**Provisional** **Title: LiCl Treatment Rescues Spine Maturation and Synaptogenesis in Layer 5 and 6 Cortical Excitatory Neurons of *Tbr1* Mutants.**

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**SUMMARY:**

*Tbr1 is* a high confidence autism spectrum disorder (ASD) gene encoding a transcription factor with distinct pre- and postnatal functions. Postnatally, *Tbr1* conditional mutants (CKOs) and constitutive heterozygotes have immature dendritic spines and reduced synaptic density. *Tbr1* regulates expression of several genes that underlie synaptic defects, including a kinesin (*Kif1a*) and a WNT signaling ligand (*Wnt7b*). Furthermore, *Tbr1* mutant corticothalamic neurons have reduced thalamic axonal arborization. LiCl, a WNT-signaling agonist, robustly rescues the synaptic and axonal defects, suggesting that this could be a therapeutic approach for some cases of ASD.

**INTRODUCTION:**

Autism spectrum disorders (ASD) are defined by deficits in social interaction/communication and restricted or repetitive patterns of behavior. Considerable genetic and phenotypic heterogeneity has complicated efforts to understand the underlying biology of ASD. However, recent progress in the genetics of ASD has revealed more than 65 high confidence ASD (hcASD) risk genes (Sanders et al., 2015), providing key substrates for unraveling the biology of this condition. Systems analyses suggest that expression ASD risk genes have important functions in mid-fetal deep layer cortical excitatory neurons and that disruption may contribute to ASD pathophysiology (Willsey et al., 2013). Among these ASD genes, analysis of the *Tbr1* transcription factor (TF) is attractive as it opens the possibility of defining a transcriptional pathway that includes other ASD genes.

*Tbr1* has a central role in the development of mouse early-born excitatory cortical neurons. *Tbr1* expression, which begins in newborn neurons, dictates layer 6 identity (Bedogni et al., 2010; Bulfone et al., 1998; Hevner et al., 2003; Hevner et al., 2001; McKenna et al., 2011). Using *Tbr1layer6* conditional CKOs we recently demonstrated that neonatal *Tbr1* function is also required for dendrite morphogenesis and synaptogenesis (Fazel Darbandi et al., 2018).

Here we delved deeper into *Tbr1’s* function in synaptogenesis in several ways. First, we identified convergent synaptic phenotypes in *Tbr1layer5* and *Tbr1layer6* CKOs, and *Tbr1constitutive* (*Tbr1+/-*) mutants, including a defect in the formation of mature dendritic spines. Next, we used single-cell RNA-sequencing (scRNA-seq) of *Tbr1layer5* mutant medial prefrontal cortex (mPFC) neurons and identified *Tbr1*-regulated genes that impact synapse formation including a kinesin motor protein (*Kif1a*) and genes in the WNT-signaling pathway (*Gsk3β, Ctnnb1,* and *Wnt7b*). We also identified a number of *Tbr1*-regulated ASD genes (*Ank2, Ap2s1, Ctnnb1, Dpysl2, Map1a, Rorb*, *Smarcc2* and *Gsk3*). Finally, we observed that LiCl, an FDA approved drug that promotes WNT-signaling, rescues the spine and synapse defects in adult *Tbr1layer5*, *Tbr1layer6* and *Tbr1constitutive* (*Tbr1+/-*) mutants. Lastly, *Tbr1layer5* mutants exhibit decreased social interactions with young mice, a phenotype that is rescued with LiCl treatment. The LiCl results raise implications for the treatment of ASD patients with *TBR1* mutations, and potentially other individuals with ASD or related neurodevelopmental disorders.

**RESULTS:**

***Tbr1* regulates neurogenesis and synaptogenesis in layer 5 pyramidal neurons of neonatal medial prefrontal cortex.**

In the frontal and motor cortex *Tbr1* is expressed in most excitatory neurons in layers 5 and 6 (Fig. 2), whereas layer 5 expression in other cortical regions is limited to a minority of neurons (Bulfone et al., 1995). Here, using a floxed allele we selectively eliminated *Tbr1* in cortical layer 5 pyramidal neurons around P0 using *Rbp4-cre*, ~8 days after *Tbr1* expression begins. We refer to these mice as *Tbr1layer5* conditional knockouts (CKO).

We focused on *Tbr1* function in the developing prefrontal cortex (PFCx), a region that is implicated in ASD (Willsey et al., 2013). To overcome the limitations caused by cellular heterogeneity of batch RNA-seq (*Tbr1* is expressed in ~60% of layer 5 pyramidal neurons at P5 and ~85% at P21; Fig. S1A, S1B), we generated single cell (sc)RNA-seq data from Fluorescent Activated Cell Sorted (FACS) layer 5 neurons isolated from postnatal day 5 (P5) medial prefrontal cortex (mPFCx). We studied the transcriptomic changes from *Tbr1wildtype, Tbr1layer5* heterozygous and homozygous mutant cells using 10X Genomics platform.

We used a t-distributed stochastic neighbor embedding (t-SNE) dimensionality reduction followed by differential expression (DEX) analysis to identify neuronal cells. We captured 11,070 neuronal cells and 7,174 genes from *Tbr1wildtype* (n=1,778 cells)*, Tbr1layer5* heterozygous (n=5,357 cells) and *Tbr1layer5* homozygous (n=3,935 cells) mutant mPFCx that were used for downstream analysis.  T-SNE plot demonstrated separation between *Tbr1wildtype* and *Tbr1layer5* CKOs (Fig. 1A). Differential expression analysis identified 470 DEX genes when comparing *Tbr1layer5* homozygous mutant to *Tbr1wildtype* and 320 DEX genes when comparing *Tbr1layer5* heterozygousto *Tbr1wildtype*, 218 of which occur in the same direction in both comparisons (false discovery rate ≤ 0.05) (Fig. 1B; Table S1). Of the DEX genes which are not shared between genotypes, 76% of unique genes exhibit a common directionality and are excluded based on significance rather than opposing directionality. Gene Ontology analysis of common DEX genes identified many terms including axon, synapse, dendrite, cell body, and neurogenesis. (Fig. 1B). <ADD direction of effet statement with resepect to unique genes)

<revise to reflect lack of enrichment> To determine whether the changes in gene expression in *Tbr1layer5* CKOs are due to direct regulation by TBR1, we used data from TBR1 ChIP-Seq from P2 wildtype cortex (Fazel Darbandi et al., 2018). Of the top 71 DEX genes highlighted in Fig. 1C, TBR1 binds to 90% of their promoters and ~57% of distal regions (within 100 Kb) (Fig. 1C, S1C, Table S2), providing evidence that TBR1 directly controls synaptogenesis and neuronal differentiation in layer 5 pyramidal neurons by activating or repressing the target genes. Furthermore, our scRNA-seq analysis aided in discovering *Mgst3*, as a new marker of prefrontal cortex layer 5 (Fig. S1D).

We used *in situ* hybridization (ISH) to study the expression of several DEX genes. To provide a histological context, we defined laminar boundaries in the prefrontal cortex at P3 using the following probes: *Cux2* (layers 2-3)*, Rorb* (layer 4)*, Etv1* (layer 5)*, Tbr1* (layers 2-3, 5, 6 and 6b)*, Nr4a2* (subplate)to (Fig. 2i). Cortical layers 2-4 appear as a single layer at this stage (Fig. 2A-F). Expression of *Calm2, Kif1a, Mgst3,* and *Wnt7b* was altered as suggested by the scRNA-seq analysis(Fig. 2ii). Thus, neonatal *Tbr1* expression in layer 5 pyramidal neurons directly regulates the expression genes involved in cytoskeletal dynamics and synapse development.

**Excitatory and inhibitory synapses are reduced in *Tbr1layer5* mutants.**

We assessed excitatory synapse numbers on apical dendrites of layer 5 neurons (within layers 2-4) in the mPFCx by analyzing VGLUT1+ presynaptic terminals that are apposed to PSD95+ postsynaptic zones at P56 (Fig. 3i) and P21 using immunofluorescence (IF) and confocal microscopy (Fig. S2i). Inhibitory synaptic density was assessed by counting the overlapping VGAT+ presynaptic inhibitory terminals and Gephyrin+ dendritic postsynaptic zones on the apical dendrites of layer 5 pyramidal neurons (n=30) at P56 (Fig. 3ii) and P21 (Fig. S2ii). Excitatory and inhibitory synapses were decreased 34% and 42% in *Tbr1layer5* heterozygous, and 70% and 73% in *Tbr1layer5* homozygous mutants at P56, respectively (Fig. 3A, 3D). A similar synaptic deficit was also present at P21 (Fig. S2G, S2M).

To assess the physiological consequences of the decrease in excitatory and inhibitory synaptic density, we measured spontaneous Excitatory/Inhibitory Post-Synaptic Current (sEPSCs/sIPSCs) using whole-cell patch clamp on the tdTomato+ layer 5 pyramidal cells in the mPFCx of brain slices at P21 and P56 (Fig. S2A-C). The sEPSC frequency was reduced 25% in *Tbr1layer5* heterozygous and 75% in *Tbr1layer5* homozygous mutants; furthermore, the frequency of sIPSCs was reduced 30% in *Tbr1layer5* heterozygous and 50% in *Tbr1layer5* homozygous mutants as compared to cells from *Tbr1wildtype* mice at P56 (Fig. 3B, 3E). Similar decreases were also present at P21 (Fig. S2I, S2O). We did not observe changes in the amplitude of sEPSCs and sIPSCs at P21 and P56 (data not shown).

Since most *de novo* ASD-risk genes are heterozygous loss-of-function rare variants, we explored the consequence of constitutive *Tbr1* haploinsufficiency on synapse numbers of layer 5 and layer 6 pyramidal neurons, using *Tbr1+/-* mice (Bulfone et al., 1995). We counted excitatory and inhibitory synapse numbers in mPFCx of *Tbr1+/-::Rbp4-cre::tdTomatof/+* (layer 5 neurons) and somatosensory cortex (SSCx) of *Tbr1+/-::Ntsr1-cre::tdTomatof/+* (layer 6 neurons) at P56 (Fig. 3i-2, 3ii-2). Layer 5 excitatory and inhibitory synapse numbers were reduced ~40% and ~35% in mPFCx of *Tbr1+/-::Rbp4-cre::tdTomatof/+* at P56 (Fig. 3C, 3F). Layer 6 neurons in SSCx of *Tbr1+/-::Ntsr1-cre::tdTomatof/+* showed ~37% and ~39% decrease in excitatory and inhibitory synaptic density, respectively (Fig. 3C, 3F). Thus, *Tbr1* haploinsufficiency results in reduced synaptic density on the excitatory neurons of cortical layers 5 and 6.

***Tbr1layer5* CKOs have increased hyperpolarization-activated cation currents (Ih).**

We next examined the intrinsic properties of layer 5 neurons in *Tbr1layer5* wildtype and CKOs using whole-cell patch clamp to measure intrinsic physiological properties of *Rbp4-cre::tdTomato+* neurons of layer 5 in mPFCx (Fig. S6A). Resting membrane potential and input resistance were not different between *Tbr1wildtype*, *Tbr1layer5* heterozygotes andhomozygotes (n=8) at P56 (Fig. S6B, S6C).

A prominent feature of many layer 5 pyramidal neurons is the presence of a hyperpolarization-activated cation current (Ih or h-current) mediated by HCN channels (Shepherd, 2013). Ih causes a characteristic “sag” and “rebound” in current clamp recordings of responses to steps of hyperpolarizing current. We examined responses to a ‑200 pA step and found that mPFCx layer 5 pyramidal neurons from P56 *Tbr1layer5* heterozygotes and homozygotesexhibited a significantly increased “sag + rebound” compared to *Tbr1wildtype* controls, suggesting increased Ih, while other intrinsic electrophysiological properties were largely unaltered (Fig. S6D).

In deep layer neocortical pyramidal neurons, the presence of Ih shifts the resonant frequency towards higher frequencies (Dembrow et al., 2010). Therefore, to further characterize potential increases in Ih in*Tbr1layer5* CKOs, we estimated the resonant frequency by injecting constant current to hold *Rbp4-cre*+ neurons in current clamp near -70mV, then introduced a sinusoidal current stimulus with constant amplitude (100 pA peak-to-peak) and a frequency that increased linearly from 0 to 20 Hz over 20 seconds (Fig. S6E). *Tbr1layer5* heterozygous and homozygous CKOs exhibited an increase in their resonant frequency compared to *Tbr1wildtype* controls at P56 (Fig. S6G).

Lastly, we blocked Ih by bath applying the specific HCN channel antagonist ZD7288 (25 μM; Fig. S6F). The resonant frequency was reduced by over 50% in the *Tbr1layer5* heterozygous and *Tbr1layer5* homozygous CKOs (Fig. S6G). Thus, both *Tbr1layer5* heterozygotes and homozygoteshave an increased Ihin layer 5 pyramidal neurons of mPFCx.

**Evidence that WNT-signaling promotes synaptogenesis in *Tbr1* CKOs through an autocrine mechanism.**

Previously, we demonstrated that restoring *in vivo* *Wnt7b* expression in *Tbr1layer6* CKOs promoted synaptogenesis onto layer 6 neurons (Fazel Darbandi et al., 2018). We have since verified this finding and included additional controls (Fig. S3A, S3B).

Towards elucidating whether WNT7B functions through autocrine and/or paracrine mechanisms, we used cortical transplantation of *Wnt7b*-expressing cortical interneurons to study synaptogenesis in *Tbr1layer6* CKO and control (WT) mice. We introduced MGE-derived cortical interneurons harboring either a *Wnt7b* expression construct or a control vector into deep cortical layers of *Tbr1wildtype* and *Tbr1layer6* CKOs at P1; we analyzed excitatory synaptic density in the cortex at P30. We quantified excitatory synapses on apical dendrites of wildtypeand *Tbr1layer6* CKOs layer 6 neurons, adjacent to the *Wnt7b-*expressing interneurons within layer 5 (Fig. S3ii). We did not observe a rescue of synapse numbers (Fig. S3C). Furthermore, we did not observe an increase of excitatory synapses onto the soma of the transplanted *Wnt7b*-expressing interneurons (Fig. S3D). Thus, this experiment provides evidence that WNT7B promotes synaptogenesis in cortical excitatory neurons through a cell-autonomous autocrine mechanism.

***Tbr1* mutants have immature dendritic spines.**

The synaptic deficits described above prompted us to investigate the state of dendritic spines in *Tbr1layer5* CKOs, *Tbr1layer6* CKOs and *Tbr1+/-* mutants (Fazel Darbandi et al., 2018). We visualized tdTomato+ spines using airyscan confocal microscopy to capture 120X magnification Z-stack images (using 2X optical zoom) from the dendrites of layer 6 and layer 5 neurons of wildtype, *Tbr1layer5* (Fig. 4), *Tbr1layer6* and *Tbr1+/-* mutant neurons at P5, P21 and P60 (Fig. S4). We used Imaris software (v9.2.1) to analyze dendritic spine morphology, density and distribution.

There were reductions in the density of mature dendritic spines in *Tbr1* heterozygotes and homozygotes in *Tbr1layer5* and *Tbr1layer6* CKOs (Figs. 4 and S4). Additionally, *Tbr1+/-* mutants have reduced mature spine density on the dendrites of layer 5 and 6 pyramidal neurons (Fig. S4). Furthermore, *Tbr1*mutant neurons had an increased filamentous spine density (Fig. S4). Thus, this defect in spine maturation may underlie the reduction in synapse numbers in *Tbr1* mutants.

***Kif1a* expression restores normal synapse numbers in *Tbr1layer5* mutant neurons *in vitro*.**

We sought to identify molecular mechanisms underlying the decrease in the excitatory and inhibitory synaptic density in *Tbr1layer5* CKO neurons using the results from the scRNA-seq analysis (Fig. 1). We assessed a subset of DEX genes that control synapse biology, including *Kif1a* (Li et al., 2016)*, Mef2c* (Barbosa et al., 2008)*, Rac3,* and *Syt4* (Barber et al., 2009). We examined whether *Kif1a, Mef2c, Rac3* and *Syt4* could rescue synapse density by expressing them in P0 primary cortical cultures derived from *Tbr1wildtype*and *Tbr1layer5* mutant neurons (n=2).

After 14 days *in vitro*, we analyzed the number of excitatory (VGLUT+ presynaptic and PSD95+ postsynaptic) and inhibitory (VGAT+ presynaptic and Gephyrin+ postsynaptic) terminals of *Tbr1*wildtype and *Tbr1layer5* homozygous mutant neurons (Fig. 3iii). The reduced excitatory and inhibitory synaptic density onto *Tbr1layer5* CKO neurons was recapitulated *in vitro* (Fig. 3G, 3H). Only *Kif1a* rescued the reduction in both excitatory (Fig. 3G) and inhibitory (Fig. 3H) synapse numbers. The *Kif1a* kinesin is implicated in the transport of vesicles for synapse development (Guedes-Dias et al., 2019), and thus may contribute, with WNT signaling (see below), to *Tbr1’s* function in promoting synapse formation.

**Restoring reduced WNT signaling in *Tbr1* CKOs rescues synaptic deficits.**

We demonstrated that *Tbr1* promotes synaptogenesis onto layer 6 neurons in part via WNT signaling through *Wnt7b* (Fazel Darbandi et al., 2018). WNT signaling promotes dendrite maturation and synapse formation (Ciani and Salinas, 2005). Here, we found several lines of evidence to further support the role of *Tbr1*-dependent WNT signaling in synapse development. First, *Wnt7b* and *Ctnnb1* expression was reduced in the mPFC of *Tbr1layer5* CKOs (Figs. 1 and 2; Table S1). *Ctnnb1* encodes -catenin, the critical intracellular transducer of canonical WNT signaling (Budnik and Salinas, 2011). Secondly, *Tbr1layer5* CKOs had increased *Gsk3β* RNA expression (Fig. 1); GSK3 negatively regulates WNT signaling through increasing the destruction of -catenin (van Noort et al., 2002).

Thus, we tested whether promoting WNT signaling rescued dendritic spine and synapse phenotypes. Among its several pharmacological effects, there is evidence that LiCl, a WNT signaling agonist, promotes synapse development (Farooq et al., 2017; Lenox and Wang, 2003; Martin et al., 2018). Thus, we administered LiCl to *Tbr1+/-, Tbr1layer5* and *Tbr1layer6* CKOs.

**LiCl treatment of *Tbr1* mutants promotes dendritic spine maturation and synapse development.**

As noted above, *Tbr1* mutants have a reduced density of mature dendritic spines (Figs. 4 and S3). We tested whether LiCl treatment at P59 could rescue spine maturation and synaptogenesis in *Tbr1* mutants. We gave a single IP injection of 400 mg/kg LiCl; control animals received a single IP injection of 4 ml/kg saline. Impressively, LiCl led to the maturation of dendritic spines within 24 hours in *Tbr1* mutants; LiCl did not affect wildtype spine morphology (Figs. 4 and S4). These results, in combination with the previously reported evidence that *Wnt7b* restores synapse numbers on *Tbr1layer6* mutant neurons (Fazel Darbandi et al., 2018), led us to test whether LiCl can rescue synapse numbers on adult *Tbr1* mutant layer 5 and 6 neurons.

We administered LiCl to *Tbr1layer5* wildtype and homozygous CKO (Fig. 5A, 5E), *Tbr1layer6* wildtype and homozygous CKO (Fig. 5B, 5F), and *Tbr1+/-* mutants (Fig. 5D, 5H). Layer 5 and layer 6 projection neurons were labeled with *Rbp4-cre::tdTomatof/+* (Fig. 5C, 5G) and *Ntsr1-cre::tdTomatof/+* (Fig. 5D, 5H). The control and LiCl treated brains were harvested either 24 hours (Fig. S5), or 4 weeks after injection at P60 (Fig. 5A-H). Confocal images of IF from mPFCx (layer 5) and SSCx (layer 6) showed a nearly complete rescue of synaptic densities, 24 hrs and 4 weeks after treatment (Fig. 5 and S5). LiCl treatment also rescued synaptic densities in mPFCx (layer 5) and SSCx (layer 6) of the constitutive *Tbr1+/-* mutants (Fig. 5).

Thus, LiCl treatment of *Tbr1layer5*, *Tbr1layer6* and *Tbr1+/-* mutant mice at P60 rescues both excitatory and inhibitory synaptic deficit in *Tbr1* mutant neurons of cortical layers 5 and 6 (Fig. 5A-H). This provides *in vivo* evidence that augmenting WNT-signaling via LiCl treatment is sufficient to restore normal synapse numbers.

**LiCl treatment at P60 improves corticothalamic axonal arborization in *Tbr1layer6* mutant.**

Layer 6 corticothalamic neurons extend their axons to the thalamus where they form synapses. Corticothalamic arborization of *Tbr1layer6* CKOs is reduced in the anteromedial thalamus (white arrowheads; Fig. 5L) (Fazel Darbandi et al., 2018). LiCl injection at P60 robustly rescued this defect 24 hours and 4 weeks after injection (yellow arrowheads; Fig. 5M, 5N). Quantification of tdTomato pixel intensity in the anteromedial thalamus (region 1 showed a significant increase; Fig. 5iv).

Thus, LiCl treatment in *Tbr1layer6* CKOs provides evidence that the decrease in synapse numbers and thalamic arborization is secondary to reduced TBR1-dependent WNT signaling that may be treated by augmenting WNT signaling via LiCl treatment.

***Tbr1layer5* CKOs** **exhibit social interaction defects that are rescued by LiCl treatment.**

We studied motor function, anxiety, and social interaction of *Tbr1layer5*mutant mice between P56-80. Motor defects were not detected based on speed in an open field or performance on a rotarod (data not shown).

The improved synaptic density of *Tbr1layer5* CKOs due to LiCl treatment prompted us to assess the impact of LiCl treatment on the social interaction of *Tbr1layer5* CKOs. We performed the novel object exploration and social interaction assays at P60 using *Tbr1layer5* wildtype and CKOs that were treated with a single IP injection of saline (control) and LiCl (experimental) 4 weeks prior to the behavioral assays.

To assay social behavior, we measured the time the experimental mouse spent exploring a novel juvenile wildtype mouse of the same sex. Subsequently, we measured the amount of time the subject mouse spent exploring a novel object. LiCl treatment of *Tbr1layer5* homozygous CKOs improved their social interaction deficit with a juvenile mouse (Fig. 6A); while LiCl treatment did not affect a novel object assay (Fig. 6B). We did not observe a social deficit between *Tbr1layer5* wildtype and *Tbr1layer5* heterozygous CKO (data not shown). Thus, LiCl rescues defects in dendritic spines, synapse density and the social behavior of *Tbr1layer5* CKOs.

**DISCUSSION:**

***Tbr1* dosage in layers 5 and 6 is essential for dendritic spine maturation and synaptogenesis.**

*Tbr1* is expressed in post-mitotic excitatory neurons in the neocortex, hippocampus, entorhinal cortex, pallial amygdala, piriform cortex, olfactory bulb, Cajal-Retzius and subplate neurons (Hevner et al., 2003; Hevner et al., 2001). *Tbr1* is best known for its expression and function in layer 6, where it is required to initiate and then maintain layer 6 identity by repressing markers of layer 5 identity (Fazel Darbandi et al., 2018; McKenna et al., 2011). There is also prominent *Tbr1* expression in layer 5 of the rostral cortex, where it is expressed in ~85% of pyramidal neurons (Bulfone et al., 1995).

Here, by deleting *Tbr1* late in gestation using a layer 5-specific Cre (*Rbp4-Cre*), we have investigated the role of *Tbr1* in medial prefrontal cortex (mPFCx) development. scRNA-seq from FACS purified layer 5 neurons of *Tbr1wildtype* and *Tbr1layer5* heterozygous and homozygous CKOsdemonstrated that *Tbr1* deletion in mPFCx layer 5 alters the expression of a subset of genes that control synaptogenesis, synaptic maturation and microtubule assembly (Tables S1 and S2).

The core phenotype of the *Tbr1layer5* CKOs is a reduction in the density of mature dendritic spines and excitatory and inhibitory synapses. The dendritic spine defect is apparent at the beginning of synaptogenesis (P5) and is maintained through adolescence and into adulthood (Fig. 4). We hypothesize that the spine maturational defect is central to the reduction of excitatory synapses and synaptic activity observed in adolescent (P21) and adult (P56) mutants. *Tbr1layer6* CKOs and *Tbr1+/-* constitutive mutants show very similar defects in dendritic spines and synapses (Fazel Darbandi et al., 2018). The fact that we observed defects in dendritic spines and synapses in *Tbr1* heterozygous CKOs and constitutive mutants implies that this phenotype could underlie behavioral phenotypes in neuropsychiatric disorders such as ASD. This hypothesis is further strengthened by investigating the phenotypes associated with mPFCx, a cortical region with critical functions in cognitive and affective processing.

**Molecular mechanisms downstream of *Tbr1* that promote synapse development.**

We have evidence that TBR1 controls synaptic development by promoting spine maturation and synaptogenesis through several mechanisms, including promoting WNT-signaling (discussed more extensively below), and through directly driving the expression of *Cyp26b1*, *Foxp2, Mef2c, Wnt7b* in layer 6 (Fazel Darbandi et al., 2018), and *Kif1a*, *Mef2c and Wnt7b* in layer 5*.* We integrated these findings in a model (Fig. 7).

Restoring *Kif1a* rescued synapses in the *Tbr1layer5* CKOs *in vitro*. *Kif1a* encodes a kinesin motor protein, that controls vesicle delivery in the assembly and function of synapses (Guedes-Dias et al., 2019). Currently, its function in the maturation of dendritic spines is unknown.

Moreover, *Tbr1* promotes the expression of the *Foxp2* (a hcASD gene) and *Mef2c* TFs (Fazel Darbandi et al., 2018). *Mef2c* promotes the development of excitatory synapses (Harrington et al., 2016). However, restoring *Mef2c* expression in *Tbr1* mutant neurons failed to rescue their synaptic deficit; suggesting that decreased expression of this TF alone doesn’t underlie *Tbr1* mutants’ synaptic deficits.

*Tbr1* also promotes *Cyp26b1* expression, a gene encoding a retinoic acid-degrading enzyme, in layer 6 pyramidal neurons. Restoring *Cyp26b1* expression in primary cortical cultures from *Tbr1layer6*CKOs rescued synaptic deficit *in vitro* (Fig. S3E, S3F). Retinoic acid (RA) acts via RARα in synapses to promote protein synthesis (Chen et al., 2014; Chen and Napoli, 2007). This suggests that *Tbr1’s* control of RA levels, via *Cyp26b1*, can impact synaptic development (Fig. 7).

While these three mechanisms appear to contribute to *Tbr1’s* orchestration of synapse development, we believe that *Tbr1’s* control of WNT signaling may be the over-riding   
*Tbr1*-dependent mechanism (Fig. 7).

***Tbr1* promotion of WNT-signaling drives dendritic spine maturation and synaptogenesis onto layer 5 and layer 6 pyramidal neurons.**

WNT signaling is essential in postsynaptic differentiation of excitatory synapses by recruiting NMDA receptors via promoting PSD95 clustering and local activation of CaMKII within dendritic spines (Ciani et al., 2011). Furthermore, CaMKII is required for WNT-mediated spine growth and increased synaptic strength; thus, promoting postsynaptic maturation and differentiation (Ciani et al., 2011). Moreover, WNTs expression increases microtubule unbundling and stability by signaling through the canonical pathways downstream of GSK3β (Ciani et al., 2004). WNT inhibition of GSK3β results in phosphorylation of microtubule-associated proteins such as MAP1B. This interaction is essential for microtubule assembly, axonal arborization and outgrowth (Ciani et al., 2004).

Transcriptomic and ISH analyses demonstrate that *Tbr1* promotes expression of *Wnt7b*, *Ctnnb1* (-catenin) and represses expression of *Gsk3β*. *Wnt7b* encodes a WNT ligand of the canonical WNT signaling pathway (Rosso et al., 2004). *Ctnnb1* encodes -catenin, the central intracellular signaling protein of the canonical WNT signaling pathway (Ciani and Salinas, 2005). GSK3 is a ubiquitously expressed kinase that represses the canonical WNT pathway by targeting β-catenin for ubiquitin-mediated proteasomal degradation (van Noort et al., 2002). Restoring *Wnt7b* expression rescued the synaptic deficit in *Tbr1layer6* mutant neurons *in vitro* and *in vivo* (Fazel Darbandi et al., 2018). To test whether *Wnt7b* is acting through an autocrine or paracrine mechanism, we introduced cortical interneurons ectopically expressing *Wnt7b* into the deep layers of *Tbr1layer6* CKOs. We measured their effect on synapse density onto apical dendrites of wild type and *Tbr1layer6* CKOs layer 6 neurons (Fig. S3). Because we did not find a statistically significant increase in synapse density, we surmise that WNT7B primarily promotes synaptogenesis cell autonomously onto pyramidal neurons.

**LiCl rescues dendritic maturation and synaptogenesis in *Tbr1* mutants.**

To further explore the hypothesis that reduced WNT-signaling in *Tbr1* mutants underlies the reduction in synapses, we tested whether a canonical WNT-signaling pathway agonist, LiCl, could rescue dendritic spine and synapse defects. Among LiCl’s best-validated mechanisms of action is inhibition of GSK3β, a central kinase in the WNT/β-catenin and AKT pathways (Lenox and Wang, 2003).

LiCl rapidly (within 24 hrs) promoted the maturation of dendritic spines in *Tbr1layer5* CKO, *Tbr1layer6* CKO and *Tbr1+/-* mutant neurons in cortical layers 5 and 6. Furthermore, LiCl treatment rescued excitatory and inhibitory synapse numbers within 24 hours. Remarkably, a single dose of LiCl at P30 led to a sustained rescue of synaptic density, measured 4 weeks after treatment. These results suggest that the *Tbr1* mutant’s dendrites have most of the machinery needed to make synapses but have a deficit of the essential signal(s) to initiate synaptogenesis. Once the LiCl-induced synapses are formed, they appear to be relatively stable.

Corticothalamic axons in the *Tbr1layer6* mutants fail to fully arborize within anterior and anteromedial regions of thalamus (Fazel Darbandi et al., 2018). This phenotype was also rescued within 24 hrs of LiCl treatment, suggesting the reduced WNT-signaling underlies the defect of axonal elongation and/or arborization in *Tbr1layer6* mutants.

In sum, we postulate that *Tbr1* mutant layer 5 and 6 cortical neurons have reduced WNT signaling that underlies their defects in dendritic spines, synapses and axonal arborization. LiCl rescues each of these defects, perhaps through promoting WNT signaling.

**LiCl treatment rescues social interaction deficit in *Tbr1layer5* CKOs.**

We eliminated *Tbr1’s* function in cortical layer 5 pyramidal neurons. In most cortical areas, a minority of layer 5 neurons express TBR1, whereas in rostral areas, including the PFCx, TBR1 is expressed in ~85% of layer 5 excitatory neurons (Fig. S1). The PFCx has a central function in distributed circuits that control higher cognitive and emotional functions that are disrupted in neuropsychiatric disorders such as ASD. *Tbr1layer5* CKOs are viable and fertile, allowing us to study the impact of *Tbr1* deletion on the behavior of heterozygous and homozygous CKOs. The *Tbr1layer5* CKOs showed no deficit in their motor functions (rotarod and open field) and interest in novel objects. However, *Tbr1layer5* homozygous CKOs showed a reduction in social interaction with a juvenile mouse. This phenotype had previously been uncovered in *Tbr1* haploinsufficiency mice (Huang et al., 2014).

Importantly, treating *Tbr1layer5* CKOs with LiCl at P30, rescued the social deficit of *Tbr1layer5* CKOs (measure at P56-P80). Thus, perhaps the LiCl-mediated rescue of synaptogenesis may underlie the rescue of the social behavior phenotype.

**Insights into how *Tbr1* may contribute to ASD pathogenesis.**

Co-expression network analysis suggests that the *de novo* mutations of ASD-risk genes are enriched in excitatory projection neurons of cortical layers 5 and 6 in the PFCx during human mid-fetal development (Willsey et al., 2013), cell types that also express *Tbr1*. The functions of many ASD-risk genes converge on pathways that control synaptogenesis, synaptic development and plasticity (Sanders et al., 2015). Thus, in this study, we deleted *Tbr1* in excitatory neurons of mouse layer 5 mPFCx at a stage similar to human mid-fetal development.

Our single-cell transcriptomic analysis of FACS-purified layer 5 neurons from mPFCx revealed that *Tbr1* regulates other ASD genes including *Ank2, Ap2s1, Ctnnb1, Dpysl2, Map1a, Rorb,* *Smarcc2* [orthologs of high confidence ASD (hcASD) genes], and *Gsk3β* [ortholog of probable ASD (pASD) gene] in either *Tbr1layer5* heterozygous and homozygous CKOs. *Tbr1layer5* heterozygous and homozygous CKOs demonstrated a decrease in dendritic spines, excitatory and inhibitory synaptic density and reduced sEPSCs and sIPSCs, phenotypes that are convergent with *Tbr1layer6* CKOs, and constitutive *Tbr1+/-*. This suggests that decreased TBR1 dosage in human may also impair synaptic development and thereby increase the risk for ASD. While some of the other phenotypes detected in *Tbr1layer5* mutants were only present in the homozygotes, including defects in social interaction, these observations could have relevance for ASD as they denote biological processes that could be altered in *Tbr1* heterozygotes.

***Tbr1* and *Shank3* mutants convergently present synaptic and physiological defects.**

The complex genetic variation underlying ASD has complicated efforts to understand the mechanism associated with ASD pathology and therapies. A possible solution for such complex diversity is to identify core mechanisms, in which ASD-risk proteins may act convergently on a common pathway (State and Šestan, 2012). Many mutations are thought to predispose to idiopathic ASDs by causing primary impairments in synaptic transmission (Rosti et al., 2014; Sanders et al., 2015).

Reduced or increased *Shank* expression in *Drosophila* reduces WNT signaling and excitatory synapses (Harris et al., 2016). In mouse reduced *Shank3* impairs synaptic function by reduction in dendritic arborization, excitatory synaptic density, synaptic transmission and Ih current (Yi et al., 2016). Similarly, *Tbr1* CKOs have evidence for reduced WNT signaling, and have reduced mature spine density and excitatory synaptic density (herein and Fazel Darbandi et al., 2018). Likewise, *Tbr1* CKOs have abnormal Ih currents in cortical layer 5 (Fig. S6) and layer 6 (Fazel Darbandi et al., 2018) although in *Tbr1* CKOs Ih is increased. TBR1 binds to the *Shank1, 2,* and *3* loci (P2 TBR1 ChIP-Seq data under accession number GEO: GSE119362,

<https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE119362>), although there are only subtle changes in *Shank* RNA expression in the *Tbr1* mutants. Thus, synaptic dysfunction and perhaps reduced WNT signaling are common features of mouse *Tbr1* and *Shank3* mutants; these defects may be the core pathophysiology of some forms of ASD.

**LiCl as a therapy for neurodevelopmental disorders that have reduced synapse development.**

Currently, there are no treatments for ASD that address its core biological defects. The ability to restore synapse numbers following lithium administration in the *Tbr1* mutant mice provides an insight to a possible human therapy, especially given that LiCl has a long history of clinical use.

While our study only directly suggests the applicability of LiCl for ASD patients with *TBR1* mutations, LiCl could be considered for other ASD syndromes where reduced synaptic development is a central feature. In a clinical case report, LiCl was reported to reverse clinical regression, stabilize behavioral abnormalities and restore brain functioning in two *SHANK3* patients with ASD (Serret et al., 2015). Additionally, for instance, one should consider treating ASD cases caused by mutations in *Tbr1*-regulated ASD genes, including *Ank2, Ap2s1, Ctnnb1, Dpysl2, Map1a, Rorb,* *Smarcc2* and *Gsk3*. We also showed that *Tbr1layer6* CKOs had arborization defects of their corticothalamic axons that were improved with LiCl, suggesting that LiCl could also treat presynaptic defects. This is consistent with the evidence that WNT signaling positively regulated presynaptic and postsynaptic development (Ahmad-Annuar et al., 2006; Stamatakou and Salinas, 2014).

Perhaps most remarkable about the properties of LiCl, regarding its ability to promote dendritic spines maturation, synaptogenesis and axon arborization, were the rapidity of its action (24 hrs) and the persistence of action of a LiCl single dose over 4 weeks. However, there were many features of the *Tbr1* mutants that did not appear to be rescued by LiCl, including increased layer 5 and 6 filamentous spines density, and layer 6 dendritic morphogenesis. Thus, while LiCl may have some promise as a therapy, it is improbable that it would fully rescue normal brain function of ASD patients with *TBR1* mutations.

**Acknowledgments**

This work was supported by the research grants to J.L.R.R. from: Nina Ireland and NINDS R01 NS34661, R01 NS099099; to V.S.S. NIMH R01MH100292 and R01MH106507; and to A.J.W the Weill Institute for Neurosciences, the Department of Psychiatry, and the Institute for Neurodegenerative Diseases at UCSF.

**Author Contributions**

Conceptualization, S.F.D., V.S.S. and J.L.R.R..; Methodology, S.F.D., S.E.R.S., A.E., E.L.-L.P., M.T., A.J.W., B.N.R.C., V.S.S. and J.L.R.R.; Investigation, S.F.D., S.E.R.S., A.E., M.T., and A.J.W.; Writing – Original Draft, S.F.D., S.E.R.S. and J.L.R.R.; Writing – Review & Editing, S.F.D., V.S.S., A.E., A.J.W., M.W.S. and J.L.R.R.; Funding Acquisition, A.J.W., M.W.S., V.S.S. and J.L.R.R.; Supervision, A.J.W., M.W.S. and J.L.R.R.

**Declaration of Interests**

J.L.R.R. is cofounder, stockholder, and currently on the scientific board of *Neurona*, a company studying the potential therapeutic use of interneuron transplantation. A.J.W. is a paid consultant for Daiichi Sankyo. M.W.S. is a consultant to BlackThorn and ArRett Pharmaceuticals.

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**FIGURE LEGENDS:**

**Figure 1: *Tbr1* regulates genes that are implicated in controlling the development of axons, synapses and dendrites in layer 5 pyramidal neurons of mPFCx**.

**(A)** *t*-distributed stochastic neighbor embedding (*t*-SNE) plot displaying 11,070 single neuronal cells, from *Tbr1layer5*wildtype (WT; red), *Tbr1layer5* heterozygous CKO (HET; green) and *Tbr1layer5* homozygous CKO (NULL; blue). *t*-SNE was performed after quality control and removal of non-neuronal cell subtypes.   
**(C)** Heat map of DEX genes (FDR < 0.05) shared between both genotypes (x-axis, n=218) over a randomly selected 1000 cells from each genotype (y-axis, n=3000).  Genes are ordered by hierarchal clustering within direction of regulation grouping and z-score of normalized gene expression data is shown. The genotype for each cell is depicted at the top and genes with membership in selected enriched GO categories are highlighted to the right.

**Figure 2: *Tbr1* regulates expression in the medial prefrontal cortex (mPFC). *In situ* hybridization defines rostral cortical lamination and validates the changes in scRNA-seq expression levels.**

**(i)** Prefrontal cortical lamination was defined using *in* *situ* hybridization (ISH) on coronal sections of neonatal mPFCx wildtype mice at P3. ISH was performed using *Cux2* (layers 2-4; A-C), *Rorb* (layer 4; D-F), *Etv1* (layer 5; G-I), *Tbr1* (layers 2/3, 5 and 6; J-L) and *Nr4a2* (subplate or layer 6b; M-O). Cortical layers in the medial and dorsal regions are labeled. MO: medial orbital cortex, PrL: prelimbic cortex, FrA: frontal association cortex, OC: orbital cortex, Cg1: cingulate cortex area1, M: motor cortex, S1: primary somatosensory cortex, IC: insular cortex. II-IV = layers 2-4, V = layer 5, VI = layer 6. VIb = Subplate. Scale bar = 300 μm.

**(ii)** ISH confirms the changes in the transcriptome changes from differential expression (DEX) analysis of scRNA-seq in *Tbr1layer5* homozygous mutants. The expression of *Mgst3* (P, Q) and *Calm2* (R, S) are increased in layer 5upper (Q, S). *Tbr1layer5* mutants exhibit reduced expression of *Wnt7b* (T, U) and *Kif1a* (V, W) in layer 5 of mPFCx at P3. Color code: downregulated (red) and upregulated (green). Red box in panels P and Q indicates the region that was dissected for scRNA-seq analyses. Cortical layers 2-4, 5upper, 5lower, 6 and 6b (subplate) are labeled. Scale bar = 100 μm.

**Figure 3: *Tbr1* mutants have reduced excitatory and inhibitory synaptic density at P56.**

Immunofluorescence (IF) was used to detect excitatory (i) and inhibitory (ii) synapses onto dendrites of **(1)** mPFCx of *Tbr1wildtype* (*Rbp4-cre::tdTomatof/+*; red), *Tbr1layer5* heterozygous (*Tbr1f/+::Rbp4-cre::tdTomatof/+*; green), and *Tbr1layer5* homozygous (*Tbr1f/f::Rbp4-cre::tdTomatof/+*; blue) mutants (n=30) and **(2)** dendrites of layer 5 neurons from mPFCx of *Tbr1wildtype*, *Tbr1+/-* and layer 6 neurons from SSCx of *Tbr1wildtype*, *Tbr1+/-* (n=15).

**(i)** Excitatory synapses were identified by colocalization of VGLUT1+ boutons and PSD95+ clusters on dendrites of layer 5 pyramidal neurons at P56. (A) Quantification of excitatory synaptic density. (B) Quantification of the sEPSC frequency from layer 5 neurons at P56 (n = 6/6/6, wildtype/ heterozygous/ homozygous; One-way ANOVA, F(2,15) = 19.76, p < 0.0001; t-test, Tukey correction, wildtype v. homozygous: q(15) = 8.582, p < 0.0001). (C) Quantification of excitatory synaptic density of *Tbr1+/-* mutants in cortical layers 5 and 6 at P56.

**(ii)** Inhibitory synapses were identified by co-localizing VGAT+ boutons and Gephyrin+ clusters. (D) Quantification of inhibitory synaptic density on dendrites of layer 5 pyramidal neurons at P56. (E) Quantification of the sIPSC frequency from layer 5 neurons at P56 (n = 7/7/7, wildtype/ heterozygous/ homozygous; One-way ANOVA, F(2,18) = 6.694, p = 0.0067; t-test, Tukey correction, wildtype v. homozygous: q(18) = 5.15, p = 0.005). (F) Quantification of inhibitory synapse numbers on dendrites of layer 5 and 6 pyramidal neurons of *Tbr1+/-* mutants at P56.

**(iii)** *In vitro* rescue assay was conducted by transfecting *Kif1a*, *Mef2c*, *Rac3* and *Syt4* expression vectors into P0 primary cortical culture from *Tbr1*wildtype (red) and *Tbr1layer5* CKOs (blue) (n=2). (G, H) Quantification of excitatory and inhibitory synaptic density *in vitro*. Two-way ANOVA was used for the statistical analysis of the control, heterozygote and null. Two-tailed T-test with tukey correction was used for pairwise comparisons. Floating bar graphs represent the min-max distribution of synaptic density and/or E/IPSC frequency measured from each genotype. Horizontal line in each box denotes the average distribution. Average distribution is numerically indicated in each box. ns = not significant. (\*\*p< 0.01) (\*\*\*p<0.001) (\*\*\*\*p<0.0001). See also Figure S2 and S3.

**Figure 4: LiCl rescues immature dendritic spines of *Tbr1layer5* CKOs.**

Imaris software was used to analyze the dendritic spine density on apical dendrites of *Tbr1layer5* wildtype and *Tbr1layer5* CKOsneurons located within layers 2-4 of mPFCx. *Rbp4-cre::tdTomatof/+* allele was used to label the dendrites of layer 5 neurons.Changes in the dendritic spine density of layer 5 neurons were examined at P5 (A, B), P21 (C-E) and P60 (F-H). (K) Quantification of dendritic spine density at P5 and P21. Spine density was improved 24 hrs after LiCl treatment at P60 (I, J), compared to the saline-injected control animals (F-H). (J) Quantification of mature dendritic spines of *Tbr1layer5* wildtype and mutant neurons 24 hrs after injection with saline (control) or LiCl. Floating bar graphs (Red: wildtype; green: heterozygous and blue: homozygous) represent min to max distribution of dendritic spine density of layer 5 neurons within layers 2-4 of mPFCx. Horizontal line in each box denotes the average spine density. Average spine density is numerically indicated in each box. ns = not significant. (\*\*\*\*p<0.0001) Scale bar = 8 μm. See also Figure S4.

**Figure 5: LiCl treatment restores synapse numbers and corticothalamic axonal arborization of *Tbr1* mutant mice.**

Excitatory (i) and inhibitory (ii) synaptic density was quantified at P60 from: (1) apical dendrites of *Tbr1layer5CKO* and *Tbr1layer6CKO*, 4 weeks after P30 injection with saline or LiCl; and from: (2) dendrites of layer 5 neurons from mPFCx of *Tbr1wildtype*, *Tbr1+/-* and layer 6 neurons from SSCx of *Tbr1wildtype*, *Tbr1+/-*, 24 hours after injection with saline or LiCl at P59 (n=15).

**(i)** Excitatory synapses were quantified from (A) layer 5 neurons of mPFCx of *Tbr1wildtype* (green) and *Tbr1layer5CKO* (orange), (B) layer 6 neurons of SSCx of *Tbr1wildtype* (red) and *Tbr1layer6CKO* (blue) mice at P60, 4 weeks after saline and/or LiCl was administered. (C) Quantification of excitatory synaptic density of layer 5 neurons of mPFCx of *Tbr1wildtype* (green) and *Tbr1+/-* (orange) and (D) layer 6 neurons of SSCx of *Tbr1wildtype* (red) and *Tbr1+/-* (blue) mice at P60, 24 hours after injection with saline or LiCl.

**(ii)** Inhibitory synapses were quantified from (E) mPFCx of *Tbr1wildtype* and *Tbr1layer5CKO* and (F) SSCx of *Tbr1wildtype* and *Tbr1layer6CKO* mice, 4 weeks after saline and/or LiCl was administered at P30. (G) Inhibitory synapses were quantified from layer 5 neurons of mPFCx of *Tbr1wildtype* and *Tbr1+/-* and (H) layer 6 neurons of SSCx of *Tbr1wildtype* and *Tbr1+/-* mice at P60, 24 hours after injection with saline or LiCl. Floating bar graphs represent the min to max distribution of all excitatory and inhibitory synapse numbers measured from each genotype. Horizontal line in each box denotes the average distribution. Average distribution is numerically indicated in each box (n=15). Two-tailed T-test with Tukey correction was used for pairwise comparisons. ns = not significant. (\*\*\*p<0.001) (\*\*\*\*p<0.0001).

**(iii)** Corticothalamic axonal arborization in the thalamus is shown by tdTomato’s endogenous fluorescence (red) of *Tbr1wildtype* (I-K) and *Tbr1layer6* CKO (L-N). The overlap between DAPI (blue) and tdTomato (red) is shown from saline-injected (I, L), 24 hrs after LiCl injection (J, M) and 4 weeks after LiCl injection (K, N). White arrowheads in panel (L) shows thalamic regions that have reduced corticothalamic axonal arborization in *Tbr1layer6* CKO. Yellow arrowheads in panels (M) and (N) correspond to improved corticothalamic axonal arborization in *Tbr1layer6* CKO following P60 LiCl treatment after 24 hrs (M) and 4 weeks (N). Thalamus, Cortex (Cx) and Corticothalamic axons (CTA) are labeled. Scale bar = 5 mm.

**(iv)** Quantification of the tdTomato pixel intensity in thalamic region 1 (panel I and L) from saline-injected *Tbr1wildtype* (WT-Saline) and *Tbr1layer6* homozygous mutant (Null-Saline) at P60. tdTomato signal intensity is improved in the thalamus of *Tbr1layer6* homozygous CKO 24 hrs (Null-LiCl-24hrs) and 4 weeks (Null-LiCl-4wks) after LiCl injection compared to LiCl treatment of *Tbr1wildtype* 24 hrs (WT-LiCl-24hrs) and 4 weeks (WT-LiCl-4wks) after injection. Two-tailed T-test with Tukey correction was used for pairwise comparisons. Floating bar graphs represent the min to max distribution of tdTomato pixel density measured from region 1 of all genotypes and treatments. Horizontal line in each box denotes the average distribution. Average distribution is numerically indicated in each box. (\*\*\*p<0.001) (\*\*\*\*p<0.0001). See also Figure S5.

**Figure 6: LiCl treatment rescues social interaction deficit of *Tbr1layer5* mutants.**

Social interaction and novel object exploration were assessed between *Tbr1layer5* wildtype (red) and *Tbr1layer5* homozygous CKOs (blue) at P56-P80. (A) *Tbr1layer5* homozygous CKOs (blue) showed reduced social interaction with a juvenile mouse. LiCl treatment of *Tbr1layer5* mutant mice rescued social deficit phenotype compared to the saline-treated mutants. (B) LiCl treatment of *Tbr1layer5* CKOs did not affect the time spent engaged in novel object exploration compared to the saline-injected control. Floating bar graphs represent the min to max distribution of interaction measured from all genotypes and treatments. Horizontal line in each box denotes the average distribution. Average distribution is numerically indicated in each box. Two-tailed T-test with Tukey correction was used for pairwise comparisons (\*p< 0.05).

**Figure 7: Model of how *Tbr1* controls spine maturation and synaptogenesis through promoting WNT-signaling: links to ASD pathogenesis.**

Schematic representation of how *Tbr1* controls spine maturation and synaptogenesis in cortical layers 5 and 6. *Tbr1* regulates WNT-signaling by promoting *Wnt7b* and *Ctnnb1* and represses *Gsk3β* expression. LiCl (blue) rescues *Tbr1* phenotypes perhaps through stimulating WNT signaling by inhibiting GSK3β activity. WNT inhibition of GSK3β results in phosphorylation of MAP1A and MAP1B.that promotes microtubule assembly and axonal outgrowth. *Tbr1* activates *Kif1a*, a kinesin motor protein involved in synaptic vesicle trafficking. Furthermore, *Tbr1* activates *Foxp2* and *Mef2c* in layer 6 pyramidal neurons*.* *Mef2c* promotes the development of excitatory synapses. Lastly, TBR1 promotes expression of *Cyp26b1* in layer 6 pyramidal neurons, that controls RA levels, and impacts synaptic development. hcASD and pASD genes that are involved in these pathways are indicated in red (reduced in *Tbr1* mutants) and green (increased in *Tbr1* mutants), respectively. Cell membrane and nuclear membrane (blue) are indicated. Pathways unique to layer 5 and layer 6 are shown in orange and yellow, respectively. Convergent pathways between layers 5 and 6 are highlighted in blue.

**SUPPLEMENTARY FIGURE LEGENDS:**

**Figure S1: Related to Figure 1. TBR1 expression in mPFC of P5 and P21 Layer 5; TBR1 genomic binding; *Mgst3* – a new layer 5 marker.**

**(A)** Immunohistochemistry (IHC) was used to determine the overlap between TBR1 and *Rbp4-cre::tdTomatof/+* reporter in wildtype mPFCx at P5 and P21. Scale bar = 100 μm.

**(B)** Quantification of tdTomato+/TBR1+ cells in wildtype mPFCx at P5 and P21. Neonatally (P5) ~60% and postnatally (P21) ~85% of reporter+ cells (layer 5 excitatory neurons) are TBR1+. Error bars represent SEM of TBR1+/tdTomato+ cells in wildtype mPFCx at each age.

**(C)** TBR1 ChIP-Seq on wildtype whole cortex at P2 (red tracks). Red boxes represent the TBR1 binding that reached statistical significance. TBR1 directly regulates a subset of genes involved in synaptogenesis, including *Wnt7b*, *Ctnnb1*, *Gsk3β* and *Kif1a*. Genes are shown in blue. Black arrow indicates the direction of transcription. Genomic scale (in Kb) are shown for each locus. **(D)** *In situ* hybridization demonstrates the expression pattern of *Mgst3* in wildtype brain at P3. At this age, *Mgst3* is a layer 5 specific marker in the neocortex. Cortical layers 2-4, 5upper, 5lower, 6 and 6b (subplate) are labeled. Scale bar = 500 μm.

**Figure S2: Related to Figure 3. Loss of *Tbr1* in layer 5 reduces excitatory and inhibitory synaptic input onto the layer 5 pyramidal neurons in mPFCx at P21.**

(A) Schematic representation of medial prefrontal cortex (mPFCx, blue boxes). The blue box represents the region of mPFCx utilized for imaging and whole-cell patch clamp experiments. (B, C) Schema of layer 5 projection neuron (red) in mPFCx of *Tbr1wildtype* (B) and *Tbr1layer5* CKOs(C). The purple rectangles indicate the zone within layers 2/3 where synapse numbers were analyzed. Pipette tip indicates that the soma was patched during the electrophysiology recordings (B, C).

**(i)** Excitatory synapses were analyzed via synaptic bouton staining onto apical dendrites of layer 5 neurons (n=30) and spontaneous EPSC (sEPSC) recordings from the soma of (D) *Tbr1wildtype*, (E) *Tbr1layer5* heterozygous, and (F) *Tbr1layer5* homozygous CKOs at P21 (D-F). *Rbp4-cre::tdTomatof/+* allele was used to label the layer 5 neurons (red)*.* ImageJ software was used to process confocal images for quantification. (G) Quantification of excitatory synaptic density. Excitatory synapse numbers were reduced by 37% in *Tbr1layer5* heterozygous (BD = 0.680, p<0.0001) and 74% in *Tbr1layer5* homozygous CKOs at P21 (BD = 0.286, p<0.0001) (H) Sample traces of sEPSC recordings at -70mV in mPFCx slices from *Tbr1wildtype* (red), *Tbr1layer5* heterozygous (green), and *Tbr1layer5* homozygous CKOs (blue) at P21. (I) Quantification of the sEPSC frequency in layer 5 neurons at P21 (n = 6/6/6, wildtype/ heterozygous/ homozygous; One-way ANOVA, F(2,15) = 23.18, p < 0.0001; t-test, Tukey correction, wildtype v. homozygous: q(15) = 9.416, p < 0.0001; heterozygous v. homozygous: q(15) = 6.455, p = 0.001).

**(ii)** Inhibitory synapses were examined by synaptic bouton staining onto apical dendrites of layer 5 neurons (J – L) and spontaneous IPSC (sIPSC) recordings from the soma of the layer 5 neurons of (J) *Tbr1wildtype*, (K) *Tbr1layer5* heterozygous, and (L) *Tbr1layer5* homozygous CKOs at P21. *Rbp4-cre::tdTomatof/+* allele was used to label the layer 5 neurons (red)*.* ImageJ software was used to process confocal images for quantification. (M) Quantification of inhibitory synaptic density at P21. Inhibitory synapse numbers were reduced ~26% in *Tbr1layer5* heterozygous CKOs (BD = 0.816, p<0.0001) and ~71% decrease in *Tbr1layer6* homozygous mutants (BD = 0.319, p<0.0001). (N) Sample traces of sIPSC recordings in voltage clamp at +10mV in SSCx slices from *Tbr1wildtype* (red), *Tbr1layer5* heterozygous (green), and *Tbr1layer5* homozygous CKOs (blue) at P21. (O) Quantification of the sIPSC frequency in layer 5 neurons at P21 (n = 7/7/7, wildtype/ heterozygous/ homozygous; One-way ANOVA, F(2,18) = 5.159, p = 0.0169; t-test, Tukey correction, wildtype v. homozygous: q(18) = 4.534, p = 0.0129). Two-way ANOVA was used for the statistical analysis of the control, heterozygous and null. Two-tailed T-test with Tukey correction was used for pairwise comparisons. Floating bar graphs represent the min-max distribution of synaptic density and/or E/IPSC frequency measured from each genotype. Horizontal line in each box denotes the average distribution. Average distribution is numerically indicated in each box. BD = Bouton Density. (\*p< 0.05) (\*\*p< 0.01) (\*\*\*p<0.001) (\*\*\*\*p< 0.0001).

**Figure S3: Related to Figure 3. Restoring WNT-signaling rescues synaptic deficit through a cell-autonomous autocrine mechanism.**

**(i)** *In vivo* rescue assay was conducted by injecting a *Cre*-dependent lentivirus expressing *CAG-Flex-IRES-GFP* (empty vector) or *CAG-Flex-Wnt7b-IRES-GFP* lentiviral constructs into layer 6 of SSCx of *Tbr1layer6* wildtype, heterozygous and homozygous CKOs at P1. **(A, B)** Quantification of excitatory and inhibitory synapse numbers onto the layer 6 neurons of *Tbr1layer6* heterozygous (Het-WNT7B-GEP) and homozygous CKOs (Null-WNT7B-GFP) expressing GFP at P21 compared to wildtype (WT-WNT7B-GFP) and empty vector-inejcted animals including *Tbr1layer6* wildtype (WT; red), heterozygous (Het; green) and homozygous (Null; blue) CKOs. Overexpressing *Wnt7b* in wildtype layer 6 neurons (located by presence of GFP) did not have an impact on excitatory and inhibitory synaptic density **(A, B)**. However, the regions expressing GFP in layer 6 cells of *Tbr1layer6* CKOs, showed an increase in excitatory and inhibitory synapse numbers **(A, B)**.

**(ii)** Transplant-mediated introduction of cortical interneurons expressing *Wnt7b* to test whether *Wnt7b* promotes synaptogenesis through a paracrine mechanism. Immature cortical interneurons (MGE donor cells; *Nkx2.1-cre::tdTomatof/+)* were transfected with lentiviral constructs encoding *Gfp* [*DlxI12b-GFP* (control)]or encoding *Wnt7b* and *Gfp* (*DlxI12b-Wnt7b-GFP*). Transfected cells were transplanted in the P1 neocortex *Tbr1layer6* wildtype (WT; red) and homozygous CKO (Null; blue) and analyzed at P30. *Ntsr1-cre::tdTomatof/+* allele was used to label the layer 6 neurons (red)*.* ImageJ software was used to process confocal images for quantification. **(C)** Quantification of excitatory synaptic density onto layer 6 dendrites of *Tbr1layer6* wildtype (WT; red) and homozygous CKO (Null; blue) in SSCx at P30. **(D)** Quantification of excitatory synapses onto the soma of transplanted interneuron expressing empty vector control (*DlxI12b-GFP*) and/or *Wnt7b* and *Gfp* (*DlxI12b-Wnt7b-GFP*).

**(iii)** *In vitro* rescue assay was conducted using *Cyp26b1* expression vector in cultured P0 neocortex from *Tbr1wildtype* (red) and *Tbr1layer6* CKOs (blue) (n=2). Excitatory and Inhibitory synapses were measured at P14. Synapses are defined by co-localization of VGLUT1+ boutons and PSD95+ clusters (excitatory) and VGAT+ boutons and Gephyrin+ clusters (inhibitory) onto endogenous tdTomato. **(E, F)** Quantification of excitatory and inhibitory synaptic density *in vitro*. Restoring *Cyp26b1* expression *in vitro* rescues excitatory and inhibitory synaptic deficit in *Tbr1layer6* CKOs (red) compared to wildtype control (blue). Floating bar graphs represent the min to max distribution of synaptic density measured from all genotypes and treatments. Horizontal line in each box denotes the average distribution. Average distribution is numerically indicated in each box. Two-tailed T-test with Tukey correction was used for pairwise comparisons. ns = not significant. (\*\*p< 0.01) (\*\*\*p<0.001) (\*\*\*\*p<0.0001).

**Figure S4: Related to Figure 4. LiCl rescues immature dendritic spines of *Tbr1* mutant neurons.**

Imaris software was used to quantify dendritic spine density from **(i)** apical dendrites of *Tbr1layer6* CKOs neurons located within layer 5 of SSCx and **(ii)** dendrites of layer 5 neurons from mPFCx of *Tbr1wildtype*, *Tbr1+/-* and layer 6 neurons from SSCx of *Tbr1wildtype* and *Tbr1+/-*. Changes in the dendritic spines were examined at P5, P21 (A) and P60 (B-D). Spine density is improved at P60, 24 hrs after LiCl treatment (B-D), compared to the saline-injected control animals. **(iii)** Filamentous spine density is increased in **(E)** *Tbr1layer5* and **(F)** *Tbr1layer6* CKO. Layer 5 neurons of *Tbr1wildtype*, *Tbr1+/-* **(G)** and layer 6 neurons of *Tbr1wildtype* and *Tbr1+/-* **(H)** showed an increase in filamentous spine density at P60. LiCl treatment of Tbr1 mutants did not rescue the increase in filamentous spine density **(E-H)**. Floating bar graphs in red (wildtype), green (heterozygotes) and blue (homozygotes) represent the min to max distribution of mature and filamentous spines in *Tbr1* CKOs. Ruby (wildtype) and orange (*Tbr1+/-*) represent the distribution of mature and filamentous spines in layer 5 and 6 *Tbr1+/-* neurons. Horizontal line in each box denotes the average distribution. Average distribution is numerically indicated in each box. Two-tailed T-test with Tukey correction was used for pairwise comparisons. ns = not significant. (\*\*p< 0.01) (\*\*\*p<0.001) (\*\*\*\*p<0.0001). Scale bar = 8 μm.

**Figure S5: Related to Figure 5. LiCl treatment at P60 restores normal synapse numbers in *Tbr1* mutant mice.**

Excitatory (i) and inhibitory (ii) synaptic density was quantified at P60 from (1) apical dendrites of *Tbr1layer5CKO* and *Tbr1layer6CKO*, 24 hrs after injection with saline or LiCl at P59 (n=10). Excitatory and Inhibitory synapses were defined by co-localization of VGLUT1+ boutons and PSD95+ clusters (excitatory) and VGAT+ boutons and Gephyrin+ clusters (inhibitory) onto endogenous tdTomato labeling layer 5 and/or layer 6 pyramidal neurons.

**(i)** Excitatory synapses are quantified from (A) layer 5 neurons of mPFCx of *Tbr1wildtype* (green) and *Tbr1layer5CKO* (orange), (B) layer 6 neurons of SSCx of *Tbr1wildtype* (red) and *Tbr1layer6CKO* (blue) mice at P60, 24 hrs after saline and/or LiCl was administered.

**(ii)** Inhibitory synapses are quantified from (E) mPFCx of *Tbr1wildtype* and *Tbr1layer5CKO* and (F) SSCx of *Tbr1wildtype* and *Tbr1layer6CKO* mice, 24 hrs after saline and/or LiCl was administered at P59. Floating bar graphs represent the min to max distribution of all excitatory and inhibitory synapse numbers measured from each genotype and treatment. Horizontal line in each box denotes the average distribution. Average distribution is numerically indicated in each box (n=10). Two-tailed T-test with Tukey correction was used for pairwise comparisons. ns = not significant. (\*\*\*p<0.001) (\*\*\*\*p<0.0001).

**Figure S6: Loss of *Tbr1* in layer 5 prefrontal cortex results in an increase in** **hyperpolarization-activated cation currents (Ih).**

Whole-cell patch clamp recordings from layer 5 mPFCx at P56 **(A-D)** show that many intrinsic electrophysiological properties were unaffected by loss of *Tbr1*, including resting membrane potential **(B)**, input resistance **(C)**, and action potential half-width (data not shown). **(D)** ‘‘Sag and rebound’’ is increased in *Tbr1layer5* mutant neurons (n = 7/6/7, wildtype/ heterozygous/ homozygous; One-way ANOVA, F(2,17) = 13.18, p = 0.0003; t-test, Tukey correction, wildtype v. heterozygous: q(17) = 3.693, p = 0.0457; wildtype v. homozygous: q(17) = 7.258, p = 0.0002). **(E)** Neurons were held in current clamp at -70mV. The resonant frequency was measured as the frequency at which the impedance profile reached its peak (arrows). Ratio of the fast Fourier transform of the voltage response (Fig. S6E top) to the fast Fourier transform of the sinusoidal current stimulus (Fig. S6E middle) to calculate the impedance amplitude profile (Fig. S6E bottom). We defined the resonant frequency as the frequency at which the impedance profile reached its peak. Scale bar = 5 mV, 5 s. **(F)** ZD7288, an HCN channel blocker, decreased resonance frequency by over 50% in *Tbr1layer5* heterozygous (green), and *Tbr1layer5* homozygous mutants (blue). **(G)** Quantification of changes in resonant frequency of *Tbr1wildtype* (red), *Tbr1layer5* heterozygous (green) and *Tbr1layer5* homozygous mutants (blue) after ZD7288 treatment (n = 7/8/8, wildtype/ heterozygous/ homozygous; One-way ANOVA, F(2,20) = 16.24, p < 0.0001; t-test, Tukey correction, wildtype v. heterozygous: q(20) = 7.075, p = 0.0002; wildtype v. homozygous: q(20) = 7.038, p = 0.0002). (\*\*p< 0.01) (\*\*\*p<0.001).

**STAR METHODS:**

**CONTACT FOR REAGENT AND RESOURCE SHARING:**

Further information and requests for resources and reagents should be directed to and will be

fulfilled by the Lead Contact, Dr. John L. Rubenstein ([john.rubenstein@ucsf.edu](mailto:john.rubenstein@ucsf.edu)).

**EXPERIMENTAL MODEL AND SUBJECT DETAILS:**

**Animals:**

All procedures and animal care were approved and performed in accordance with the University of California San Francisco Laboratory Animal Research Center (LARC) guidelines. All strains were maintained on a C57Bl/6 background. Animals were housed in a vivarium with a 12hr light, 12hr dark cycle. Postnatally, experimental animals were kept with their littermates.For timed pregnancies, noon on the day of the vaginal plug was counted as embryonic day 0.5.

The *Tbr1flox*allele was generated by inGenious Targeting Laboratory (Ronkonkoma, NY). LoxP sites were inserted into introns 1 and 3, flanking *Tbr1* exons 2 and 3 (Fazel Darbandi et al., 2018). To enable selection of homologous recombinants, the LoxP site in intron 3 was embedded in a *neo* cassette that was flanked by *Flp* sites. The *neo* cassette was removed by mating to a *Flp*-expressing mouse to generate the *Tbr1flox* allele.Cre excision removes exons 2 and 3, including the T-box DNA binding region, similar to the constitutive null allele (Bulfone et al., 1998).*Rbp4-cre* mice (Gensat KL100) were used to delete *Tbr1* in layer 5 projection neurons. *tdTomatofl/+* (*Ai14*) mice were crossed with *Tbr1f/f*mice and used as an endogenous reporter. *Tbr1* layer 5 knockout mice (*Tbr1layer5* mutant) were generated by crossing *Tbr1f/f::tdTomatof/+* mice with *Tbr1f/+::Rpb4-cre+*. The specific gender and age of experimental animals can be found in the Results section and corresponding figure legends.

**TRANSGENIC ANIMAL MODELS:**

The mouse strains used for this research project, B6.FVB(Cg)-Tg(Ntsr1-cre)GN220Gsat/Mmucd, RRID:MMRRC\_030648-UCD and B6.FVB(Cg)-Tg(Rbp4-cre)KL100Gsat/Mmucd, RRID:MMRRC\_037128-UCD, were obtained from the Mutant Mouse Resource and Research Center (MMRRC) at University of California at Davis, an NIH-funded strain repository, and was donated to the MMRRC by MMRRC at UCD, University of California, Davis. Made from the original strain (MMRRC:032081) donated by Nathaniel Heintz, Ph.D., The Rockefeller University, GENSAT <https://protect2.fireeye.com/url?k=c19cddd3-9ddce8ed-c19cface-0cc47ad9c120-2678b1e782f452c7&u=http://www.gensat.org/> and Charles Gerfen, Ph.D., National Institutes of Health, National Institute of Mental Health.

Information about the generation and genotyping of the transgenic lines used in this study can be found in the corresponding original studies: *Rbp4-Cre* (Gong et al., 2007), lox-STOP-lox-tdTomato (Ai14;(Madisen et al., 2010)). Mice were maintained on C57BL/6J background.

**METHOD DETAILS:**

**Genomic DNA extraction and genotyping:**

Tissue samples were digested in a solution containing 1 mg/mL of proteinase K, 50 mM Tris-HCl pH 8.0, 100 mM EDTA, 100 mM NaCl and 1% SDS. Genomic DNA was extracted using a standard ethanol precipitation protocol. Genotyping was performed with PCR-based assays using purified genomic DNA, and primer-pair combinations flanking the deleted region and detecting *Cre* and *tdTomato* alleles.

**RNA extraction and cDNA synthesis:**

Total RNA was extracted from the cortices of wildtype mice at P0 using RNeasy Plus® Mini Kit (QIAGEN) following the manufacturer’s protocol. First strand cDNA was synthesized using Superscript reverse transcriptase II following manufacturer’s protocol (Thermofisher). cDNA library was used as template to clone and generate *in situ* probes.

**Single-Cell RNA-sequencing (scRNA-seq) on FAC-Sorted Cells:**

Layer specific transcriptome profiling was conducted by using 10X Chromium scRNA-seq on FAC-Sorted cells from medial prefrontal cortex of *Tbr1wildtype* *and Tbr1layer5* heterozygous and homozygous mutants at P5. The medial prefrontal cortex was dissected in HBSS from P5 mice. Cortices were dissociated using a Papain Dissociation System (Worthington Biochemical Corporation) following manufacturer’s protocol. tdTomato+ cells were sorted using BD FACS Aria II Cell Sorter at Center for Advanced Technology (UCSF). Approximately 20,000 *tdTomato+* cells were collected from each sample. Following FAC-sorting, the cell suspensions were centrifuged at 300 ×g for 5 min. Cells were washed for a total of 3 times with 1 mL 1X PBS supplemented with 0.04% BSA. Following the final wash, the cell pellet was resuspended with 25 μL of 1X PBS supplemented with 0.04% BSA. Cell concentration for each sample was determined using trypan blue and a hemocytometer. We targeted to capture approximately 5000 cells per each genotype to generate scRNA-seq libraries. Single cell RNA-seq was performed using 10X Chromium Single Cell 3’ Reagent Kit v2 following manufacturer’s protocol. Library concentration was assessed with Qubit dsDNA HS Assay Kit following manufacturer’s protocol (Thermofisher). Library fragment size distribution was examined on the Agilent Bioanalyzer 2100 (Agilent Technologies) and Agilent High Sensitivity DNA Kit (Agilent Technologies) following manufacturer’s protocol. Libraries were sequenced on Hiseq4000 at Center for Advanced Technology (UCSF).

**Bioinformatics analysis of FAC-Sorted Layer 5 scRNA-seq data:**

***Read pre-processing*:** Single cell RNA-sequencing libraries were sequenced on Illumina Hiseq4000 to an average depth of 45K reads per cell. Read quality control, UMI counting, barcode counting, and alignment to the mouse reference genome (mm10) were performed using the “cellranger 2.0.1” pipeline provided by the manufacturer.

***Filtering and Normalization*:** The initial dataset contained 17,823 cells with an average of 892 genes per cell. Cells with greater than 30% of mitochondrial genes were removed as this is indicative of poor-quality cells (n=82). Cells with fewer than 500 or more than 10,000 unique-molecular-identifier (UMI) counts were removed as this often represents sequencing errors (n=163). Cells with fewer than 500 or more than 3,000 genes were removed based on the distribution (n=182). Genes which occurred in less than 0.01% of cells were also removed (n=13065). The remaining 17,396 cells and 14,933 genes were used for downstream analysis.  No experimental factors were determined to explain a disproportionate of expression variance using the Single Cell Analysis Toolkit for gene Expression in R (scater; v 1.9.15).

Using the R package Seurat (v 2.3.4), the data was log normalized for each cell by the total expression and scaled to 10,000 transcripts per cell. Variable genes were identified using the *FindVariableGenes()* function which calculates the average expression and dispersion for each gene, then bins genes and calculates a z-score for dispersion within each bin. The data was scaled, centered, and regressed on the percent of mitochondrial gene content, number of UMI counts, and the number of genes.

***Cell-type Identification and Clustering*:** TSNE was generated using all principal components accounting for more than 2% of the variance and a clustering resolution of 0.3 which resulted in 12 clusters (average silhouette width 0.16). Three clusters were identified as neuronal cells using known markers *Nrgn, Rorb*, and *Cnih2.*

The raw data from the three identified neuronal clusters was retained and filtered again based on the distribution of UMI counts and the number of genes per cell (N= 11,943). We applied more stringent filtering to genes by removing mitochondrial genes, ribosomal genes, pseudogenes, genes that did not occur in 1% of neuronal cells, and genes with a variation below the median variation across all genes (N = 7,174). The data normalized as described above and TSNE was generated using all principal components accounting for more than 4% of the variance and a clustering resolution of 0.3 which resulted in 6 clusters (average silhouette width 0.15). Two clusters were identified as atypical cells due to a reduced expression in excitatory neuronal markers and subsequently removed from downstream DEX analysis.

***Differential Gene Expression (DEX) Analysis and Gene Ontology Enrichment*:**To identify gene signatures of each genotype, we used MAST (Finak et al., 2015) and the zero-inflated regression (zlm) method to compare raw UMI counts (i.e. non-normalized counts) per gene across the cells in the population (FDR < 0.05). Gene Ontology enrichment analysis of common differentially expressed genes was performed using the R package goseq (v 1.34.1) using all expressed genes (N = 7,174) as background.

**Primary Cell Culture and *in vitro* Rescue Assay:**

***Primary Cell Culture:*** Cortex was dissected from P0 *Tbr1wildtype* and *Tbr1layer5* homozygous mutants and dissociated using papain dissociation kit following manufacturer’s protocol (Worthington). A total of 300,000 cells were seeded into tissue culture slides pre-coated with poly-L-lysine (10 mg/ml, Sigma) and then laminin (5 mg/ml, Sigma), and grown *in vitro* with media containing DMEM-H21 with 5% fetal bovine serum for 3 hrs. After the cells recovered, DMEM-H21 media was replaced by Neurobasal medium containing B27 supplement, 25% glucose, and glutamax overnight.

***In vitro Rescue Assay:*** *Tbr1layer5* mutant cells were transfected with *Syt4, Mef2c, Kif1a, Rac3* expression vectors and *Tbr1wildtype* were transfected with mock empty vector using Lipofectamine 3000 (Invitrogen) for 6 hrs. Following incubation, the media was replaced by Neurobasal medium containing B27 supplement, Penicillin/Streptomycin, 25% glucose, and glutamax. Cultures were grown for 14 days *in vitro*. After 14 days, cultures were washed 3 times with 0.5 mL 1X PBS for 5 min each and fixed for 15 min with 4% PFA in 1X PBS at RT. Fixed cells were washed 3 times with 0.5 mL 1X PBS and blocked in 1X PBS containing 10% Normal Serum, 0.1% Triton X- 100 and 2% BSA for 1 hr at RT. Primary antibodies including mouse anti-Vglut1 (1:200, Synaptic Systems) and rabbit anti-PSD95 (1:200, Cell Signaling; excitatory synapses), rabbit anti-Vgat (1:500, Synaptic Systems) and mouse anti-gephyrin (1:200, Synaptic Systems; inhibitory synapses) were diluted 1:200 in blocking solution. Cells were stained for excitatory and inhibitory synapses with primary antibodies for 48 hrs at 4°Cwith gentle shaking. On a shaker, the cells were washed 3 times with 0.5 mL 1X PBS for 5 min each and incubated with the secondary antibody for 2 hrs (room temperature), washed 3X with 1X PBS, and mounted. This experiment was repeated twice (n=2).

***In vivo* Synapse Rescue Assays:**

We performed *in vivo* rescue assay of synaptic deficit in *Tbr1* mutant mice using three different approaches. First, we directly injected a lentivirus harboring WNT7B. Secondly, we utilized a transplantation assay to deliver the protein of interest (WNT7B) by introducing MGE progenitor cells, following previously published MGE transplantation assay (Vogt et al., 2015). Lastly, we used a single intraperitoneal injection of LiCl to rescue the decrease in synapse numbers in *Tbr1* mutants.

***Direct lentiviral injection*:***In vivo* rescue assay was carried out by cloning *Wnt7b* into a *Cre*-dependent lentiviral backbone (*pLenti-CAG-Flex-IRES-GFP*). *CAG-Flex-GFP* (empty vector) and *Wnt7b-IRES-GFP* expressing lentivirus (*pLenti-CAG-Flex-Wnt7b-IRES-GFP*) were generated in HEK293T cells as previously reported (Vogt et al., 2015) using Polyplus jetPRIME® transfection reagent following manufacturer’s protocol.

Lentivirus containing *CAG-Flex-GFP* or *Wnt7b-IRES-GFP* were injected in the SSCx of *Tbr1layer6* wildtype as well as *Tbr1layer6* heterozygous and homozygous CKO pups at P1. For injections, a glass micropipette of 50 μm diameter (with a beveled tip) was preloaded with sterile mineral oil and viral suspension was front-loaded into the tip of the needle using a plunger connected to a hydraulic drive (Narishige) that was mounted to a stereotaxic frame. P1 pups from *Tbr1layer6* wildtype and *Tbr1layer6* heterozygous and homozygous CKOs were anesthetized on ice for 1–2 min before injections. Each pup received 2–3 viral injections (150 nl per site) in the right hemisphere. These sites were about 1 mm apart along the rostral to caudal axis. Viral suspensions were injected into layer 6 of the neonatal SSCx. After injections, pups were put back with the mother to recover after they began to move around on their own. Mice were sacrificed 21 days after injection and transcardially perfused with PBS followed by 4% PFA.

***MGE-Derived Interneuron Transplantation Assay:*** A detailed protocol for the MGE transplantation assay has been previously described (Vogt et al., 2015). First, E13.5 MGEs from *Nkx2.1-cre::tdTomatof/+* embryos were dissected in ice-cold HBSS. Next, cells were mechanically dissociated by repeated pipetting (10–15 times) through a 1000 μL plastic pipette tip in DMEM media that contained 10% fetal bovineserum. Cells were dissociated in DMEM with 10% FBS that was preconditioned in a tissue culture incubator at 37 °C and with 5% CO2 to achieve a physiological pH. The cells were then transfected with either *DlxI12b-GFP* (control) or *DlxI12b-Wnt7b-GFP* (WNT7B-GFP expressing). Cells were transfected for 30 min at 37°C then pelleted by centrifugation (3 min, 700 ×g), and resuspended in 2-3 μL of DMEM, put on ice, and then remaining media containing the transfected MGE cells was removed before loaded into the injection needle. For injections, a glass micropipette of 50 μm diameter (with a beveled tip) was preloaded with sterile mineral oil and cells were front-loaded into the tip of the needle using a plunger connected to a hydraulic drive (Narishige) that was mounted to a stereotaxic frame. *Tbr1layer6*Wildtype and *Tbr1layer6* homozygous CKO P1 pups were anesthetized on ice for 1-2 min before being placed into a molded surface (modeling clay) for injections. Each pup received 2-3 injections of cells (~100 nL per site) in the right hemisphere. These sites were about 1mm apart along the rostral to caudal axis; cells were injected into layers 5/6 of the neocortex. After injections, pups were put back with the mother to recover after they began to move around on their own. Mice were sacrificed 28 days after transplantation and transcardially perfused with PBS followed by 4% PFA.

***Lithium chloride (LiCl) injection:*** P59 and P30 mice were administered a single intraperitoneal (IP) injection of 400 mg/kg LiCl or saline in a volume of 4 ml/kg (Martin et al., 2018). Treated mice were anesthetized at P60, 24 hrs or 4 weeks after LiCl injection with intraperitoneal injection of 100 mg/kg Ketamine containing 15 mg/kg Xylazine. A separate cohort of P58 mice were administered a single IP injection of 400 mg/kg LiCl or saline in a volume of 4 ml/kg. Treated mice were anesthetized 24 hrs after LiCl injection with intraperitoneal injection 100 mg/kg Ketamine containing 15 mg/kg Xylazine. All brains were processed at P60. Animals were perfused transcardially with ice-cold 1X PBS and then with 4% PFA in 1X PBS, followed by brain isolation, 1-2 hr post-fixation, cryoprotected in 30% sucrose in PBS, and cut frozen (coronally or sagittally) on a sliding microtome at 40µm for immunohistochemistry.

**Histology:**

For P0 and P3 experiments, neonatal animals were anesthetized on ice. For P21 and P56 experiments, animals were anesthetized with intraperitoneal injection of 100 mg/kg Ketamine containing 15 mg/kg Xylazine. Animals were perfused transcardially with cold PBS and then with 4% PFA in PBS, followed by brain isolation, 1-2 hr post-fixation, cryoprotected in 30% sucrose in PBS, and cut frozen (coronally or sagittally) on a sliding microtome at 40µm for immunohistochemistry or *in situ* hybridization. All primary and secondary antibodies were diluted in PBS containing 10% Normal Serum, 0.25% Triton X-100 and 2% BSA. The following primary antibodies were used: Chicken anti-GFP (1:2000, Aves), mouse anti-Vglut1 (1:200, Synaptic Systems), rabbit anti-Vgat (1:500, Synaptic Systems), rabbit anti-PSD95 (1:200, Cell Signaling), mouse anti-gephyrin (1:200, Synaptic Systems). The secondary antibodies for immunofluorescence were Alexa Fluor-conjugated and purchased from Thermofisher. For *in vivo* synapse immunohistochemistry, a total of n=30 apical dendrites were counted from each of *Tbr1wildtype*, *Tbr1layer5* heterozygous and *Tbr1layer5* homozygous mutants. The coronal sections were pre-treated with pepsin to enhance the staining. Immunofluorescence specimens were counterstained with 1% DAPI to assist the delineation of cortical layers. For *in situ* hybridization a rostro-caudal coronal series of at least ten sections from n=2 brains from *Tbr1wildtype* and *Tbr1layer5* heterozygous and homozygous mutants were examined. Anti-sense riboprobes for *Calm2, Kif1a, Wnt7b,* and *Mgst3* were prepared as previously described (Cobos et al., 2005; Fazel Darbandi et al., 2016). We also investigated cortical lamination within rostral cortex including PFCx of wildtype brain at P3 and P21 using anti-sense riboprobes for lamination markers *Cux2, Rorb, Etv1, Tbr1* and *Nr4a2*. ISH was performed using digoxigenin-labeled riboprobes.

**Image Acquisition and Analysis:**

Fluorescent and bright-field images were taken using a Coolsnap camera (Photometrics) mounted on a Nikon Eclipse 80i microscope using NIS Elements acquisition software (Nikon). Confocal imaging experiments were conducted at the Cancer Research Laboratory (CRL) Molecular Imaging Center, supported by Helen Wills Neuroscience Institute at UC Berkeley. Confocal images were acquired using Zeiss LSM 880 with Airyscan with a 63X objective at 1,024×1,024 pixels resolution with 2.0X optical zoom using ZEN 2.0 software. Brightness and contrast were adjusted, and images merged using Photoshop or ImageJ software. ImageJ software was used for image processing. For synapse counting (presynaptic and postsynaptic boutons), confocal image stacks (0.4µm step size) were processed with ImageJ software. In brief, background subtraction and smooth filter were applied to each stack. Using a threshold function, each stack was converted into a ‘masks’ image. Furthermore, the channels were co-localized with the Image Calculator plugging. Lastly, the number of co-localizations were counted, and the length of each dendrite was measured in each of the focal plane. Staining for control and mutant were done in parallel as well as the image capturing.

**Electrophysiology:**

Coronal brain slices (250 µm) including medial prefrontal cortex were made from three mice (n=3) at age p21-28 and at p56-p80. Slicing solution was chilled to 4°C and contained (in mM): 234 sucrose, 26 NaHCO3, 11 glucose, 10 MgSO4, 2.5 KCl, 1.25 NaH2PO4, 0.5 CaCl2, bubbled with 5% CO2/ 95% O2. Slices were incubated in artificial cerebrospinal fluid (aCSF) at 32°C for 30 minutes and then at room temperature until recording. aCSF contained (in mM): 123 NaCl, 26 NaHCO3, 11 glucose, 3 KCl, 2 CaCl2, 1.25 NaH2PO4, 1 MgCl2, also bubbled with 5% CO2/ 95% O2. Neurons were visualized using differential interference contrast or DODT contrast microscopy on an upright microscope (Olympus). *Rbp4-cre* positive neurons were identified by fluorescent visualization of cre-dependent tdTomato. We obtained somatic whole-cell patch clamp recordings using a Multiclamp 700B (Molecular Devices) amplifier and acquired with pClamp. Patch pipettes (2-5 MΩ tip resistance) were filled with the following (in mM): 130 KGluconate, 10 KCl, 10 HEPES, 10 EGTA, 2 MgCl2, 2 MgATP, 0.3 Na3GTP. All recordings were made at 32-34°C. Series resistance was compensated in all current clamp experiments and monitored throughout recordings. Recordings were discarded if Rs changed by >25%. For spontaneous EPSC and IPSC recordings cells were held in voltage clamp at -70 mV and +10mV, respectively. In both cases patch pipettes were filled with the following (in mM): 135 Cesium Methanesulfonate, 8 NaCl, 10 HEPES, 0.3 EGTA, 5 QX314, 4 MgATP, 0.3 Na3GTP.

**Behavioral Assays:**

Experiments were conducted during the light cycle (8am to 8pm). Mice were habituated to investigator handling for 1-2min on three consecutive days. On the testing day, mice were transferred to experimental room and allowed to habituate for at least 45 minutes prior to testing. All behavior assays were performed on mice age P56 to P80. We were blind to the genotypes during scoring of videos.

***Open-field test:***An individual mouse was placed near the wall-side of 50 x 50 cm open-field arena, and the movement of the mouse was recorded by a video camera for 10 min. The recorded video file was analyzed with Any-Maze software (San Diego Instruments). Time in the center of the field (a 25 x 25 cm square) was measured. The open field arena was cleaned with 70% ethanol and wiped with paper towels between each trial.

***Elevated plus maze test:***An individual mouse was placed at the junction of the open and closed arms, facing the arm opposite to the experimenter, of an apparatus with two open arms without walls (30 x 5 x 0.5 cm) across from each other and perpendicular to two closed arms with walls (30 x 5 x 15 cm) with a center platform (5 x 5 cm), and at a height of 40 cm above the floor. The movement of the mouse was recorded by a video camera for 10 min. The recorded video file was analyzed with Any-Maze software and time in the open arms of the apparatus was measured. The arms of the elevated plus maze apparatus was cleaned with 70% ethanol and wiped with paper towels between each trial.

***Rotarod test:***The assay consisted of four trials per day over the course of 2 days with the rotarod set to accelerate from 4rpm to 45rpm over 5 minutes. The trial started once five mice were placed on the rotarod rotating at 4rpm in separate partitioned compartments. Each trial ended when a mouse fell off, made three complete revolutions while hanging on, or reached 300 s. Digital videos of the mice on the rotarod were recorded from behind. The rotarod apparatus was cleaned with 70% ethanol and wiped with paper towels between each trial.

***Social interaction and novel object task:***An individual mouse was allowed to habituate for 5 minutes in their home cage prior to starting the trial. A juvenile (3-4 weeks old) mouse of the same strain and sex was introduced to the home cage. After 5 minutes, the juvenile was removed from the home cage. After a 5 min break a novel object (typically a plastic test tube cap) was introduced into the home cage for five minutes. We scored videos offline, blind to genotype. We measured the number of seconds the mouse spent with its nose in direct contact with the novel object or engaged in social interaction with the juvenile (defined as sniffing, close following, or allo-grooming) in the 300 seconds following the time the juvenile or object was introduced into the cage. In addition, we noted any aggressive-appearing behaviors toward the juvenile, freezing, and grooming behaviors. We repeated this behavioral assay on adult wildtype and mutant mice that were treated with a single IP injection of LiCl and compared to vehicle treated animals injected with saline.

**Quantification and Statistical Analysis:**

All individual data points are shown as well as mean ± SEM. All statistical analyses were performed using GraphPad Prism 7.0 software. Statistical significance was accepted at the level p < 0.05. We used student’s t-test to compare pairs of groups if data were normally distributed (verified using Lillie test). If more than two groups were compared, we used one-way ANOVA with post-hoc tests between groups corrected for multiple comparisons (Holm-Sidak or Tukey). For the ISH experiments reported in this paper n=2 represents two biological replicates for each of the reported genes. We examined the changes in synapse numbers of n=30 different dendrites from n=2 animals for each genotype. Whole-cell patch clamp experiments at P21 and P56 were conducted from n=3 different animals for each age and genotype. Lastly, behavioral analysis was conducted from n = 11/8/9, wildtype/ heterozygous/ homozygous animals. The specific n for each experiment as well as the post-hoc test, exact F and corrected p values can be found in the Results section.

**Data and Software Availability:**

Data and MATLAB analysis scripts are available upon request from the Lead Contact.

**LIST OF TABLES:**

**Table S1:** Complete list of differentially expressed (DEX) genes in the *Tbr1layer5* CKOs compared to *Tbr1wildtype* at P5 and enriched Gene Ontology terms. Related to Figure 1.

**Table S2:** scRNA-Seq analysis of FACS purified Layer 5 neurons at P5 identified different classes of DEX genes that are directly regulated in the *Tbr1layer5* CKOs compared to *Tbr1*wildtype at P5. Related to Figure 1C.