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Disruption of TCF4 regulatory networks leads to abnormal cortical development and mental disabilities

Hong Li[1,2,3], **Ying Zhu**[4], **Yury M. Morozov**[1], **Xiaoli Chen**[5], **Stephanie Cerceo Page**[6], **Matthew D. Rannals**[6], **Brady J. Maher**[6,7,8], **Pasko Rakic**[1,9]

[1]Department of Neuroscience, Yale University School of Medicine, New Haven, CT 06510, USA

[2]School of Basic Medical Sciences, Anhui Medical University, Anhui, China

[3]Biopharmaceutical Institute, Anhui Medical University, Anhui, China

[4]Department of Biostatistics, School of Public Health, Yale University, New Haven, CT 06510, USA

[5]Department of Medical Genetics, Capital Institute of Pediatrics, 100020 Beijing, China

[6]Lieber Institute for Brain Development, Johns Hopkins Medical Campus, Baltimore, MD 21205, USA

[7]Department of Psychiatry and Behavioral Sciences, Johns Hopkins School of Medicine, Baltimore, MD 21205, USA

[8]Solomon H. Snyder Department of Neuroscience, Johns Hopkins School of Medicine, Baltimore, MD 21205, USA

[9]Kavli Institute for Neuroscience, Yale University, New Haven, CT 06510, USA

Abstract

The TCF4 gene is the subject of numerous and varied investigations of it's role in the genesis of neuropsychiatric disease. The gene has been identified as the cause of Pitt–Hopkins syndrome (PTHS) and it has been implicated in various other neuropsychiatric diseases, including schizophrenia, depression, and autism. However, the precise molecular mechanisms of the gene's involvement in neurogenesis, particularly, corticogenesis, are not well understood. Here, we present data showing that TCF4 is expressed in a region-specific manner in the radial glia and stem cells of transient embryonic zones at early gestational ages in both humans and mice. TCF4 haploinsufficiency mice exhibit a delay in neuronal migration, and a significant increase in the number of upper-layer cortical neurons, as well as abnormal dendrite and synapse formation. Our research also reveals that TCF3 up-regulates *Tcf4* by binding to the specific "E-box" and its flank sequence in intron 2 of the *Tcf4* gene. Additionally, our transcriptome study substantiates that *Tcf4* transcriptional function is essential for locomotion, cognition, and learning. By activating expression of TCF4 in the regulation of neuronal proliferation and migration to the overlaying

Pasko Rakic, Pasko.Rakic@yale.edu.

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neocortex and subsequent differentiation leading to laminar formation TCF4 fulfills its normal function, but if not, abnormalities such as those reported here result. These findings provide new insight into the specific roles of *Tcf4* molecular pathway in neocortical development and their relevance in the pathogenesis of neuropsychiatric diseases.

Introduction

During development of the cerebral cortex in all mammalian species, including humans, projection neurons are generated in the proliferative ventricular zone (VZ) and the subventricular zone (SVZ) of the embryonic telencephalon (reviewed in Bystron et al. [1]). After final cell division, these neurons migrate along radial glia (RG) fibers through the intermediate zone (IZ) in an inside-out manner to the cortical plate (CP) and, once there, form six-layer laminar structures [2-4]. Functionally distinct cortical areas are determined by gradients of morphogens and transcriptional factors in the neuronal proliferative zones [5-7]. Subsequently, properly positioned neurons undergo differentiation and synapse formation [8-10]. Current evidence indicates that abnormalities in the achievement of any of the above-mentioned processes are very likely involved in the pathogenesis of neurodevelopmental disorders, such as autism and intellectual disability and schizophrenia [6, 11-13].

Given this overview, there is a wide body of evidence identifying various types of mutations in the *Tcf4* gene that lead to abnormalities in brain development, It is well established that TCF4 protein is a member of the class B basic helix-loop-helix (bHLH) transcription factor family and that haploinsufficiency due to rare variants of *Tcf4*, such as nonsense, missense, or microdeletion mutations cause Pitt–Hopkins syndrome (PTHS) [14-17]. In some cases, the majority of mutations produce truncated proteins or missense mutations that disrupt the bHLH-binding domain necessary for the functional transcription activity of *Tcf4* [17, 18]. *Tcf4* is also associated with many other psychiatric disorders. Several publications, for example, provide evidence that common variants in the *Tcf4* gene, including single nucleotide polymorphisms (SNPs) in the intron exert risks for schizophrenia, bipolar disease, and autism [19-22]. Indeed, the existence of a single-nucleotide polymorphism (SNP rs9960767) and an extended CTG triple repeat in the third intron of the *Tcf4* gene have been identified in patients with schizophrenia and bipolar disease respectively [23, 24]; Wirgenes et al. reported *Tcf4* sequence variants and elevated levels of *Tcf4* mRNA associated with neurodevelopmental characteristics in psychotic disorders [25], and Page et al. reported that overexpression of TCF4 severely disrupts the columnar organization of rat medial prefrontal cortex in a transcription and activity-dependent manner [26]. Studies such as these underscore the importance of understanding the basic mechanisms governing how the *Tcf4* gene is regulated during corticogenesis.

Also, in conjunction with genetic mutations such as those mentioned above, there is accumulated evidence that abnormalities in cell migration leading to neuron misplacement contributes to psychiatric disease. Misplaced or ectopic clusters of neurons, for example, have been observed in the neocortex of schizophrenia patients [27] and animal models of the disease [28, 29], for example, that disruption of *Reln*, the gene encoding reelin, a laminar organizer in neocortex, causes cognitive defects in rodents [30]. Indeed, evidence

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of cell disorganization in the neocortex of children with autism has been documented [31], indicating that abnormal neuronal migration contributes to psychiatric diseases. Thus, despite early or late onset of symptoms, serious mental dysfunction appears to stem from abnormalities occurring during various stages of corticogenesis.

In the present study, it is our intention to expand our knowledge of the role that TCF4 plays in the developing cerebrum of both mouse and human. Specifically, we aim to explore regulation of *Tcf4* gene expression in developing mouse and human neocortex and determine how disruption of the *Tcf4* regulatory network affects corticogenesis and contributes to neuropsychiatric disease.

Material and methods

Human fetal tissue

Human fetal tissue was obtained from Anhui Medical University Affiliated Hospital (Anhui, China) in compliance with the Ethical Regulation on Research Using Human Tissue. Brains (1 for 14 post conception week (pcw); 1 for 30pcw) were cut into 2 cm coronal blocks and fixed in 4% PFA for 2–3 days before implementing the standard dehydration procedure for paraffin embedding. We cut 7 μm-thick paraffin sections for subsequent immunostaining experiments.

Animals

All experiments on animals were performed in compliance with Yale IACUC and U. S. Department of Health and Human Services. We obtained *Tcf4* heterozygotes (*Tcf4Het*, Jackson Laboratories, stock #:013598) and generated *Tcf4KO* and *Tcf4Het* pups (Supplementary Figure 4e). *Tcf3*flox/+ female mice (Jackson Laboratories, stock #: 008303) were crossed with Emx1cre+/− males to generate *Tcf3* brain-specific knock out (*Tcf3KO*) which were maintained routinely in the lab. Sample size for each experiment to gain sufficient size (alpha: 0.05) used in this study were determined on the basis of previous similar experiments. Numbers of animals used in each experiment are indicated in the figure legends.

mRNA-sequencing

mRNA preparation and library construction—Dorsal telencephalon from each of three samples of *Tcf4KO*, *Tcf4Het*, and WT genotypes from three different litters were isolated by standard Trizol method and cDNA library was prepared as previously described [32]. Quantification of gene expression level was performed in HTSeq v0.6.1, which counted the read numbers mapped for each gene, and then reported as FPKM (fragment per kilobase of exon model per Million mapped reads) values for each gene [32].

Results

TCF3 regulates *Tcf4* expression in early human and mouse embryonic telencephalon

We examined expression of *Tcf4* in human fetal telencephalon at 14 pcw and found that TCF4 protein is highly expressed in the VZ/SVZ and moderately in the IZ. At this

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embryonic age, the expression is highest in the VZ/SVZ of the frontal lobe (Fig. 1a) and lowest in the VZ/SVZ of the occipital lobe. The lower expression level in the CP is similar between the frontal and the occipital lobes. At 30 pcw, TCF4 is expressed only by migrating neurons in the IZ and differentiating neurons in the CP of both frontal and occipital lobes (Fig. 1a). To investigate transcriptional control of human *Tcf4* gene expression in early embryonic brain, we employed a bioinformatic search for potential transcriptional-binding sites with 5 kb up-stream and 5 kb down-stream sequences of the transcription start site (TSS). We then manually validated these transcription factors, the expression of which is temporally and spatially correlated to *Tcf4* expression in the dorsal telencephalon with search tools from the Developing Mouse Brain Atlas (Allen Brain Institute) and the high-resolution transcriptome map (Rakic lab: http://medicine.yale.edu/lab/rakic/transcriptome/).

We found that TCF3, another member of the bHLH transcription factor family, has an evolutionary conserved binding site within the second intron of the *Tcf4* gene. Luciferase assay showed robust transcriptional activation of the luciferase gene downstream of the binding site (Fig. 1b, upper panel, in red) when co-expressed with species specific TCF3 compared to a scrambled sequence (Fig. 1b, lower panel). We then examined the expression of TCF3 in human fetal brain sections adjacent to those we used in Fig. 1a for TCF4. Similar to TCF4, the VZ/SVZ in the frontal cortex exhibited the highest expression of TCF3 compared to moderate expression in the CP and IZ of both the frontal and occipital lobes at 14 pcw and still remains detectable at 30 pcw (Fig. 1c).

As a next step, we examined expression of *Tcf4* and *Tcf3* mRNA in 9 subdivisions of mouse dorsal telencephalon at embryonic day 13 (E13) (Fig. 1d, left). Both *Tcf4* and *Tcf3* mRNA are expressed in a gradient of caudal-medial high to rostral-lateral low across the tangential surface of the dorsal telencephalon (Fig. 1d, right). TCF4 is expressed throughout the neuroepithelium layer at E12 and in selected populations of cells in the VZ/SVZ, IZ, and CP at E14 (Fig. 1e). However, *Tcf3* mRNA is confined to the VZ/SVZ at E13 (Fig. 1f) when expression of its transcripts reaches peak levels (Fig. 1g). *Tcf3* mRNA is abundant at the early to mid-neurogenesis stages while *Tcf4* is gradually up-regulated from early to the mid-neurogenesis stage, and remains at a high level at late embryonic stages. Both transcripts are decreased significantly after birth (Fig. 1g). To determine whether TCF3 regulates *Tcf4* in vivo, we examined TCF4 expression in *Tcf3KO* and found that TCF4 is down-regulated significantly in the telencephalon of *Tcf3KO* at E12 (Fig. 1h), but not at E14 and P0 (Fig. 1h), indicating that regulation of *Tcf4* expression by TCF3 in the dorsal telencephalon is limited to the early embryonic stage.

Increased cell proliferation in the VZ/SVZ in the absence of TCF4 and TCF3

To further investigate these various observations, we analyzed aspects of cortical development in *Tcf4* heterozygotes (*Tcf4Het*) and knockout mice (*Tcf4KO*) at embryonic, neonatal, and postnatal stages along with *Tcf3KO*.

We observed a significant increase in the number of TBR2-positive intermediate progenitors in the VZ/SVZ of the *Tcf4KO* (Fig. 2a, b) and *Tcf3KO* (Fig. 2e, f) mice compared to their WT littermate controls. The general proliferation marker, Ki67, was also increased in *Tcf4KO* at E12 (Fig. 2a, b). We further injected IdU intraperitoneally to E12–E14 mothers

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and fixed the embryos 3 h after injection to analyze cycling cells. We found increased IdU-positive cells in early and mid-neurogenesis stages of *Tcf4KO* (Fig. 2c, d) and *Tcf3KO* (Fig. 2e, f) in comparison to controls. In addition, we found that phospho-histone 3 (PH3), a mitotic marker, is also increased in *Tcf4KO* at E12 and E14 (Fig. 2c, d). Collectively, our observations that the loss of either TCF4 or TCF3 in the dorsal telencephalon results in above abnormal cell division in the VZ/SVZ provides evidence that TCF4 and TCF3 are involved in regulating neuronal proliferation primarily at an early stage of neurogenesis.

Abnormal neuronal migration and laminar formation in the neocortex of TCF4 haploinsufficency mice

To determine whether the change in neuronal proliferation leads to abnormalities in neuronal migration, we double-labeled early-born and late-born neurons with IdU at E12 and with CldU at E14 and examined their final position in postnatal *Tcf4Het* (Fig. 2g, h). In comparison to their littermate WT controls at P4, P7, and P15, *Tcf4Het* mice exhibited a significantly increased number of CldU-labeled neurons stuck in the deep layers of the telencephalic wall while still on their migratory route to the CP (Fig. 2g, h). In addition, we observed total CldU-labeled cells in *Tcf4Het* outnumbered their WT littermate controls, but not in the case of the IdU-positive cells (Fig. 2h). Similar results were obtained in *Tcf3KO* mice at P4 (Fig. 2j). These results showed that generation and migration of upper layer neurons are significantly affected in *Tcf4Het* and *Tcf3KO*. To further confirm the migration defect observed in *Tcf4Het*, we performed in utero electroporation of GFP in *Tcf4Het* embryos and their WT littermate controls at gestational stage E14. In WT controls, the majority of GFP-positive neurons are CUX1 upper layer neurons, but the multitude of GFP-positive neurons in *Tcf4Het* mice are in the CUX1-negative deep layers at P15 (Fig. 2k).

We then performed TCF4 gain of function experiments and measured what effect overexpression of TCF4 had on neuronal migration by electroporating either GFP alone, TCF4 full length (TCF4B), or TCF4R582P [26], a PTHS-causing mutation in which TCF4 is unable to bind to DNA. We observed that overexpression of TCF4B significantly enhanced the rate of neuronal migration such that 2 days after electroporation 32.5% of migrating neurons had reached the IZ and CP compared to only 13.9% in the R582P condition and 3.6% in the GFP condition (Fig. 2l).

Next, we examined expressions of typical neocortex laminar markers. CUX1, for example, is a homeobox transcription factor normally expressed in layer 2/3 and 4 in sensory cortices [33, 34] and is nearly absent at P0 in *Tcf4KO*, but normal in *Tcf4Het* (Fig. 3a, upper panel; b). CUX1 immunoreactivity is largely reduced in the primary visual cortical area and parietal cortex *Tcf4*KO, but still exhibits a weak signal in the frontal/motor cortex (Supplementary Figure 1a, b). This may be due to the high caudal-medial to low rostral-lateral gradient distribution of TCF4 expression in the mouse telencephalon (Fig. 1d). However, the number of neurons labeled by SATB2 and BRN2, also superficial neuron markers [35-37], are increased in both *Tcf4Het* and *Tcf4KO* telencephalon (Fig. 3a, middle and lower panels; b). The majority of increased neurons are found in the deep layers of cortical columns (Fig. 3a, b, arrows in a) indicating migration defects of these

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neurons. Increased numbers of SATB2 and BRN2-positive neurons are more prominent in the occipital cortex than the frontal cortex in *Tcf4KO* (Supplementary Figure 1c, d). In parallel with *Tcf4Het* and *Tcf4KO* neocortical defects, the absence of TCF3 causes general increases of superficial layer neurons including SATB2, BRN2, and CUX1-positive neurons, most significantly observed in the occipital cortex at P0 (arrows in Fig. 3c, d; Supplementary Figure 2c, d).

Layer 5 neurons marked with CTIP2 are generally normal in *Tcf4Het*, *Tcf4KO*, and *Tcf3KO* mice, but the number of TBR1-positive layer 6 neurons is significantly reduced in TCF4 insufficiency mice including *Tcf4Het* and *Tcf4KO*, but not in *Tcf3KO*. A thinner TBR1-positive layer 6 is found throughout the neocortex of *Tcf4Het* with reduction in total cell number (Fig. 3e, f). Notably, there is about only half of TBR1-positive cells present in *Tcf4KO* compared to WT (Fig. 3e, f). However, we did not observe a gradient effect of the reduction in TBR1-positive cells along the anterior–posterior axis of the neocortex in *Tcf4KO* (Supplementary Figure 2a, b). The disorganized laminar structure of *Tcf4KO* mice is effectively demonstrated by triple staining of SATB2, CTIP2, and TBR1 as shown in Fig. 3e, f.

TCF4 haploinsufficiency impairs cortical neuron dendrite development and synapse formation

Beyond structural issues, we also investigated postnatal dendrite and synapse development of cortical neurons in *Tcf4Het*. We performed scattered in utero electroporation with GFP in *Tcf4Het* embryos and their WT littermate controls at E14, and examined dendritic morphology of the posterior neocortex at P15. In WT controls, GFP-positive neurons display long and ramified basal dendrites and apical dendrites. However, many GFP-positive neurons in *Tcf4Het* mice are not only misplaced in the deep layers but also exhibit abnormal dendrite morphology with short and sparse basal dendrites and long apical dendrites with scarce ramification (Fig. 4a-d). These results were further confirmed by MAP2 immunostaining of cultured neurons from *Tcf4KO*, *Tcf4Het*, and WT neocortex (Supplementary Figure 3). In addition, we analyzed synapse formation on primary cultured *Tcf4Het* cortical neurons with synaptophysin immunostaining and observed a general decrease in the density of synaptophysin-positive puncta on dendrites of *Tcf4Het* neurons compared to WT (Fig. 4e, f). These results indicate again that a sufficient expression level of TCF4 protein is necessary for dendrite development and synapse formation of neocortical neurons.

TCF4 down-stream gene regulatory network in developing neocortex

In order to identify molecular mechanisms responsible for abnormal corticogenesis in the absence of TCF4, we performed RNA-seq from dorsal telencephalon of *Tcf4KO*, *Tcf4Het*, and WT mice at P0 and deposited the data to GEO (GEO#: GSE79663; NCBI tracking system #17817994) (Fig. 5a). Differentially expressed genes (DEGs) were pairwise compared between each genotype (Fig. 5b, Supplementary Figure 4a-c). Cluster analysis of the three genotypes showed that *Tcf4Het* exhibits significant alterations in gene expression levels in the dorsal telencephalon similar to *Tcf4KO* (Fig. 5c-e). In the absence of TCF4 the majority of up-regulated genes are normally VZ/SVZ expressed genes involving

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in regulating neuronal proliferation, cell fate determination, and migration (Fig. 5d, f; Supplementary Table 1). Further, a large number of genes normally expressed in the CP or IZ of the developing dorsal telencephalon are down-regulated in response to a dosage level change of TCF4 (Fig. 5f). These results indicate that TCF4 normally acts as a repressor of proliferation genes and activator of neuronal differentiation genes in the developing neocortex. A repetition of the same analyses with additional samples of each knockout, heterozygote and WT genotypes validated these results (Supplementary Figure 5).

Down-regulation of neuronal differentiation genes may cause a delay in corticogenesis. To determine whether the developmental stage of the neocortex in TCF4 insufficiency mice lies along the normal developmental temporal axis, we combined transcriptome data from WT RNAseq data at different developmental stages available from the GEO database (GSE84803) with our transcriptome data from *Tcf4Het*, *TCf4KO* and their WT controls. As shown in the PCA analysis, the development of *Tcf4Het* and *Tcf4KO* neocortex shifts towards an immature state compared to WT littermate controls (Fig. 5g). Additional functional enrichment analysis of top 100 genes underlying the shift along the PC1 axis (Fig. 5g,h; for gene lists: see Supplementary tables 2 and 3) shows that up-regulated genes are functional enriched in RNA and DNA binding, cell cycle, and proliferation regulation, whereas down-regulated genes are enriched in schizophrenia disease- related genes, neuro-transmitter release, and sodium transporter activity.

Taken together, regulation of the *Tcf4* gene by TCF3 and possibly other transcription factors yet to be identified is critical for normal corticogenesis and, if disrupted, can result in mental deficits or disabilities. Utilizing the *Tcf4Het* and *KO* mouse models and a variety of biotechnology tools, we investigated and documented cortical structural abnormalities occurring during corticogenesis under conditions wherein normal TCF4 expression was altered in the presence or absence of regulation by TCF3.

Discussion

Although the T*cf4*KO mouse model was created half a decade ago [17, 22, 38], we show here that this model together with the *Tcf4Het* model are appropriate models for investigating whether impairment of cognitive and behavioral functions in PTHS and schizophrenia caused by mutations in the *Tcf4* gene are due to malformation of neocortex or other part of brain. By utilizing these models, we were able to show that TCF4 is expressed in dorsal telencephalon at an early embryonic stage in both human and mouse and plays a significant role in neurogenesis, migration, and neuronal maturation. Further, we did not detect TCF4 protein is expressed in other part of brain other than neocortex and hippocampus at P0 in mouse (Supplementary Figure 4f). Specifically, TCF4 is found at significant levels in the prefrontal cortex of human fetal brain; whereas in the mouse we found that TCF4 displays a rostral–caudal gradient distribution with TCF4 prominently expressed in the posterior cortex. These differences between human and mouse TCF4 cortical distribution may possibly be attributed to evolution, but otherwise remain unexplainable. Nevertheless, by utilizing *Tcf4KO* and *Tcf4Het*, we were able to show that pre-natal and post-natal development is sensitive to the presence and level of TCF4 expression.

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In conjunction with the aforementioned studies, we also obtained data showing that *Tcf4Het* failed to meet the normal ratio of upper and lower layer cortical neurons and that migration of postmitotic upper layer neurons to their proper position is disrupted (Figs. 2 and 3). In humans, misplaced neurons and disorganized laminar structure subsequently fail to form proper circuits during the critical developmental window required for normal cognitive and language development. In our postnatal study, we observed that dendritic arborization and synapse development were impaired in projection neurons of the *Tcf4Het* neocortex. This result may seem surprising considering TCF4 expression is down-regulated after birth (Fig. 1g). However, our transcriptome data for *Tcf4Het* and *Tcf4KO* showed that a large spectrum of genes that regulate neurite outgrowth and synapse formation are down-regulated in both *Tcf4Het* and *Tcf4KO* at P0 (Fig. 5d). In this regard, it is noteworthy that D'Rosario et al. found that TCF4 inhibits neurite outgrowth and synapse formation in drosophila and mouse postmitotic neurons by suppressing the *Neurexin* family genes (*nrxn1*, *nrxn2*, *nrxn3*) [39]. It is intrigue that we detect *Neurexin* family genes down-regulated in *Tcf4Het* and *Tcf4KO*. Future confirmation study will be required to clarify the disparity. It is not often the case that heterozygote mouse models display phenotypes enabling demonstration of a dominant role for TCF4 or any other factor in corticogenesis, a process that is conserved across species. It is noteworthy, also, in a genetic study of autism spectrum disorder (ASD) conducted by De Rubeis et al. [40], it was determined that the majority of ASD genes function mainly at mid-fetal term, the period during which both neuronal differentiation and maturation is taking place. Notably, many of the genes associated with ASD, such as Shank3, C4b, Syngap1, and Pten, overlapped with many of the genes affected in *Tcf4Het* and *Tcf4KO* and in schizophrenia [41-43], as well. These suggest that TCF4 may be an up-stream gene regulator of the molecular network governing cognitive function and that *Tcf4* could be one of the higher level transcription factors of a hierarchical molecular network regulating cortical development. Although *Tcf4* and *Tcf3* knockout mice show coordinated cortical abnormalities, the *Tcf4KO* mice display more adverse malformations demonstrating a larger spectrum of genes under the control of the *Tcf4* gene network in early and later stages of prenatal development. It is important to recall that TCF3 regulates neuronal genesis at an early embryonic stage. The observed restoration of *Tcf4* expression in the *Tcf3*KO mice at later embryonic stages by unknown mechanisms is a subject for further investigation

It is important to mention here that TCF4 is not exclusively expressed in neocortex as many studies have shown that TCF4 is also expressed in other parts of the brain, as well as in the immune system. However, our gene expression profile showed that the most abundant expression of TCF4 protein is in the neocortex, and data identifying TCF4 as a critical disease factor in other brain structures is relatively sparse compared to the neocortex.

In summary, our results support a fundamental role for TCF4 protein in the development of the cerebral cortex in humans and mice. In this report, we provide evidence in the mouse of abnormal neuronal proliferation, cell migration defects, subsequent laminar disorganization, as well as dendritic and synapse abnormalities leading to inappropriate synaptic connections during embryonic as well as early postnatal stages. By utilizing the heterozygote and knockout mouse models, we were able to expand our current knowledge of detailed anatomical and genetic mechanisms underlying the function or dysfunction of TCF4, thus

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mimicking cortical deficits recognized in PTHS and some forms of schizophrenia, bipolar disease, and autism.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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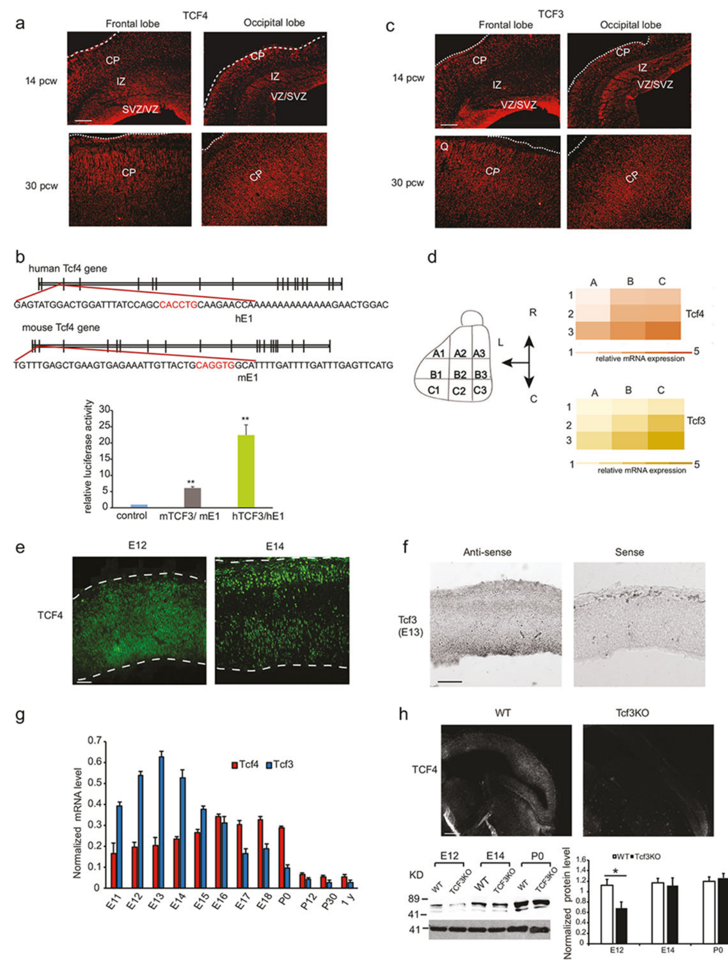

Fig. 1.

TCF3 is an up-stream regulator of TCF4. **a** Expression of TCF4 in human fetal telencephalon is shown in VZ/SVZ, IZ, and CP at 14 pcw and in IZ, CP at 30 pcw. Scale bar: 200 μm. Five replicates. **b** Human and mouse TCF3 binds to the second intron of the human and mouse *Tcf4* gene shown by the Luciferase Assay. The upper panel shows the binding sequences including the ‘E-box’ (‘CANNTG’, in red) and flanking sequences in the mouse (mE1) and human (hE1) *Tcf4* genes. The lower panel shows luciferase reporter activity. $n = 9$ for each construct. $^{**}P < 0.01$. **c** Similar expression pattern of TCF3 in human dorsal telencephalon revealed by immunostaining of adjacent sections in (**a**) at 14 pcw and 30 pcw. Scale bar: 200 μm, five replicates. **d** Quantitative DDPCR shows the spatial distribution of *Tcf4* (upper panel) and *Tcf3* (lower panel) mRNA across the surface of dorsal telencephalon from E13 WT mouse embryos. The surface of dorsal telencephalon was divided into 9 subdivisions with respect to the rostral (R)–caudal (C), medial–lateral (L) axis (indicated in the left and middle panels in (**c**). Data is presented as mean value (color coded) of relative expression level to GAPDH, $n = 9$ for both probs. **e** In the mouse, TCF4 protein is expressed throughout the cortical wall at E12 and in selected cell populations within VZ/SVZ, IZ, and CP at E14. Scale bar: 30 μm, $n = 6$. **f** In situ hybridization shows *Tcf3* mRNA in transverse sections of the mouse E13 telencephalon. Scale bar: 100 μm, $n =$

3. **g** Temporal dynamic expression of *Tcf4* and *Tcf3* mRNA in dorsal telencephalon across developmental ages examined by DDPCR. Data presented as mean value ± s.e.m. of relative expression level to GAPDH. $n = 18$. **h** Down-regulation of TCF4 expression in *Tcf3KO* demonstrated by immunostaining (upper panel, scale bar: 200 μm) and western-blot (lower panel). Data presented as mean value ± s.e.m. of relative expression level to actin. $^*P < 0.05$

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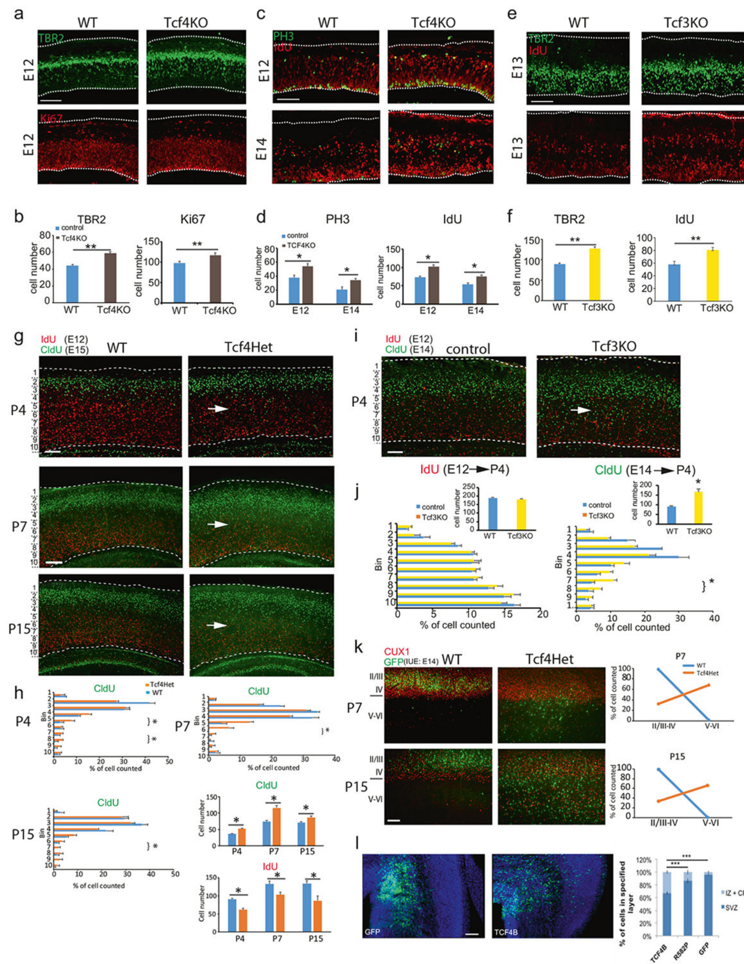

Fig. 2.

Increased proliferation and delayed migration of cells in the VZ/SVZ of neocortex in TCF4 and TCF3 insufficiency mice. **a** TBR2-positive intermediate progenitor (upper panel) and Ki67- positive proliferating cells (lower panel) are increased in the *Tcf4KO* telencephalon at E12. Scale bar: 100 μm. **b** Quantification of total TBR2 and Ki67-positive cells in *Tcf4KO* telencephalon. Data presented as mean ± s.e.m. $n = 6$. **c** PH3 and IdU immune-positive cells (3 h after intraperitoneal injection) are increased in *Tcf4KO* at E12 and E14. Scale bar: 100 μm. **d** Quantification of PH3 and IdU-positive cells in *Tcf4KO* at E12 and E14. $n = 6$. **e** Increased TBR2-positive cells are also found in *Tcf3KO*, as well as increased IdU-labeled cells in the VZ/SVZ of telencephalon of *Tcf3KO* at E13 embryos 3 h after intraperitoneal injection of pregnant mothers. Scale bar: 100 μm, $n = 3$. **f** Quantification of total TBR2 and IdU-positive cells in *Tcf3KO*. TBR2: $n = 9$, IdU: $n = 3$. **g** Superficial layer neuron migration defects observed with CldU (injected at E14) and IdU (injected at E12) double labeling in the neocortex of *Tcf4Het* at P4, P7, and P15. Note increased CldU-positive neurons in deep layers of *Tcf4Het* (white arrows). Scale bar: P4: 100 μm; P7, P15: 200 μm. **h** Quantification of laminar distribution of CldU-positive cells from *Tcf4Het* and littermate controls. Ten bins were divided evenly from the pial surface to the ventricular surface of each image from experiments in **g**. $n = 3$. Note total number of CldU-labeled cells is increased whereas IdU-

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positive cells are decreased in *Tcf4Het*. **i** and **j** CldU and IdU double-labeling experiments performed in *Tcf3KO* show superficial neurons delayed migration and increased number. Scale bar: 100 μm. **k** Migration defect of superficial neurons in *Tcf4Het* were observed by electroporating GFP in utero at E14. Scale bar: 100 μm. **l** TCF4 overexpression by in utero electroporation of TCF4B (full length) construct causes an increase in cells in the intermediate zone and cortical plate (IZ+CP) and corresponding reduction in cells in the SVZ, when compared to GFP or TCF4B-R582P (GFP $n = 6$, TCF4B $n = 17$, R582P $n = 14$). Scale bar: 100 μm

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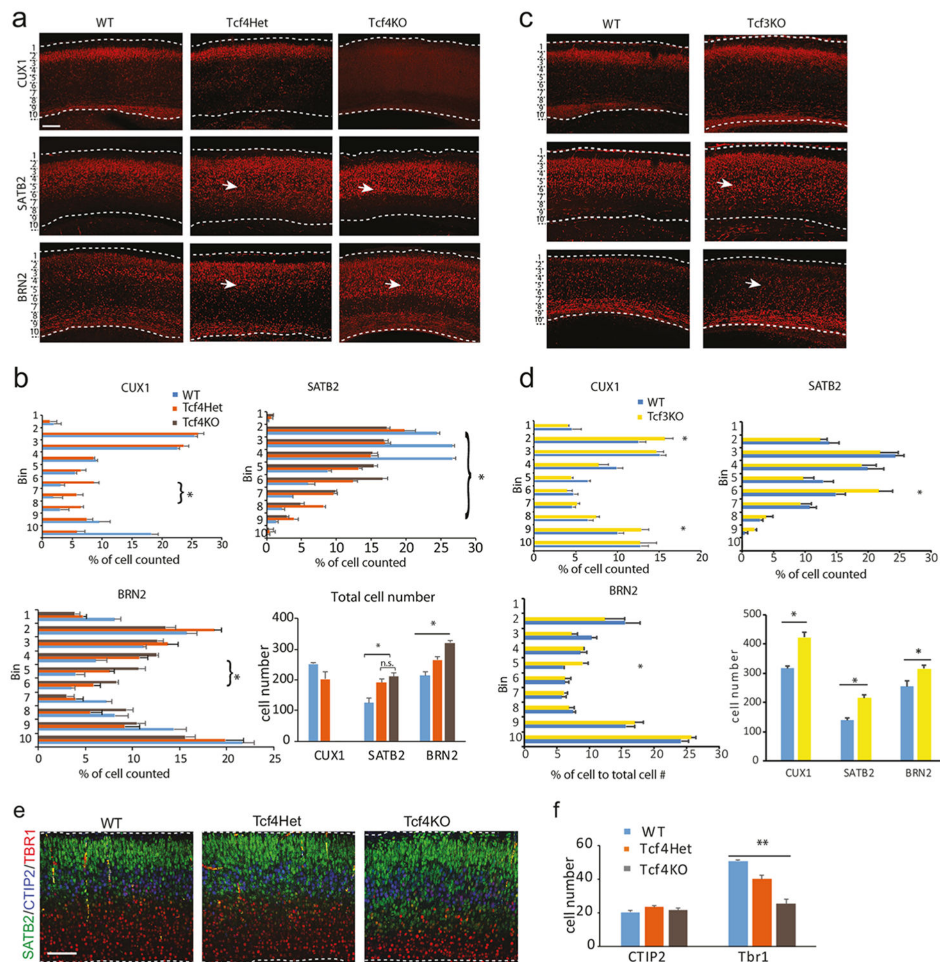

Fig. 3.

Cortical laminar disorganization in TCF4 and TCF3 insufficiency mice at P0. **a** Immunostaining of CUX1, SATB2, and BRN2 in posterior neocortex of *Tcf4Het*, *Tcf4KO*, and littermate WT controls. Note: dramatic reduction of CUX1 immunostaining signal in *Tcf4KO*, but not *Tcf4Het*; abnormal positions of SATB2 and BRN2-positive neurons (white arrows) in *Tcf4Het* and *Tcf4KO*. Scale bar: 100 μm. **b** Quantification of layer distribution and total numbers of the marked superficial neurons in *Tcf4Het* ($n = 7$) and *Tcf4*KO ($n = 10$). **c** Immunostaining of CUX1, SATB2, and BRN2 in neocortex of *Tcf3KO* and littermate controls at P0. Scale bar: 100 μm. **d** Quantification of layer distribution and total numbers of marked superficial neurons from *Tcf3KO*. $^{*}P < 0.05$. Defect in deep layer neurons only found in *Tcf4KO*. **e, f** Triple staining of SATB2, CTIP2, and TBR1 on *Tcf4Het*, KO and WT show there is no change in CTIP2-positive layer 5 neurons between genotypes, but decreased TBR1-positive layer 6 neurons in both *Tcf4Het* and KO. Scale bar: 100 μm. *Tcf4Het*, $n = 7$; *Tcf4KO*, $n = 4$, $^{**}P < 0.01$

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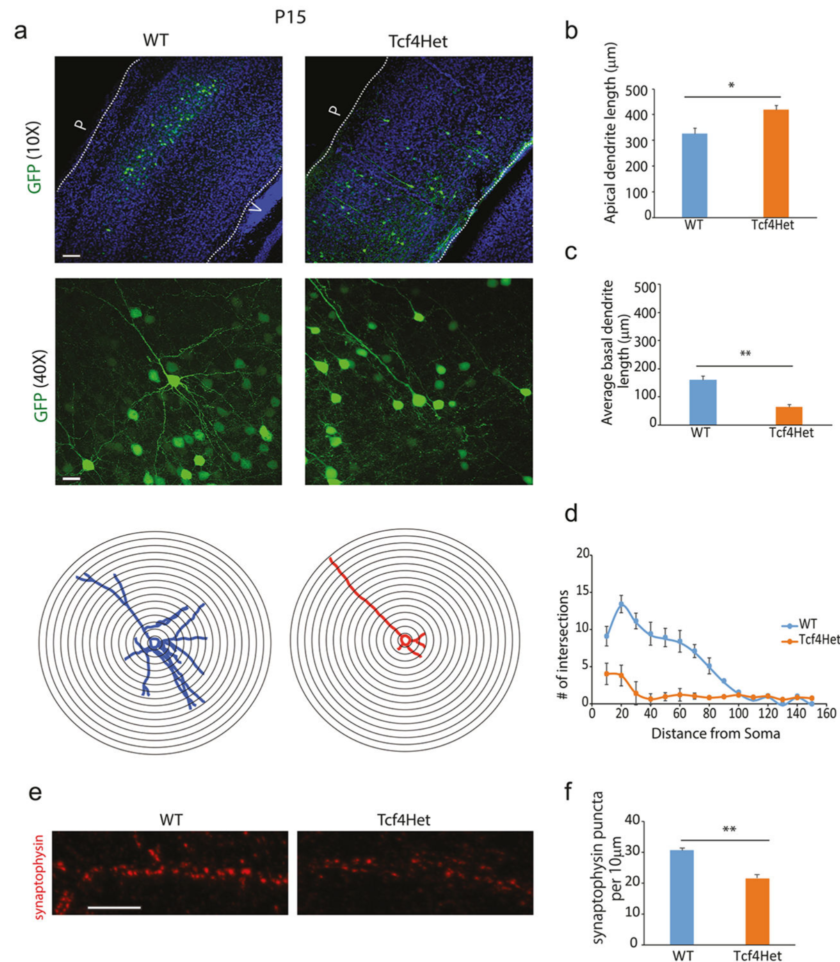

Fig. 4.

Impaired dendritic and synapse development in *Tcf4Het*. **a** GFP was electroplated in utero to *Tcf4Het* embryos and WT littermate controls at E14 and analyzed at P15. The upper panel are low magnification images of neocortex expressing GFP. P: pial surface; V: ventricular surface, scale bar = 100 μm. The middle panel are high magnification images showing dendritic morphology of GFP-positive single neurons, scale bar: 20 μm. The lower panel are samples of shell divisions in the sholl analysis of 3-D reconstructed single neuron dendritic arborization from WT and *Tcf4Het*. **b** Quantification of average apical dendritic length of cortical neurons from two genotypes. WT: $n = 15$; *Tcf4Het*: $n = 24$. **c** Average basal dendritic length quantification. WT: $n = 27$; *Tcf4Het*: $n = 25$. **d** Sholl analysis shows that dendritic complexity of cortical neurons in *Tcf4Het* is significantly reduced. WT: $n = 27$ (four animals); *Tcf4Het*: $n = 25$ (four animals). **e** Synaptophysin immunostaining of low density neuronal culture shows reduced synapse puncta in *Tcf4Het* neurons at DIV15, scale bar: 5 μm. **f** Synapse was quantified as number of puncta per 10 μm dendrite. $^{**}P < 0.001$

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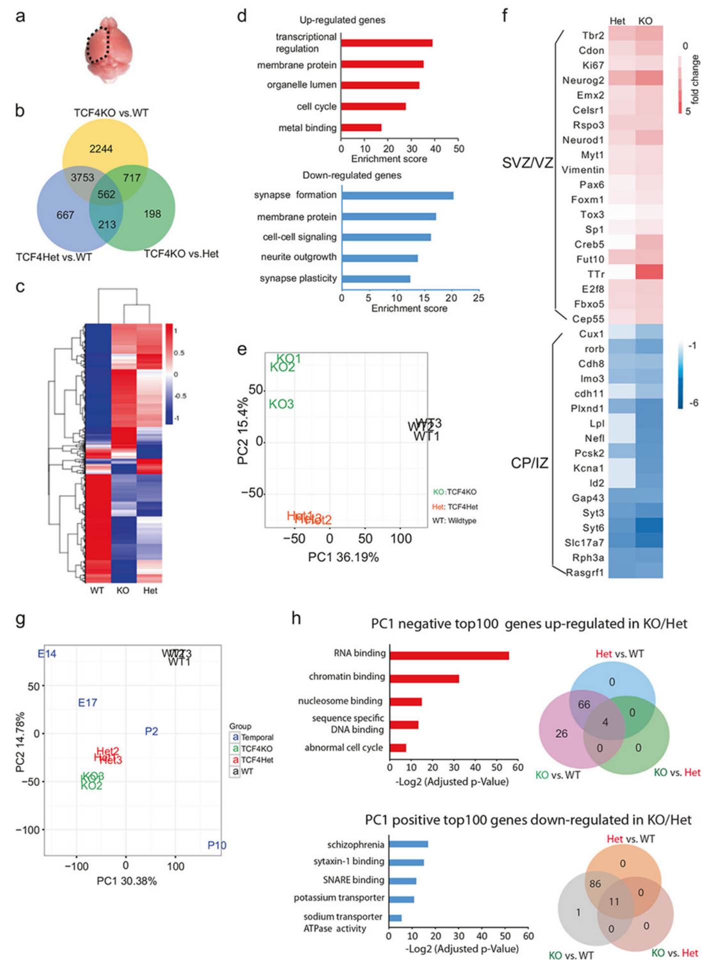

Fig. 5.

TCF4 insufficiency impairs cognitive gene regulatory networks. **a** Dorsal telencephalons from *Tcf4Het*, *TCF4KO* and WT are dissected out for RNA extraction. **b** Venn diagram shows DEGs distribution of *Tcf4KO* vs. WT, *Tcf4Het* vs. WT, and *Tcf4KO* vs. *Tcf4Het*. **c** Unsupervised hierarchical clustering analysis according to FPKM values from *Tcf4KO*, *Tcf4Het*, and WT. **d** Clusters of up-regulated and down-regulated genes that have the highest enrichment scores from DEGs of *Tcf4KO* vs. WT annotated by the DAVID Bioinformatics Resources Tool. **e** PCA plot of *Tcf4Het*, KO and WT control shows two component differences between genotypes. **f** Example of VZ/SVZ zone-specific genes that are up-regulated (pink) in *Tcf4KO* and *Tcf4Het* compared to their WT littermate controls and CP/IZ zone-specific expressed genes that are down-regulated (blue). **g** PCA plot combined *Tcf4Het*, *TCF4KO*, and littermate controls with WT transcriptome data at different developmental ages (batch effect corrected, see supplementary methods). **h** Functional analyzed genes underlying immature shift in *Tcf4Het* and *TcfKO* mice along PC1 axis in **g**