0308$) and diminished action potential amplitude (Fig. 9I; unpaired two-sample *t* test with Welch's correction; $n = 14$ R579W and 13 control; $t_{(20.73)} = 3.461$, $p = 0.0024$), but were similar in half-width (Fig. 9J; unpaired two-sample *t* test; $n = 14$ R579W and 13 control; $t_{(25)} = 1.874$, $p = 0.0727$).

When we examined the cHet mutant mice for similar alterations in firing properties, we found no reduction in intrinsic excitability between cHet and controls (Fig. 10A; two-way repeated-measures ANOVA; $n = 10$ cHet and 11 control; genotype effect, $F_{(1,19)} = 4.077$, $p = 0.0578$; current effect, $F_{(7,133)} = 229.8$, $p < 0.0001$; genotype $\times$ current interaction, $F_{(7,133)} = 2.188$, $p = 0.0392$) and no differences in firing threshold (Fig.

10B; unpaired two-sample *t* test; $n = 10$ cHet and 11 control; $t_{(19)} = 0.4806$, $p = 0.6363$). We also observed that there were no differences in the rate of action potential rise (Fig. 10C; unpaired two-sample *t* test; $n = 10$ cHet and 11 control; $t_{(19)} = 1.424$, $p = 0.1707$), action potential amplitude (Fig. 10D; unpaired two-sample *t* test; $n = 10$ cHet and 11 control; $t_{(19)} = 0.9052$, $p = 0.3767$), and half-width (Fig. 10E; unpaired two-sample *t* test; $n = 10$ cHet and 11 control; $t_{(19)} = 0.6051$, $p = 0.5523$). At the same time, we observed differences in input resistance (unpaired two-sample *t* test; cHet, $n = 10$, $89.76 \text{ M}\Omega \pm 2.9 \text{ M}\Omega$, control, $n = 10$, $105.6 \text{ M}\Omega \pm 3.8 \text{ M}\Omega$; $t_{(18)} = 3.478$, $p = 0.0025$) and membrane time constant (two-way repeated-measures ANOVA; $n = 10$ cHet and 10 control; genotype effect, $F_{(1,18)} = 5.432$, $p = 0.0316$; injected current effect, $F_{(1,18)} = 0.01903$, $p = 0.8918$; genotype $\times$ injected current interaction, $F_{(1,18)} = 0.1214$, $p = 0.7316$; *post hoc* unpaired *t* tests with Bonferroni corrections; –400 pA, cHet $25.42 \pm 1.0 \text{ ms}$ vs control $31.17 \pm 2.4 \text{ ms}$, $t_{(36)} = 2.219$, $p = 0.0658$; +400 pA, cHet $25.31 \pm 1.21 \text{ ms}$ vs control $31.41 \pm 2.3 \text{ ms}$, $t_{(36)} = 2.354$, $p = 0.0483$). Resting membrane potential was similar in cHet and control mice (unpaired two-sample *t* test; $n = 10$ cHet and 11 control; cHet, $-69.8 \pm 1.6 \text{ mV}$, control, $-67.2 \pm 1.2 \text{ mV}$; $t_{(19)} = 1.284$, $p = 0.2146$).

These results suggest that the precise genetic alteration of *Tcf4* might dictate the consequences on intrinsic excitability and, moreover, that changes in excitability are unlikely to underlie the consistent LTP phenotype observed in all four PTHS mouse models. Therefore, we investigated whether changes in NMDAR-mediated signaling might provide a common deficit to account for the observed LTP enhancement. To test this, we first used field potential recordings to determine whether the enhanced LTP was

Figure 10. Active membrane properties in CA1 pyramidal neurons are largely unaffected by heterozygous deletion of *Tcf4*. Shown are current-clamp measurements from CA1 pyramidal cells from cHet mice and control littermates of intrinsic excitability and action potential features. **A**, Action potential firing rate to a depolarizing (200 pA) current step. Sample trace scale bar, 250 ms $\times$ 25 mV. **B**, Firing threshold. Left, Example action potentials. Scale bar, 20 ms $\times$ 25 mV. Right, Membrane voltage at which action potentials (APs) are initiated. **C–E**, Maximum AP dV_m/dt (**C**), height (**D**), and half-width (**E**). Error bars indicate mean $\pm$ SEM.

NMDAR dependent in R579W mice; the NMDAR antagonist *D,L*-APV blocked all LTP in R579W mice (Fig. 11A; unpaired two-sample *t* test; $n = 6$ R579W + APV and 6 control + APV; $t_{(10)} = 0.3654$, $p = 0.7224$), demonstrating that the R579W mice did not gain an NMDAR-independent form of LTP in CA1. Subsequently, we conducted whole-cell voltage-clamp recordings to assess NMDAR function in CA1 pyramidal neurons and found that the NMDA/AMPA current ratio was enhanced in R579W mice compared with control littermates (Fig. 11B; unpaired two-sample *t* test; $n = 28$ R579W and 31 control; $t_{(57)} = 2.146$, $p = 0.0361$). Likewise, we found that the NMDA/AMPA current ratio was enhanced in cHet mice compared with control littermates (Fig. 11F; unpaired two-sample *t* test; $n = 18$ cHet and 21 control; $t_{(37)} = 2.655$, $p = 0.0116$). Because AMPAR-mediated synaptic transmission appeared intact (Fig. 7), this finding suggests that NMDAR-mediated currents are selectively enhanced in R579W and cHet mice.

In addition, we measured the decay kinetics of the NMDAR-mediated component of the EPSCs and found a significant increase in the decay time in R579W mice compared with control littermates (Fig. 11C; unpaired two-sample *t* test; $n = 27$ R579W and 30 control; $t_{(55)} = 2.618$, $p = 0.0114$) and in cHet mice compared with control littermates (Fig. 11G; unpaired two-sample *t* test; $n = 18$ cHet and 21 control; $t_{(37)} = 2.077$; $p = 0.0448$). This finding was confirmed in R579W mice by measurements of the decay kinetics of pharmacologically isolated NMDAR-mediated currents (Fig. 11D; $n = 14$ R579W and 12 control; unpaired two-sample *t* test, $t_{(23)} = 2.317$, $p = 0.0298$). Because prolonged NMDAR-mediated currents may result from alterations in NMDAR subunit composition (Carmignoto and Vicini, 1992; Flint et al., 1997; Paoletti et al., 2013), we measured the sensitivity of NMDAR-mediated currents in CA1 pyramidal cells to the NR2B-selective antagonist ifenprodil (de Marchena et al., 2008). After a 20 min period of ifenprodil wash-on in the presence of DNQX, the amplitude of NMDAR-mediated currents were suppressed in neurons from both R579W mutants and control littermates; however, the reduction was significantly enhanced in the mutants (Fig. 11E; unpaired two-sample *t* test; $n =$

7 mutant and 10 control; $t_{(15)} = 2.563$, $p = 0.0216$). This suggests that CA1 neurons in R579W mice have a relative increase in synaptic NR2B-containing NMDARs. Collectively, our findings suggest that NMDAR function is enhanced in R579W and cHet mice and potentially all PTHS mouse models.

Discussion

We used an intersectional approach to reveal common pathophysiological deficits arising from distinct classes of PTHS-linked *Tcf4* mutations. We compared preexisting mouse models of *Tcf4* disruption (cHet and CNS-cHet mice) (Bergqvist et al., 2000) with novel point mutation and indel mouse models (R579W and $\Delta 574$ –579 mice) and found that these models consistently present with certain phenotypes observed in PTHS individuals (Amiel et al., 2007; Zweier et al., 2007; de Pontual et al., 2009). Furthermore, these *Tcf4* mutations had convergent impacts on behavior: both cHet and R579W mice exhibited hyperactivity, decreased anxiety, and deficient spatial learning and memory. The reported deficits, many of which could arise from altered hippocampal function (Bast and Feldon, 2003; Bannerman et al., 2004; Leussis and Bolivar, 2006), were accompanied by enhanced hippocampal LTP in every mouse model tested. The enhanced LTP was linked to NMDAR hyperfunction in R579W mice and cHet mice, the only mouse models we studied in this aspect, providing one mechanistic basis for deficits observed in PTHS and a potential therapeutic opportunity. Moreover, our data identify a number of consistent behavioral and electrophysiological phenotypes across PTHS model mice, which provide a robust platform for preclinical studies aiming to treat PTHS.

Identifying penetrant consequences of *TCF4* disruption is critical for guiding future PTHS therapeutics, a goal that is particularly important given the challenges in identifying consistent phenotypes across mutations of a single gene for other neurodevelopmental disorders. This issue is perhaps best exemplified by the high-confidence autism risk gene *SHANK3*, in which the type of mutation and the exon affected dramatically affect the severity of intellectual disability, the precise nature of autism- and schizophrenia-like symptoms, and other clinical features (Jiang and

Figure 11. The R579W and cHet mutations result in hippocampal NMDA receptor hyperfunction. **A**, The NMDAR antagonist *D,L*-APV (50 μ M) blocked LTP induced by 3×1 s 100 Hz bursts and measured in fEPSPs in hippocampal area CA1 in R579W mice and controls. **B**, NMDAR-mediated current to AMPAR-mediated current ratio (NMDA/AMPA) in R579W mice and controls. Sample traces represent AMPA majority (−70 mV) and NMDA majority (+40 mV). Scale bars, 100 ms $\times$ 100 pA. **C**, Decay time constants (τ_{decay}) measured at +40 mV of NMDAR majority currents in R579W mice and controls. Amplitude-normalized traces are shown with scale bar, 100 ms. **D**, Decay time constants (τ_{decay}) in R579W mice and controls measured from pharmacologically isolated NMDAR-mediated currents (shaded indicates DNQX). Amplitude-normalized traces are shown with scale bar, 100 ms. **E**, Sensitivity of NMDAR-mediated currents to the NR2B subunit-selective antagonist ifenprodil (5 μ M) in R579W mice and controls. **F**, NMDA/AMPA ratio in cHet mice and controls. Sample traces represent AMPA-majority (−70 mV) and NMDA-majority (+40 mV). Scale bars, 100 ms $\times$ 100 pA. **G**, Decay time constants (τ_{decay}) measured at +40 mV of NMDAR-majority currents in cHet mice and controls. Amplitude-normalized traces are shown with scale bar, 100 ms. Error bars indicate mean $\pm$ SEM. $p \leq 0.05$.

Ehlers, 2013). This diversity has complicated the preclinical study of *SHANK3*-related disorders such as Phelan–McDermid syndrome (Wilson et al., 2003) because *Shank3* mouse models have yielded inconsistent neurobehavioral, synaptic, and molecular phenotypes (Jiang and Ehlers, 2013). However, the issue may be clarified by a recent study of the shared and distinct features of autism- or schizophrenia-causing point mutations on exon 21 (Zhou et al., 2016), highlighting the need to produce accurate disease models. Likewise, PTHS-relevant mutations of *TCF4* span a wide spectrum (Sepp et al., 2012; Whalen et al., 2012) and *TCF4* has been linked to both autism (Stessman et al., 2017) and schizophrenia

(Stefansson et al., 2009; Navarrete et al., 2013; Quednow et al., 2014). Furthermore, it is unclear to what degree PTHS-related phenotypes vary with genotype in patients due to sparse clinical data, although previous studies suggest that there are both highly penetrant and variable phenotypes (Giurgea et al., 2008; Rosenfeld et al., 2009; Whalen et al., 2012). Different PTHS mouse models had the potential to vary in behavioral and synaptic phenotypes, yet we found largely homogenous phenotypes among the mice harboring point mutations or small deletions compromising the function of the critical bHLH domain. The observed overlapping phenotypes across PTHS mouse models provide insights for identifying potential drug targets and outcome measures for assessing preclinical therapeutic efficacy.

Our finding of reduced adult brain weight and body weight in our mouse models is consistent with the features of microcephaly and hypotonia in PTHS. Qualitatively, the reductions in brain size appear to be uniform, consistent with the observation that *TCF4* is expressed in nearly every brain area (Sepp et al., 2011). Interestingly, previous work in adult *Tcf4*^{+/-} mice (Rannals et al., 2016a, b) showed no apparent changes in body weight (Grubišić et al., 2015); brain weight was not assessed. This discrepancy may be due to mouse strain differences: the *Tcf4*^{+/-} mice were on a mixed C57BL/6-129SF1/J background, whereas our mouse models were on a congenic C57BL/6 background. Our behavioral characterization of cHet and R579W mice showed that these mice expressed hyperactivity in the open field and decreased anxiety in the elevated plus maze, but also lacked sociability deficits and reliable long-lasting impairment to auditory startle. In addition, both R579W and cHet mice displayed learning and memory deficits in the Morris water maze; however, subtle genotypic differences were apparent in their performance in this hippocampus-dependent task that might merit further investigation using additional spatial memory tasks. Consistent with our data, *Tcf4*^{+/-} mice are also hyperactive in the open field and demonstrate learning and memory deficits in the Morris water maze, as well as other hippocampus-dependent tasks (Kennedy et al., 2016). Likewise, both cHet and *Tcf4*^{+/-} mice (but not R579W mice) demonstrate deficits in habituation to novel environments at least 30 min after entering the open field (Kennedy et al., 2016). Our findings across PTHS mouse models, combined with data from other investigators in the *Tcf4*^{+/-} mice (Kennedy et al., 2016), emphasize the penetrance of the open-field and hippocampus-dependent cognition deficits as central features of PTHS mouse models. However, *Tcf4*^{+/-} mice also have behaviors that

were inconsistent with our findings: the *Tcf4*^{+/-} mice are not only anxious in the elevated plus maze, but also asocial and hyperresponsive to auditory stimuli (Kennedy et al., 2016). The disparities between these findings, particularly between *Tcf4*^{+/-} and cHet mice, which both model a similar disruption of *Tcf4*, may be due to potential but untested differences in these tasks because they commonly appear between backgrounds and among substrains of the similar backgrounds (Balogh et al., 1999; Moy et al., 2007; Bryant et al., 2008; Matsuo et al., 2010). Alternatively, there may be variations in how each task was performed between studies. Such variations should be investigated closely in further work, possibly as a basis for examining variability in molecular targets downstream of TCF4 in PTHS. Despite subtle differences across PTHS mouse models, our data demonstrate that there is a large core set of behavioral deficits around which preclinical studies can be designed.

Enhanced hippocampal LTP was a particularly penetrant feature across our four PTHS mouse models, which, together with a similar finding in *Tcf4*^{+/-} mice (Kennedy et al., 2016), comprises the most consistent phenotype in the PTHS literature to date. Consistent with the concept that neurodevelopmental disorders may largely be a disease of the synapse (Zoghbi and Bear, 2012), we found a strong synaptic phenotype, enhanced NMDAR-mediated LTP, that may underlie cognitive dysfunction observed in *Tcf4* mouse models (Brzózka et al., 2010; Kennedy et al., 2016) and in individuals with PTHS (Amiel et al., 2007; Brockschmidt et al., 2007; Zweier et al., 2007; Rosenfeld et al., 2009). There is a complex relationship between LTP and cognitive performance (Lee and Silva, 2009), as evidenced by studies showing enhanced LTP impairs learning in mouse models with genetic alterations in *IRSp53* (Kim et al., 2009), *LIMK1* (Meng et al., 2002), PSD-95 (Migaud et al., 1998), syndecan-3 (Kaksonen et al., 2002), dystrophin (Vaillend et al., 2004), or PTPδ (Uetani et al., 2000), or in rodents exposed prenatally to valproic acid (Rinaldi et al., 2007; Rouillet et al., 2013). Therefore, our finding suggests that PTHS-related *Tcf4* disruption broadly perturbs the mechanisms underlying LTP induction, negatively affecting learning and memory. Upon closer investigation of the electrophysiology of CA1 pyramidal cells in R579W mice, we found reduced intrinsic excitability in the R579W mice, but not the cHet mice, indicating a potentially divergent phenotype among the models. The excitability defect in the R579W mice, also recently linked to *Tcf4* perturbation in prefrontal layer 2/3 pyramidal neurons (Rannals et al., 2016a, b), is surprising in light of the observed LTP enhancement. However, this defect may be overcome by enhanced NMDAR function, which was suggested by the increased NMDA/AMPA current ratio and intact AMPAR-mediated synaptic transmission. Likewise, this NMDAR hyperfunction appears sufficient to enhance LTP in the cHet mice. This shared alteration may be due to NMDAR subunit dysfunction, as suggested by the prolonged NMDAR-mediated current decay time and increased ifenprodil sensitivity, which may favor *Grin2b* function over *Grin2a* function, as well as the observed changes in *Grin2a* expression in *Tcf4*^{+/-} mice (Kennedy et al., 2016). Together, these results pinpoint a key mechanistic insight that may apply broadly across PTHS-relevant *Tcf4* mutations.

Our finding of hippocampal NMDAR hyperfunction in R579W mice, coupled with findings of prefrontal cortex NMDAR hyperfunction in *Tcf4*^{+/-} mice (Page et al., 2017), suggests that NMDAR antagonism may be a feasible therapeutic strategy for many PTHS individuals. NMDAR hyperfunction has been observed in many neurodevelopmental disorders (Chez et al., 2007; Rinaldi et al., 2007; Kim et al., 2009; Etherton et al., 2011), raising the possibil-

ity that drugs correcting NMDAR function may rescue core disease features (Lee et al., 2015; Katz et al., 2016a, b). For example, a recent study of Rett syndrome, which has a clinical presentation similar to PTHS (Brockschmidt et al., 2007; Zweier et al., 2007, 2008; Giurgea et al., 2008; Tan et al., 2014), showed that chronic administration of a subsaturating dose of the noncompetitive NMDAR antagonist ketamine was sufficient to extend lifespan and correct anatomical and physiological abnormalities (Patrizi et al., 2016). These results have led to the idea that NMDAR antagonists such as ketamine might offer a treatment for some features of Rett syndrome (Kron et al., 2012; Katz et al., 2016b). In PTHS, there has been informal clinical interest in the effectiveness of the NMDAR antagonist amantadine (Pitt–Hopkins Research Foundation, personal communications). Our data support an assessment of the efficacy of amantadine or the pharmacologically similar NMDAR antagonist memantine (Kos and Popik, 2005; Rammes et al., 2008) in treating the core features of PTHS in model mice. Our study further outlines penetrant and reproducible core features of PTHS mouse models, including enhanced hippocampal LTP, hyperactivity, and deficient hippocampus-dependent learning and memory, representing potential biomarkers of the preclinical effectiveness of therapeutic approaches, including NMDAR antagonism.

References

- Amiel J, Rio M, de Pontual L, Redon R, Malan V, Boddaert N, Plouin P, Carter NP, Lyonnet S, Munnich A, Colleaux L (2007) Mutations in TCF4, encoding a class I basic helix-loop-helix transcription factor, are responsible for Pitt–Hopkins syndrome, a severe epileptic encephalopathy associated with autonomic dysfunction. *Am J Hum Genet* 80:988–993. [CrossRef Medline](#)
- Balogh SA, McDowell CS, Stavnezer AJ, Denenberg VH (1999) A behavioral and neuroanatomical assessment of an inbred substrain of 129 mice with behavioral comparisons to C57BL/6J mice. *Brain Res* 836:38–48. [CrossRef Medline](#)
- Bannerman DM, Rawlins JN, McHugh SB, Deacon RM, Yee BK, Bast T, Zhang WN, Pothuizen HH, Feldon J (2004) Regional dissociations within the hippocampus—memory and anxiety. *Neurosci Biobehav Rev* 28:273–283. [CrossRef Medline](#)
- Bast T, Feldon J (2003) Hippocampal modulation of sensorimotor processes. *Prog Neurobiol* 70:319–345. [CrossRef Medline](#)
- Bergqvist I, Eriksson M, Saarikettu J, Eriksson B, Corneliussen B, Grundström T, Holmberg D (2000) The basic helix-loop-helix transcription factor E2-2 is involved in T lymphocyte development. *Eur J Immunol* 30:2857–2863. [CrossRef Medline](#)
- Berrios J, Stamatakis AM, Kantak PA, McElligott ZA, Judson MC, Aita M, Rougie M, Stuber GD, Philpot BD (2016) Loss of UBE3A from TH-expressing neurons suppresses GABA co-release and enhances VTA–NAc optical self-stimulation. *Nat Commun* 7:10702. [CrossRef Medline](#)
- Bischofberger J, Engel D, Li L, Geiger JR, Jonas P (2006) Patch-clamp recording from mossy fiber terminals in hippocampal slices. *Nat Protoc* 1:2075–2081. [CrossRef Medline](#)
- Blake DJ, Forrest M, Chapman RM, Tinsley CL, O'Donovan MC, Owen MJ (2010) TCF4, schizophrenia, and Pitt–Hopkins syndrome. *Schizophr Bull* 36:443–447. [CrossRef Medline](#)
- Brockschmidt A, Todt U, Ryu S, Hoischen A, Landwehr C, Birnbaum S, Frenck W, Radlwimmer B, Lichter P, Engels H, Driever W, Kubisch C, Weber RG (2007) Severe mental retardation with breathing abnormalities (Pitt–Hopkins syndrome) is caused by haploinsufficiency of the neuronal bHLH transcription factor TCF4. *Hum Mol Genet* 16:1488–1494. [CrossRef Medline](#)
- Bryant CD, Zhang NN, Sokoloff G, Fanselow MS, Ennes HS, Palmer AA, McRoberts JA (2008) Behavioral differences among C57BL/6 substrains: implications for transgenic and knockout studies. *J Neurogenet* 22:315–331. [CrossRef Medline](#)
- Brzózka MM, Radyushkin K, Wichert SP, Ehrenreich H, Rossner MJ (2010) Cognitive and sensorimotor gating impairments in transgenic mice overexpressing the schizophrenia susceptibility gene *Tcf4* in the brain. *Biol Psychiatry* 68:33–40. [CrossRef Medline](#)

- Carmignoto G, Vicini S (1992) Activity-dependent decrease in NMDA receptor responses during development of the visual cortex. *Science* 258:1007–1011. [CrossRef Medline](#)
- Chez MG, Burton Q, Dowling T, Chang M, Khanna P, Kramer C (2007) Memantine as adjunctive therapy in children diagnosed with autistic spectrum disorders: an observation of initial clinical response and maintenance tolerability. *J Child Neurol* 22:574–579. [CrossRef Medline](#)
- de Pontual L, et al. (2009) Mutational, functional, and expression studies of the TCF4 gene in Pitt–Hopkins syndrome. *Hum Mut* 30:669–676. [CrossRef Medline](#)
- de Marchena J, Roberts AC, Middlebrooks PG, Valakh V, Yashiro K, Wilfley LR, Philpot BD (2008) NMDA receptor antagonists reveal age-dependent differences in the properties of visual cortical plasticity. *J Neurophysiol* 100:1936–1948. [CrossRef Medline](#)
- Dembrow NC, Chitwood RA, Johnston D (2010) Projection-specific neuromodulation of medial prefrontal cortex neurons. *J Neurosci* 30:16922–16937. [CrossRef Medline](#)
- de Winter CF, Baas M, Bijlsma EK, van Heukelingen J, Routledge S, Hennekam RC (2016) Phenotype and natural history in 101 individuals with Pitt–Hopkins syndrome through an internet questionnaire system. *Orphanet J Rare Dis* 11:37. [CrossRef Medline](#)
- Etherton M, Földy C, Sharma M, Tabuchi K, Liu X, Shamloo M, Malenka RC, Südhof TC (2011) Autism-linked neuroligin-3 R451C mutation differentially alters hippocampal and cortical synaptic function. *Proc Natl Acad Sci U S A* 108:13764–13769. [CrossRef Medline](#)
- Flint AC, Maisch US, Weishaupt JH, Kriegstein AR, Monyer H (1997) NR2A subunit expression shortens NMDA receptor synaptic currents in developing neocortex. *J Neurosci* 17:2469–2476. [Medline](#)
- Giurgea I, Missirian C, Cacciagli P, Whalen S, Fredriksen T, Gaillon T, Rankin J, Mathieu-Dramard M, Morin G, Martin-Coignard D, Dubourg C, Chabrol B, Arfi J, Giuliano F, Claude Lambert J, Philip N, Sarda P, Villard L, Goossens M, Moncla A (2008) TCF4 deletions in Pitt–Hopkins Syndrome. *Hum Mut* 29:E242–E251. [CrossRef Medline](#)
- Grubišić V, Kennedy AJ, Sweatt JD, Pappas V (2015) Pitt–Hopkins mouse model has altered particular gastrointestinal transits in vivo. *Autism Res* 8:629–633. [CrossRef Medline](#)
- Hasi M, Soileau B, Sebold C, Hill A, Hale DE, O'Donnell L, Cody JD (2011) The role of the TCF4 gene in the phenotype of individuals with 18q segmental deletions. *Hum Genet* 130:777–787. [CrossRef Medline](#)
- Hsu PD, Lander ES, Zhang F (2014) Development and applications of CRISPR–Cas9 for genome engineering. *Cell* 157:1262–1278. [CrossRef Medline](#)
- Jiang YH, Ehlers MD (2013) Modeling autism by SHANK gene mutations in mice. *Neuron* 78:8–27. [CrossRef Medline](#)
- Kaksonen M, Pavlov I, Voikar V, Lauri SE, Hienola A, Riekk R, Lakso M, Taira T, Rauvala H (2002) Syndecan-3-deficient mice exhibit enhanced LTP and impaired hippocampus-dependent memory. *Mol Cell Neurosci* 21:158–172. [CrossRef Medline](#)
- Katz DM, Menniti FS, Mather RJ (2016a) N-methyl-D-aspartate receptors, ketamine, and Rett syndrome: something special on the road to treatments? *Biol Psychiatry* 79:710–712. [CrossRef Medline](#)
- Katz DM, Bird A, Coenraads M, Gray SJ, Menon DU, Philpot BD, Tarquinio DC (2016b) Rett syndrome: crossing the threshold to clinical translation. *Trends Neurosci* 39:100–113. [CrossRef Medline](#)
- Kennedy AJ, Rahn EJ, Paulukaitis BS, Savell KE, Kordasiewicz HB, Wang J, Lewis JW, Posey J, Strange SK, Guzman-Karlsson MC, Phillips SE, Decker K, Motley ST, Swayze EE, Ecker DJ, Michael TP, Day JJ, Sweatt JD (2016) Tcf4 regulates synaptic plasticity, DNA methylation, and memory function. *Cell Rep* 16:2666–2685. [CrossRef Medline](#)
- Kim MH, Choi J, Yang J, Chung W, Kim JH, Paik SK, Kim K, Han S, Won H, Bae YS, Cho SH, Seo J, Bae YC, Choi SY, Kim E (2009) Enhanced NMDA receptor-mediated synaptic transmission, enhanced long-term potentiation, and impaired learning and memory in mice lacking IRSp53. *J Neurosci* 29:1586–1595. [CrossRef Medline](#)
- Kos T, Popik P (2005) A comparison of the predictive therapeutic and undesired side-effects of the NMDA receptor antagonist, memantine, in mice. *Behav Pharmacol* 16:155–161. [CrossRef Medline](#)
- Kron M, Howell CJ, Adams IT, Ransbottom M, Christian D, Ogier M, Katz DM (2012) Brain activity mapping in Mecp2 mutant mice reveals functional deficits in forebrain circuits, including key nodes in the default mode network, that are reversed with ketamine treatment. *J Neurosci* 32:13860–13872.
- Lee EJ, Choi SY, Kim E (2015) NMDA receptor dysfunction in autism spectrum disorders. *Curr Opin Pharmacol* 20:8–13. [CrossRef Medline](#)
- Lee YS, Silva AJ (2009) The molecular and cellular biology of enhanced cognition. *Nat Rev Neurosci* 10:126–140. [CrossRef Medline](#)
- Leussis MP, Bolivar VJ (2006) Habituation in rodents: a review of behavior, neurobiology, and genetics. *Neurosci Biobehav Rev* 30:1045–1064. [CrossRef Medline](#)
- Marangi G, Zollino M (2015) Pitt–Hopkins syndrome and differential diagnosis: a molecular and clinical challenge. *J Pediatr Genet* 4:168–176. [CrossRef Medline](#)
- Matsuo N, Takao K, Nakanishi K, Yamasaki N, Tanda K, Miyakawa T (2010) Behavioral profiles of three C57BL/6 substrains. *Front Behav Neurosci* 4:29. [CrossRef Medline](#)
- Meng Y, Zhang Y, Tregoubov V, Janus C, Cruz L, Jackson M, Lu WY, MacDonald JF, Wang JY, Falls DL, Jia Z (2002) Abnormal spine morphology and enhanced LTP in LIMK-1 knockout mice. *Neuron* 35:121–133. [CrossRef Medline](#)
- Migaud M, Charlesworth P, Dempster M, Webster LC, Watabe AM, Makhinson M, He Y, Ramsay MF, Morris RG, Morrison JH, O'Dell TJ, Grant SG (1998) Enhanced long-term potentiation and impaired learning in mice with mutant postsynaptic density-95 protein. *Nature* 396:433–439. [CrossRef Medline](#)
- Moy SS, Nadler JJ, Young NB, Perez A, Holloway LP, Barbaro RP, Barbaro JR, Wilson LM, Threadgill DW, Lauder JM, Magnuson TR, Crawley JN (2007) Mouse behavioral tasks relevant to autism: phenotypes of 10 inbred strains. *Behav Brain Res* 176:4–20. [CrossRef Medline](#)
- Navarrete K, Pedrosa I, De Jong S, Stefansson H, Steinberg S, Stefansson K, Ophoff RA, Schalkwyk LC, Collier DA (2013) TCF4 (e2-2; ITF2): a schizophrenia-associated gene with pleiotropic effects on human disease. *Am J Med Genet B Neuropsychiatr Genet* 162B:1–16. [CrossRef Medline](#)
- Page SC, Hamersky GR, Gallo RA, Rannals MD, Calcaterra NE, Campbell MN, Mayfield B, Briley A, Phan BN, Jaffe AE, Maher BJ (2017) The schizophrenia- and autism-associated gene, transcription factor 4 regulates the columnar distribution of layer 2/3 prefrontal pyramidal neurons in an activity-dependent manner. *Mol Psychiatry*. Advance online publication. Retrieved on Mar 14, 2017. [CrossRef](#)
- Paoletti P, Bellone C, Zhou Q (2013) NMDA receptor subunit diversity: impact on receptor properties, synaptic plasticity and disease. *Nat Rev Neurosci* 14:383–400. [CrossRef Medline](#)
- Patrizi A, Picard N, Simon AJ, Gunner G, Centofante E, Andrews NA, Fagiolini M (2016) Chronic administration of the N-methyl-D-aspartate receptor antagonist ketamine improves Rett syndrome phenotype. *Biol Psychiatry* 79:755–764. [CrossRef Medline](#)
- Philpot BD, Weisberg MP, Ramos MS, Sawtell NB, Tang YP, Tsien JZ, Bear MF (2001) Effect of transgenic overexpression of NR2B on NMDA receptor function and synaptic plasticity in visual cortex. *Neuropharmacology* 41:762–770. [CrossRef Medline](#)
- Quednow BB, Brzózka MM, Rossner MJ (2014) Transcription factor 4 (TCF4) and schizophrenia: integrating the animal and the human perspective. *Cell Mol Life Sci* 71:2815–2835. [CrossRef Medline](#)
- Rammes G, Danysz W, Parsons CG (2008) Pharmacodynamics of memantine: an update. *Curr Neuropharmacol* 6:55–78. [CrossRef Medline](#)
- Rannals MD, Page SC, Campbell MN, Gallo RA, Mayfield B, Maher BJ (2016a) Neurodevelopmental models of transcription factor 4 deficiency converge on a common ion channel as a potential therapeutic target for Pitt Hopkins syndrome. *Rare Dis* 4:e1220468. [CrossRef Medline](#)
- Rannals MD, Hamersky GR, Page SC, Campbell MN, Briley A, Gallo RA, Phan BN, Hyde TM, Kleinman JE, Shin JH, Jaffe AE, Weinberger DR, Maher BJ (2016b) Psychiatric risk gene transcription factor 4 regulates intrinsic excitability of prefrontal neurons via repression of SCN10a and KCNQ1. *Neuron* 90:43–55. [CrossRef Medline](#)
- Rinaldi T, Kulangara K, Antonietti K, Markram H (2007) Elevated NMDA receptor levels and enhanced postsynaptic long-term potentiation induced by prenatal exposure to valproic acid. *Proc Natl Acad Sci U S A* 104:13501–13506. [CrossRef Medline](#)
- Rosenfeld JA, Leppig K, Ballif BC, Thiese H, Erdie-Lalena C, Bawle E, Sastry S, Spence JE, Bandholz A, Surti U, Zonana J, Keller K, Meschino W, Bejjani BA, Torchia BS, Shaffer LG (2009) Genotype-phenotype analysis of TCF4 mutations causing Pitt–Hopkins syndrome shows increased seizure activity with missense mutations. *Genet Med* 11:797–805. [CrossRef Medline](#)
- Roulet FI, Lai JK, Foster JA (2013) In utero exposure to valproic acid and

- autism—a current review of clinical and animal studies. *Neurotoxicol Teratol* 36:47–56. [CrossRef Medline](#)
- Routh BN, Johnston D, Harris K, Chitwood RA (2009) Anatomical and electrophysiological comparison of CA1 pyramidal neurons of the rat and mouse. *J Neurophysiol* 102:2288–2302. [CrossRef Medline](#)
- Sepp M, Kannike K, Eesmaa A, Urb M, Timmusk T (2011) Functional diversity of human basic helix-loop-helix transcription factor TCF4 isoforms generated by alternative 5' exon usage and splicing. *PLoS One* 6:e22138. [CrossRef Medline](#)
- Sepp M, Pruunsild P, Timmusk T (2012) Pitt–Hopkins syndrome-associated mutations in TCF4 lead to variable impairment of the transcription factor function ranging from hypomorphic to dominant-negative effects. *Hum Mol Genet* 21:2873–2888. [CrossRef Medline](#)
- Stefansson H, et al. (2009) Common variants conferring risk of schizophrenia. *Nature* 460:744–747. [Medline](#)
- Stessman HA, et al. (2017) Targeted sequencing identifies 91 neurodevelopmental-disorder risk genes with autism and developmental-disability biases. *Nat Genet* 49:515–526. [CrossRef Medline](#)
- Tamberg L, Sepp M, Timmusk T, Palgi M (2015) Introducing Pitt–Hopkins syndrome-associated mutations of TCF4 to *Drosophila* daughterless. *Biol Open* 4:1762–1771. [CrossRef Medline](#)
- Tan WH, Bird LM, Thibert RL, Williams CA (2014) If not Angelman, what is it? A review of Angelman-like syndromes. *Am J Med Genet A* 164A:975–992. [Medline](#)
- Uetani N, Kato K, Ogura H, Mizuno K, Kawano K, Mikoshiba K, Yakura H, Asano M, Iwakura Y (2000) Impaired learning with enhanced hippocampal long-term potentiation in PTPdelta-deficient mice. *EMBO J* 19:2775–2785. [CrossRef Medline](#)
- Vaillend C, Billard JM, Laroche S (2004) Impaired long-term spatial and recognition memory and enhanced CA1 hippocampal LTP in the dystrophin-deficient Dmd(mdx) mouse. *Neurobiol Dis* 17:10–20. [CrossRef Medline](#)
- Wallace ML, Burette AC, Weinberg RJ, Philpot BD (2012) Maternal loss of Ube3a produces an excitatory/inhibitory imbalance through neuron type-specific synaptic defects. *Neuron* 74:793–800. [CrossRef Medline](#)
- Whalen S, et al. (2012) Novel comprehensive diagnostic strategy in Pitt–Hopkins syndrome: clinical score and further delineation of the TCF4 mutational spectrum. *Hum Mut* 33:64–72. [CrossRef Medline](#)
- Wilson HL, Wong AC, Shaw SR, Tse WY, Stapleton GA, Phelan MC, Hu S, Marshall J, McDermid HE (2003) Molecular characterisation of the 22q13 deletion syndrome supports the role of haploinsufficiency of SHANK3/PROSAP2 in the major neurological symptoms. *J Med Genet* 40:575–584. [CrossRef Medline](#)
- Zhou Y, Kaiser T, Monteiro P, Zhang X, Van der Goes MS, Wang D, Barak B, Zeng M, Li C, Lu C, Wells M, Amaya A, Nguyen S, Lewis M, Sanjana N, Zhou Y, Zhang M, Zhang F, Fu Z, Feng G (2016) Mice with Shank3 mutations associated with ASD and schizophrenia display both shared and distinct defects. *Neuron* 89:147–162. [CrossRef Medline](#)
- Zhuang Y, Cheng P, Weintraub H (1996) B-lymphocyte development is regulated by the combined dosage of three basic helix-loop-helix genes, E2A, E2–2, and HEB. *Mol Cell Biol* 16:2898–2905. [CrossRef Medline](#)
- Zoghbi HY, Bear MF (2012) Synaptic dysfunction in neurodevelopmental disorders associated with autism and intellectual disabilities. *Cold Spring Harb Perspect Biol* 4: pii: a009886. [CrossRef Medline](#)
- Zweier C, et al. (2008) Further delineation of Pitt–Hopkins syndrome: phenotypic and genotypic description of 16 novel patients. *J Med Genet* 45:738–744. [CrossRef Medline](#)
- Zweier C, Peippo MM, Hoyer J, Sousa S, Bottani A, Clayton-Smith J, Reardon W, Saraiva J, Cabral A, Gohring I, Devriendt K, de Ravel T, Bijlsma EK, Hennekam RC, Orrico A, Cohen M, Dreweke A, Reis A, Nurnberg P, Rauch A (2007) Haploinsufficiency of TCF4 causes syndromal mental retardation with intermittent hyperventilation (Pitt–Hopkins syndrome). *Am J Hum Genet* 80:994–1001. [CrossRef Medline](#)