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ORIGINAL ARTICLE

DIXDC1 contributes to psychiatric susceptibility by regulating dendritic spine and glutamatergic synapse density via GSK3 and Wnt/β-catenin signaling

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Mice lacking DIX domain containing-1 (DIXDC1), an intracellular Wnt/β-catenin signal pathway protein, have abnormal measures of anxiety, depression and social behavior. Pyramidal neurons in these animals' brains have reduced dendritic spines and glutamatergic synapses. Treatment with lithium or a glycogen synthase kinase-3 (GSK3) inhibitor corrects behavioral and neurodevelopmental phenotypes in these animals. Analysis of *DIXDC1* in over 9000 cases of autism, bipolar disorder and schizophrenia reveals higher rates of rare inherited sequence-disrupting single-nucleotide variants (SNVs) in these individuals compared with psychiatrically unaffected controls. Many of these SNVs alter Wnt/β-catenin signaling activity of the neurally predominant DIXDC1 isoform; a subset that hyperactivate this pathway cause dominant neurodevelopmental effects. We propose that rare missense SNVs in *DIXDC1* contribute to psychiatric pathogenesis by reducing spine and glutamatergic synapse density downstream of GSK3 in the Wnt/β-catenin pathway.

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INTRODUCTION

Advances in human genomics are revolutionizing knowledge of molecules conferring susceptibility to psychiatric disorders, but have simultaneously highlighted the complexity of genetic contributions and underscored a need to define common biological pathways.[1,2] One pathway proposed to have a role based on such evidence is Wnt/β-catenin signaling, a biochemical cascade conserved in all metazoans by which nearby cells communicate during and after development.[3,4]

DIX domain containing-1 (DIXDC1), a positive cytoplasmic transducer of the Wnt/β-catenin pathway,[5,6] is of additional interest because it interacts with *DISC1* (disrupted in schizophrenia 1), a gene separately implicated in the genetics of psychiatric disorders including schizophrenia (Scz), major depression, bipolar disorder (BD) and autism spectrum disorder (ASD).[7,8] Compared with some core Wnt/β-catenin pathway components, DIXDC1 has a relatively restricted tissue distribution including in the late developmental and postnatal central nervous system,[6,9] suggesting that it might have specialized roles in neurons and that its functional sequence variants might manifest as behavioral syndromes in the human population.

Here we describe a multifaceted analysis of DIXDC1 in neurodevelopment and psychopathogenesis. Using behavioral, neurodevelopmental, biochemical and pharmacological analyses of a knockout mouse model combined with human genetic analyses across several psychiatric disorders and functional analyses of rare missense mutations found in one set of such patients (ASD), we show that DIXDC1 participates in the regulation of dendritic spine and glutamatergic synapse density downstream of Wnt/β-catenin signaling and upstream of behavior, particularly depression- and anxiety-like behaviors potentially relevant to affective disorders and reciprocal social interactions potentially relevant to ASD.

MATERIALS AND METHODS

Animals

The *Dixdc1* knockout (Dixdc1KO) mouse line was created by gene targeting that replaced several critical exons of the *Dixdc1* locus with a *neo* interrupter cassette, causing loss of *Dixdc1* gene products confirmed at both the mRNA[10] and protein levels (Supplementary Figure 1a). Products of the original gene-targeting event were outcrossed >10 times to

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Figure 1. Phenotypes in Dixdc1KO mice. (**a**–**d**) Behavioral phenotypes. (**a**) Despair (forced swim test (FST)). (**b**) Anxiety (time in center versus surround in an open field). (**c**) Drive to eat versus anxiety over unfamiliar (hyponeophagia assay). (**d**) Social behavior (social interactions in pairs (SIP)). (**e**–**s**) Dendritic spine phenotypes: Primary dendrites of cultured neurons transfected with green fluorescent protein (GFP) (**e** quantified in (**f** and **g**)). Top, WT; bottom, KO; arrow, mature spine; asterisk, filopodial projection. Cortical L5/6 apical dendrites in brain tissue labeled by *Tg(Thy1-EGFP)M* (**h** quantified in (**i** and **j**)). Top, WT; bottom, KO; arrows and asterisks as in (**e**). (**k**–**m**) Imaging of spine density and spine dynamics *in vivo*. Two-photon images of the same dendritic branch over 7 days labeled by *Tg(Thy1-YFP)H* in the primary somatosensory cortex of living adult mice (**k** quantified in (**l** and **m**)). Top, WT; bottom, KO; arrow, eliminated spine; arrowhead, newly formed spine. (**n**–**q**) Recording of I_h-dependent voltage and current. (**n**) Representative whole-cell current clamp recordings in L5/6 prefrontal corticothalamic neurons; black, WT; orange, KO. (**o**) Quantification of voltage sag and rebound (after-depolarization (ADP)) dependent of I_h. (**p**) Direct measurement of hyperpolarization-activated currents and inhibition by ZD7288, a specific I_h antagonist; black, WT; orange, KO; gray, ZD7288-treated WT; light orange, ZD7288-treated KO. (**q**) I_h current activation at − 120 mV potential step; colors as in (**p**). (**r**–**s**) Immunohistological visualization of HCN1 channel protein: Representative micrographs of cortical L5/6 apical dendrites (**r** quantified in **s**). Top, WT; bottom, KO; far red, HCN1; green (*Tg (Thy1-EGFP)M)*. (**t**–**w**) Glutamatergic synapse phenotypes. Colocalized immunohistochemical markers for glutamatergic synapses on dendrites in GFP-transfected cultured neurons (**t** quantified in (**u**)), and cortical L5/6 apical dendrites labeled by *Tg(Thy1-EGFP)M* (**v** quantified in (**w**)). Top, WT; bottom, KO; blue, VGLUT; red, PSD95; green, GFP; arrowheads, colocalized green/blue/red puncta. Scale bars, 5 μm. $*P \leqslant 0.05$, $**P \leqslant 0.01$ and $***P \leqslant 0.001$. KO, knockout; WT, wild-type.

different wild-type (WT) mice to eliminate flanking allele effects, and mice for this study were maintained in an outbred mixed (~75% CD-1; Charles River, Hollister/San Diego, CA, USA) genetic background. All comparisons were made in cohorts of littermate mice, separated by genotype blind to experimenter.

Statistical analysis

Data were analyzed by Student's *t*-test, two-way analysis of variance followed by multiple comparisons, χ^2 test or Fisher's exact test using GraphPad Prism software (GraphPad Software, La Jolla, CA, USA) and are displayed as mean ± s.e.m. Variance was similar between all groups compared; all reported differences minimally reflect significance of $P \leqslant 0.05$ for experimental versus control.

Details are provided in Supplementary Materials and methods.

RESULTS

Depression, anxiety and social behavior in Dixdc1KO mice

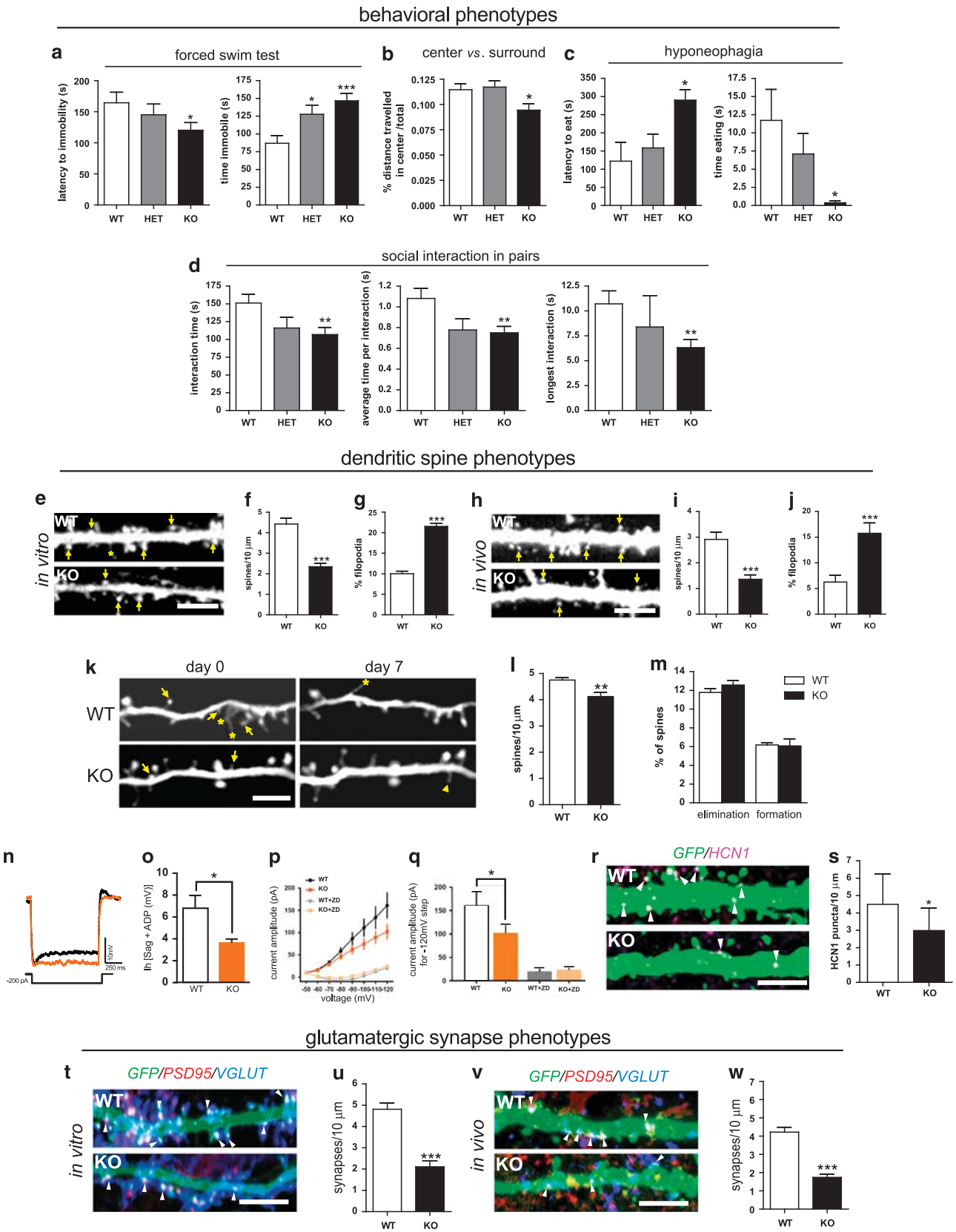
We showed previously that homozygous Dixdc1KO mice maintained in an isogenic C57Bl/6 background had behavioral differences potentially relevant to psychiatry, but were also generally hypoactive.[10] Neurodevelopmental and behavioral phenotypes in mice can be sensitive to isogenic background and this has occasionally confounded interpretation.[11–13] Accordingly, we reprobed Dixdc1KO mice for behavioral and neurodevelopmental phenotypes that remained robust even in a mixed outbred (primarily *CD-1*) genetic background and used these mice for all behavioral and neurodevelopmental phenotyping in this study.

Outbred Dixdc1KO mice were no different from WT littermates in general activity or several other behavioral measures (Supplementary Table 1), yet had decreased latency to immobility (Figure 1a, left) and spent more total time immobile (Figure 1a, right) in the forced swim test (FST), an assay for behavioral despair regarded as a rodent model for depression.[14] These mice also had a trend toward increased total time immobile in the tail suspension test, an independent assay for behavioral despair (Supplementary Figure 2). Homozygous Dixdc1KO mice also spent less time than WT littermates in the center versus surround of an open field (Figure 1b), an indication of increased anxiety.[15] Furthermore, in the hyponeophagia assay, which measures balance between opposing drives to feed versus to avoid the unfamiliar,[16] Dixdc1KO mice had an increased latency to begin feeding (Figure 1c, left) and spent less total time feeding (Figure 1c, right).

In the social interactions in pairs (SIP) test, where two males of the same genotype freely interact within a closed arena,[17] Dixdc1KO mice spent less time together than WT littermates (Figure 1d, left). Interestingly, this decrease in total interaction time was not attributable to a reduced number of mouse–mouse interactions. Instead Dixdc1KO mice spent less time on average during each interaction (Figure 1d, middle) and their longest interaction was shorter (Figure 1d, right). Interactions of longer duration in this assay were characterized by reciprocal social behavior including nose-to-nose sniffing, nose-to-anogenital sniffing or one animal following close behind the other, suggesting that reciprocal social interactions are deficient in this mouse model.

Pyramidal neurons in Dixdc1KO mice have reduced spines and glutamatergic synapses

Dixdc1KO mice are viable and fertile and have grossly normal brains with typical regional architecture[10] (Supplementary Figures 1b–h). We therefore searched for other neuronal phenotypes underlying behavioral differences in these animals. Studies of other neurodevelopmentally expressed Wnt pathway components and DISC1 have supported roles in dendrite, dendritic spine and glutamatergic synapse formation and function.[18–22] To initially probe for these phenotypes in the Dixdc1KO mouse line, we used cultured hippocampal neurons because of their established validity for modeling these aspects of neurodevelopment.[22] Both visual inspection and Sholl analysis[23] revealed no significant differences in dendrite arborization in neurons from Dixdc1KO neonates in the mixed outbred genetic background (Supplementary Figures 3a–c); however, mutant neurons had significantly reduced spine density along primary dendrites (Figures 1e and f) and a significantly increased percentage of immature (filopodial) spines (Figures 1e and g). We confirmed these same spine phenotypes within forebrain cortical tissue by crossing in transgenic Thy1-GFP alleles that sparsely label deep layer (L5/6) pyramidal neurons,[24] then collecting tissue and quantifying density and maturity of spines along apical dendrites of individual GFP-expressing neurons;[18] here again Dixdc1KO mutants had reduced spine density and an increased percentage of filopodial spines (Figures 1h–j). In the living brain, we imaged apical dendrites and spines in 1-month-old transgenic Thy1-YFP mice using transcranial two-photon imaging, once more finding that spine density on L5 apical dendrites in the somatosensory cortex was reduced in the Dixdc1KO (Figures 1k and l). Interestingly, spine dynamics (proportion of spines formed and eliminated over 7 days) were no different between Dixdc1KO and WT littermates at this stage (Figure 1m). To substantiate imaging findings at the level of neuronal function, we conducted internal electrophysiological recordings from L5/6 corticothalamic pyramidal neurons in the prefrontal cortex. In Dixdc1KO mice these neurons had a reduced hyperpolarization-activated cationic current (h-current; I_h) as estimated from the reduction in voltage 'sag' and rebound after-depolarization in response to a hyperpolarizing current pulse (Figures 1n and o). We confirmed this by directly measuring I_h current in response to hyperpolarizing voltage steps: this current was reduced in Dixdc1KO L5/6 corticothalamic pyramidal neurons and inhibited by the I_h channel blocker ZD7288 (Figures 1p and q). The I_h current is mediated by hyperpolarization-activated cyclic nucleotide-gated (HCN)



channels localized primarily to mature spines.[25] We therefore corroborated the electrophysiological finding of reduced Ih current via the methodologically independent strategy of fluorescent immunohistochemistry, visualizing the HCN1 channel protein using a specific antibody and counting puncta along dendrites of GFP-expressing L5/6 pyramidal neurons in Thy1-GFP mice. This method confirmed a significant reduction in HCN1 puncta on Dixdc1KO L5/6 pyramidal neuron dendrites (Figures 1r

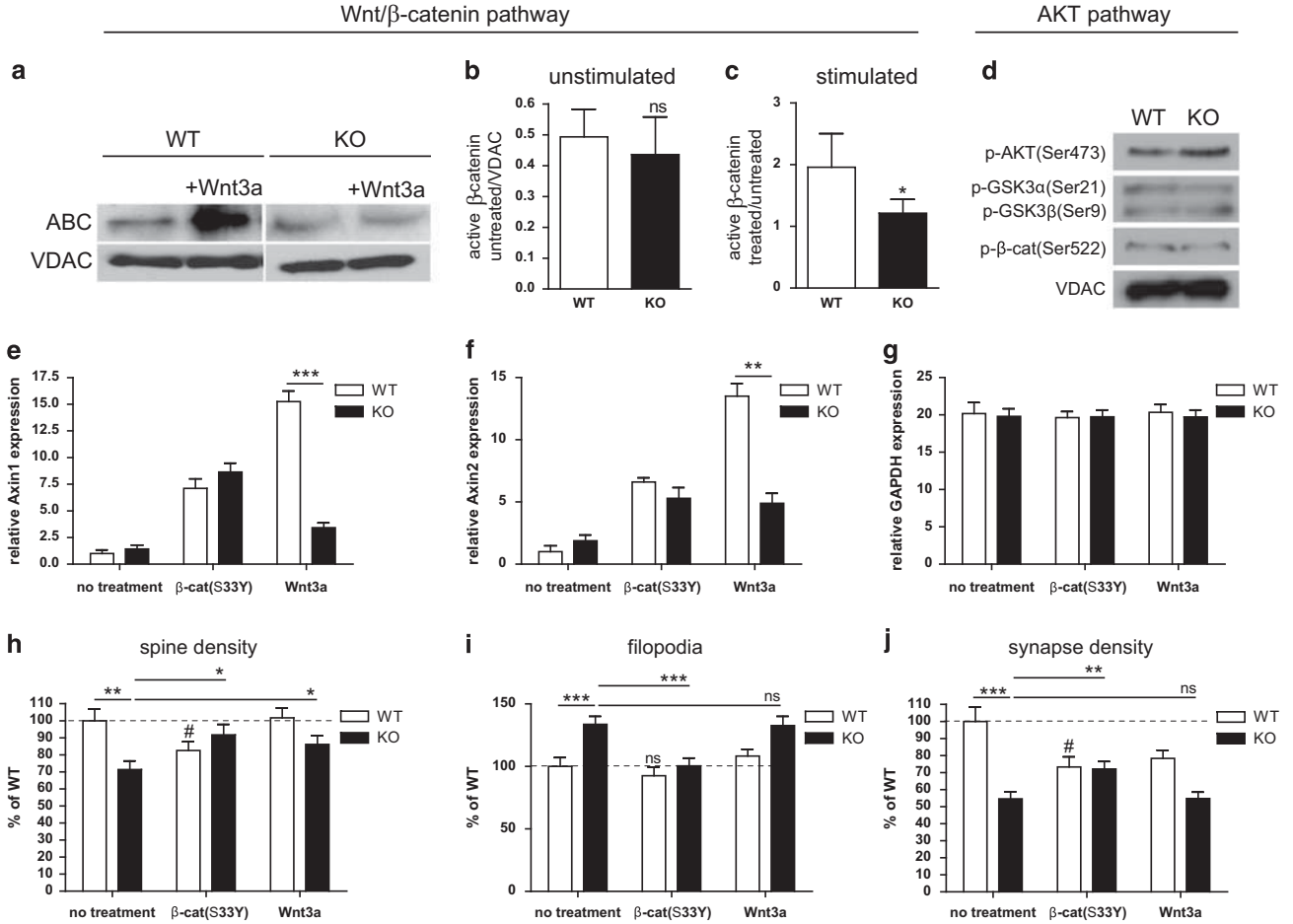


Figure 2. Decreased Wnt/β-catenin signal transduction in Dixdc1KO neurons. (**a**) Immunoblot of active β-catenin (ABC) from cultured neurons. (**b** and **c**) Quantification in unstimulated (**b**) and Wnt3a-stimulated (**c**) WT versus KO neurons. (**d**) AKT pathway: immunoblots of phosphorylated AKT (p-AKT(Ser473)), AKT-phosphorylated glycogen synthase kinase-3 (GSK3) (p-GSK3α(Ser21) top, p-GSK3β(Ser9) bottom and AKT-phosphorylated β-catenin (p-β-cat(Ser522)), in WT versus KO neurons. (**e–g**) Quantitative reverse-transcriptase PCR for Wnt/β-catenin pathway transcriptional targets in neurons: *Axin1* (**e**) and *Axin2* (**f**); *GAPDH* (control) (**g**); KO (black) versus WT (white). Left, untreated; middle, transfected with β-cat(S33Y); right, treated with Wnt3a. (**h–j**) Neurodevelopmental responses to Wnt/β-catenin pathway stimulation. Quantification (as in Figure 1): (**h**) spine density, (**i**) percentage of filopodia and (**j**) glutamatergic synapse density; white, WT; black, KO, all conditions normalized to WT untreated (left-most bar). Comparisons as in (**e–g**). $^{*}P \leqslant 0.05$, $^{**}P \leqslant 0.01$, $^{***}P \leqslant 0.001$ and $^{\#}P \leqslant 0.05$ versus untransfected WT. β-cat, β-catenin; KO, knockout; WT, wild-type.

and s). In sum, the electrophysiological and immunohistochemical data agree and strongly support our other measures of reduced mature spine density in these animals.

Spines correspond to the postsynaptic compartment of most glutamatergic inputs to pyramidal neuron dendrites.[26] We therefore asked whether spine deficits in Dixdc1KO pyramidal neurons were accompanied by deficits in glutamatergic synapse density. Cultured neurons from Dixdc1KO neonates had reduced glutamatergic synapse density along dendrites as measured by fluorescent confocal colocalization of pre- and postsynaptic molecular markers (Figures 1t and u). We confirmed this within cortical tissue, observing a deficit in glutamatergic synapse density along apical dendrites of individual pyramidal neurons in Thy1-GFP Dixdc1KO mice (Figures 1v and w). In contrast, using similar methodologies we detected no differences in GABAergic synapse density (Supplementary Figures 3d–g).

Neurons from Dixdc1KO mice have impaired Wnt/β-catenin signal transduction

Given that Dixdc1 has a conserved subdomain that directly interacts with the core Wnt/β-catenin pathway components Dvl and Axin,[5,6,27,28] we hypothesized that Wnt/β-catenin signal transduction would be impaired at the level of these cytoplasmic signal-transduction proteins in differentiating Dixdc1KO pyramidal neurons. We tested this via several complementary approaches: first, we tested whether Wnt/β-catenin signal pathway activity was altered in developing neurons of Dixdc1KO mice by directly measuring the levels of β-catenin unphosphorylated at residue S33 ('active β-catenin'), the molecular target of glycogen synthase kinase-3 (GSK3) in the Wnt/β-catenin pathway. There was no difference in levels of active β-catenin in unstimulated Dixdc1KO versus WT cultured forebrain neurons (Figures 2a and b). However, treatment of these neurons with Wnt3a, an extracellular activator of this pathway,[29] resulted in markedly different responses in Dixdc1KO versus WT neurons: in WT neurons the level of active β-catenin rose markedly in response to Wnt3a, indicating Wnt/β-catenin signal transduction via regulation of GSK3-mediated phosphorylation (Figures 2a and c). Contrastingly, in Dixdc1KO neurons there was no effect of Wnt3a treatment on active β-catenin level (Figures 2a and c). Because GSK3 is independently regulated by the AKT pathway, we asked whether markers for this pathway were altered in Dixdc1KO versus WT neurons. In contrast to deficiencies observed in Wnt/β-catenin signal transduction,

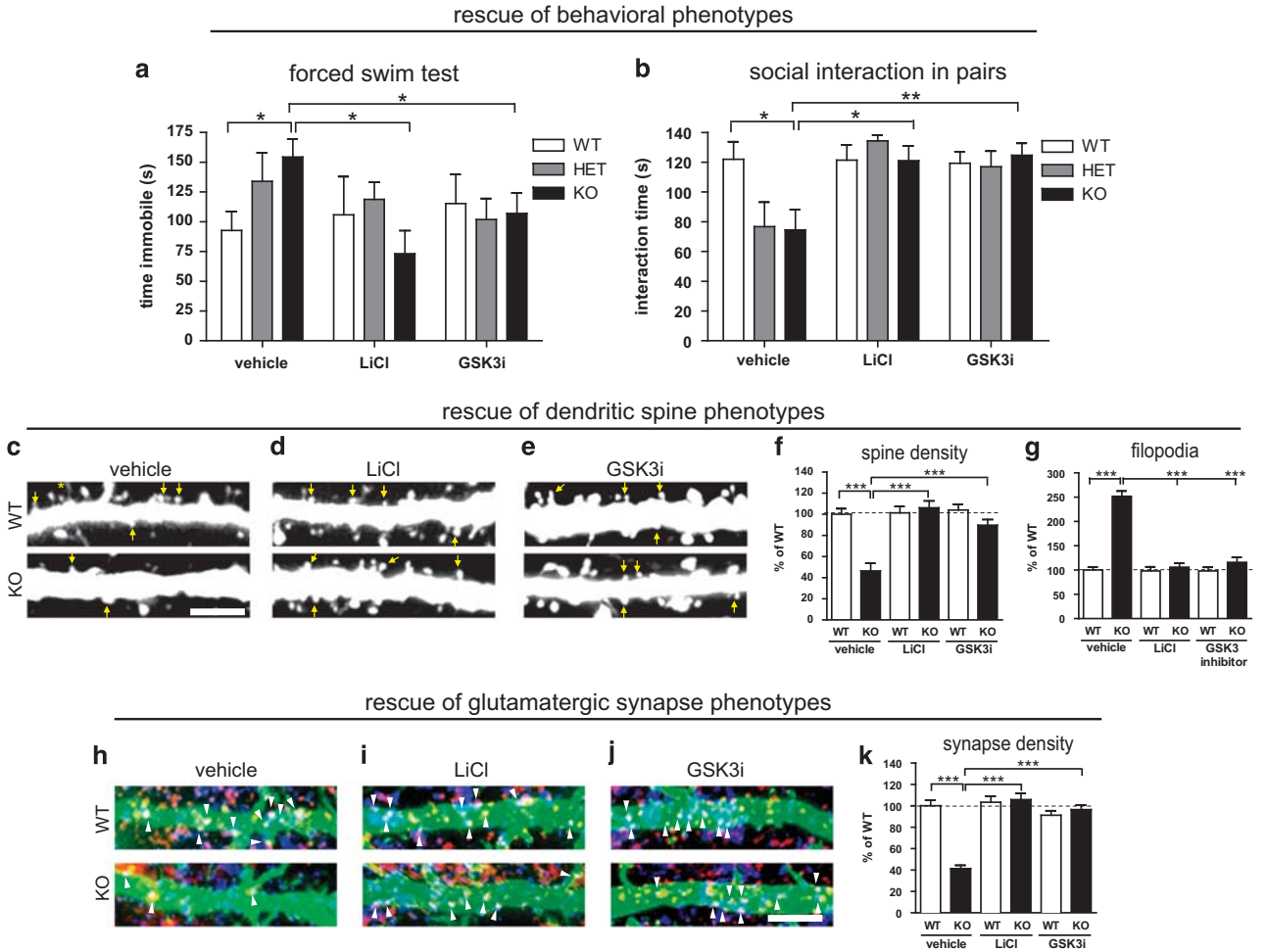


Figure 3. Glycogen synthase kinase-3 (GSK3) inhibition rescues Dixdc1KO phenotypes. (**a** and **b**) Pharmacologic rescue of behavioral phenotypes. The forced swim test (FST) phenotype (increased time immobile) (**a**) and social interactions in pairs (SIP) phenotype (reduced social interaction) (**b**) are rescued by injection of either lithium (middle) or GSK3 inhibitor (GSK3i) (right). (**c–g**) Pharmacologic rescue of dendritic spine phenotypes. Cortical L5/6 apical dendrites labeled by *Tg(Thy1-EGFP)M* in mice treated with vehicle (**c**), lithium (**d**) or GSK3i (**e**). Top, WT; bottom, KO; arrows and asterisks as in Figure 1e. Quantification, all conditions normalized to WT vehicle (left-most bar) (**f** and **g**). (**h–k**) Pharmacologic rescue of glutamatergic synapse phenotypes. Colocalized immunohistochemical markers for glutamatergic synapses on cortical L5/6 apical dendrites labeled by *Tg(Thy1-EGFP)M* in mice treated with vehicle (**h**), lithium (**i**) or GSK3i (**j**). Top, WT; bottom, KO; colors as in Figure 1r. Quantification with all conditions normalized to WT vehicle (left-most bar) (**k**). Scale bars, 5 μm. $*P \leqslant 0.05$, $**P \leqslant 0.01$ and $***P \leqslant 0.001$. KO, knockout; WT, wild-type.

there were no differences in biochemical markers for the AKT pathway, including levels of phosphorylated AKT (p-AKT(Ser473)) or its specifically phosphorylated targets GSK3 (p-GSK3α(Ser21) and p-GSK3β(Ser9)) and β-catenin (p-β-cat(Ser522)) in Dixdc1KO neurons (Figure 2d).

The most universal transcriptional targets of the Wnt/β-catenin pathway are *Axin2* and its molecular relative *Axin1*. We confirmed Wnt/β-catenin signal pathway disruption in Dixdc1KO neurons by directly measuring levels of these transcriptional targets using the quantitative reverse-transcriptase PCR. There was no difference in mRNA levels of *Axin1* or *Axin2* in unstimulated Dixdc1KO versus WT cultured forebrain neurons (Figures 2e–g), demonstrating that these targets are basally transcribed normally in Dixdc1KO neurons. As expected, recombinant expression of a point-mutant-stabilized form of β-catenin (β-cat(S33Y)) that bypasses Wnt signal transduction increased *Axin1* and *Axin2* transcription in both Dixdc1KO and WT neurons with no effect on the control GAPDH (glyceraldehyde 3-phosphate dehydrogenase) transcript (Figures 2e–g). In contrast, treatment with Wnt3a resulted in markedly different responses in Dixdc1KO versus WT neurons: levels of both target transcripts rose markedly in WT neurons, whereas this signaling response was greatly attenuated in Dixdc1KO neurons (Figures 2e and f).

To determine whether dendritic spine and glutamatergic synapse phenotypes arise from decreased Wnt/β-catenin signaling efficiency in Dixdc1KO pyramidal neurons, we sought to rescue neurodevelopmental phenotypes by stimulating the pathway upstream and downstream of the putative pathway block. As anticipated, treatment with the upstream ligand Wnt3a had no or minimal effect on spine maturity and glutamatergic synapse density, although it did partially rescue spine density in Dixdc1KO cultured hippocampal neurons (Figures 2h–j, right-most bars). In contrast, bypassing the pathway via transfection with β-cat(S33Y) rescued all these phenotypes (Figures 2h–j, middle bars). Interestingly, recombinant expression of β-cat(S33Y) in WT neurons also significantly decreased spine and synapse density ('#' in Figures 2h and j). These data suggest that either Wnt/β-catenin pathway hyperactivity or hypoactivity during neural differentiation can lead to grossly similar spine and glutamatergic synapse reductions.

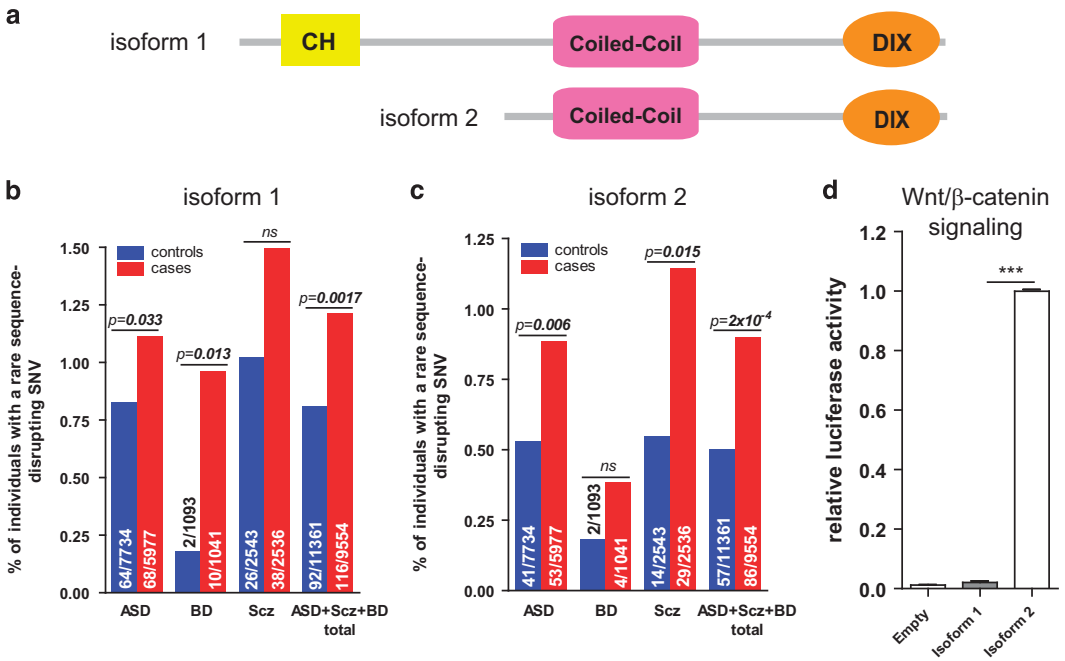


Figure 4. Increased rare sequence-disrupting *DIXDC1* single-nucleotide variants (SNVs) in autism spectrum disorder (ASD), bipolar disorder (BD) and schizophrenia (Scz). (**a**) Schematic representation of human DIXDC1 isoform 1 and isoform 2. Yellow, calponin homology domain (CH); pink, coiled-coil domain; orange, DIX domain. (**b** and **c**) Percentage of individuals carrying a rare sequence-disrupting (nonsense, missense or splice-site disrupting) SNV in *DIXDC1* isoform 1 (**b**) and 2 (**c**). (**d**) Relative TOPflash (Wnt/β-catenin signaling) activity for wild-type (WT) human DIXDC1 isoform 1 versus isoform 2; values normalized to isoform 2 (white bar). ***$P \leqslant 0.001$.

Behavioral and neurodevelopmental phenotypes are rescued by lithium or GSK3 inhibitor

Supporting a strong genotype–phenotype correlation between the Dixdc1KO allele and behavioral phenotypes in the Dixdc1KO mouse line, we noticed that behavioral differences on the FST, hyponeophagia assay and SIP were gene-dose sensitive, with heterozygous Dixdc1KO mice displaying an intermediate phenotype (Figures 1a, c and d, gray bars). We hypothesized that this reflects Dixdc1-dose-sensitive reductions in Wnt/β-catenin signal transduction. To test this hypothesis, we asked whether these behavioral phenotypes could be corrected by treatment with the mood-stabilizing agent lithium chloride, which among other cell biological effects activates the Wnt/β-catenin pathway through direct inhibition of GSK3.[30] Indeed, systemic injection of either lithium chloride or a selective small-molecule GSK3 inhibitor (GSK3i) corrected behavioral phenotypes in Dixdc1KO animals, including in the FST (Figure 3a) and SIP (Figure 3b). Combined with reversible pharmacology (Supplementary Figure 4), these data support that behavioral phenotypes in this animal model occur secondary to *Dixdc1* gene-dose-sensitive changes in the regulation of GSK3, specifically increased GSK3 activity.

We hypothesized that lithium/GSKi-responsive behavioral abnormalities in Dixdc1KO mice correspond to spine and glutamatergic synapse deficits and tested this by using a drug administration protocol identical to that which rescued behavior, assessing neurodevelopmental phenotypes instead. We found that both lithium and GSK3i rescued dendritic spine density, spine morphology and glutamatergic synapse density in L5/6 pyramidal neurons within these animals' brains (Figures 3c–k).

Inherited rare missense *DIXDC1* SNVs are more prevalent in psychiatric cases

Given the preceding results, we asked whether sequence variation at the *DIXDC1* locus, which encodes two major isoform classes (Figure 4a),[6,31] might contribute to psychiatric disorders. We first analyzed two data sets of exome sequences (Supplementary Figure 5), totaling nearly 6000 ASD cases and over 7000 controls, focusing on 'sequence-disrupting' single-nucleotide variants (SNVs) most likely to affect Dixdc1 function—that is, nonsense, missense and conserved splice-donor/acceptor-disrupting SNVs. This revealed a greater burden of rare sequence-disrupting SNVs at this locus in ASD versus unaffected controls (Figures 4b and c, left-most bars; Supplementary Figures 5a and b).

Next, we analyzed a sample of ~1000 BD patients and a similarly sized ethnically matched control cohort. There was once again a greater burden of rare sequence-disrupting SNVs in both isoforms in BD cases versus controls (Figures 4b and c, middle-left bars). Finally, we analyzed a data set of over 2500 exome sequences from Scz patients and a similar number of ethnically matched controls.[32] Here again, a greater burden of rare sequence-disrupting SNVs was present in cases versus controls (Figures 4b and c, middle-right bars).

In each psychiatric data set (2 ASD data sets, 1 BD data set and 1 Scz data set), there was a higher burden of rare sequence-disrupting SNVs in each of two major DIXDC1 isoforms in those affected, compared with those unaffected, by the disorder. Combining these data sets—totaling over 9000 cases and over 11 000 controls—there was a higher burden of rare sequence-disrupting SNVs for each isoform across these three psychiatric disorders; this association was greater for isoform 2 than for isoform 1 (isoform 1, $P = 1.7 \times 10^{-3}$; isoform 2, $P = 2 \times 10-^{4}$; Figures 4b and c, right-most bars; Supplementary Tables 2 and 3).

Rare missense SNVs from ASD patients alter Wnt/β-catenin signaling function

The two major *DIXDC1* isoforms have distinct expression patterns, the longer isoform 1 has a relatively widespread embryonic tissue

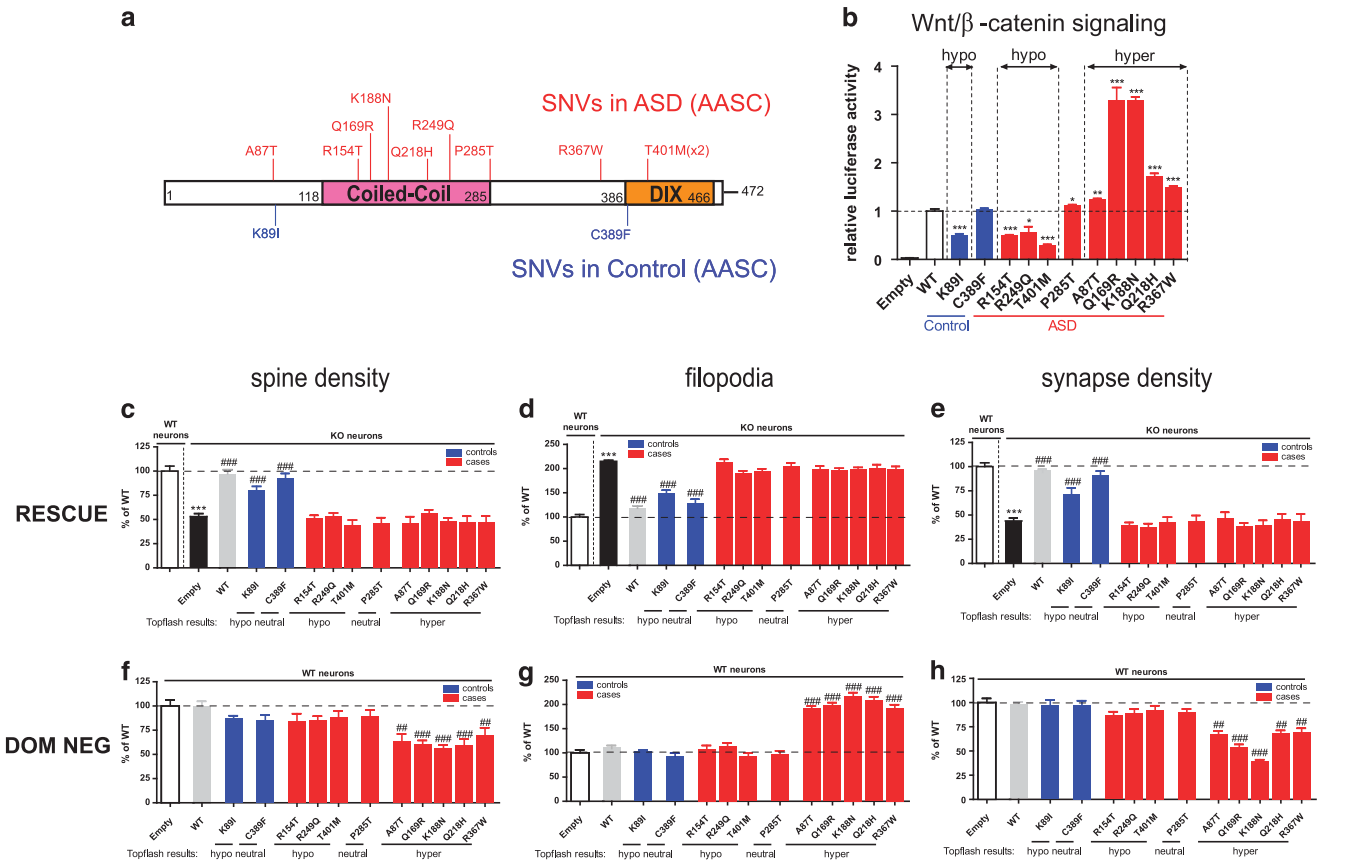


Figure 5. Rescue and dominant neurodevelopmental effects of missense single-nucleotide variants (SNVs) with functional effects on the Wnt/β-catenin pathway. (**a**) Schematic diagram of isoform 2 indicating positions of rare missense SNVs found in autism spectrum disorder (ASD) (red, top) versus ethnically matched controls (blue, bottom) in the discovery (AASC) data set. (**b**) TOPflash for alleles shown in (**a**); values normalized to wild-type (WT) isoform 2 (white bar); alleles grouped as hypoactive, neutral or hyperactive based on $\Delta \geqslant 10\%$ versus WT. (**c–e**) Neurodevelopmental rescue: WT human DIXDC1 isoform 2 (gray bar) restores spine density (**c**), percentage of immature spines (**d**) and glutamatergic synapse density (**e**) to WT levels (white bar) when expressed in knockout (KO) neurons. Control-derived SNVs (K89I, C389F) (blue bars) but no case-derived SNVs (red bars), similarly rescue these KO phenotypes. (**f–h**) Dominant neurodevelopmental effects: WT human DIXDC1 isoform 2 (gray bar) or cDNAs containing hypoactive or activity-neutral SNVs (K89I, C389F, R154T, R249Q, T401M, P285T) do not cause neurodevelopmental phenotypes, but cDNAs containing hyperactive SNVs found in cases (A87T, Q169R, K188N, Q218H, R367W) decrease spine density (**f**), increase immature spine percentage (**g**) and decrease glutamatergic synapse density (**h**) when overexpressed in WT neurons. $^{*}P \leqslant 0.05$, $^{**}P \leqslant 0.01$, $^{***}P \leqslant 0.001$ versus WT in (**b–e**); $^{\#\#}P \leqslant 0.01$ and $^{\#\#\#}P \leqslant 0.001$ versus identical genotype+empty vector in (**c–h**).

distribution, whereas the shorter isoform 2 predominates in the developing nervous system at later stages and in neurons of the mature brain.[6,9,33] Isoform 2 also has far more robust activity in standard cell-based Wnt/β-catenin signaling assays (Figure 4d).[6] We accordingly tested rare missense SNVs in isoform 2 from our discovery data set (Figure 5a and Supplementary Table 3, AASC) for functional effects on this pathway by engineering each SNV into a human DIXDC1 isoform 2 cDNA and testing signaling activity.[34] We found that, compared with WT, most of these SNVs either reduced or increased Wnt/β-catenin pathway activation by the encoded protein (Figure 5b).

Rare missense SNVs from psychiatric patients fail to rescue spine and synapse deficits

To confirm functional conservation between the mouse and human DIXDC1 proteins, we tested whether WT human DIXDC1 could rescue neurodevelopmental phenotypes in Dixdc1KO cultured hippocampal neurons by recombinantly expressing the WT human isoform 2 protein beginning at 12 days *in vitro* (DIV12), assessing neurodevelopmental phenotypes 6 days later (DIV18). WT human isoform 2 completely rescued spine density, spine maturity and glutamatergic synapse density in Dixdc1KO neurons (Figures 5c–e, gray bar).

Given our phenotypic data in the Dixdc1KO animal model, we hypothesized that rare sequence-disrupting SNVs discovered in psychiatric patients might alter Wnt/β-catenin signal transduction upstream of effects on spine and glutamatergic synapse density. To test this, we assessed SNV-containing cDNAs for their ability to rescue Dixdc1KO neurodevelopmental phenotypes similar to the WT protein. Isoform 2 cDNAs containing control-derived SNVs (K89I and C389F) rescued neurodevelopmental phenotypes in cultured neurons much like the WT protein (Figures 5c–e, blue bars). Remarkably, no cDNAs containing SNVs found in the ASD cases rescued these neurodevelopmental phenotypes (Figures 5c–e, red bars).

Wnt/β-catenin pathway hyperactivating SNVs cause dominant spine and synapse deficits

With allele frequencies $\leqslant 0.1\%$, each of the SNVs we studied is present in a single copy in patients; to contribute to psychiatric pathogenesis they must therefore have a dose-dependent or dominant effect over the WT allele. To test for this, we assessed

whether SNV-containing isoform 2 cDNAs caused *dominant* neurodevelopmental effects when recombinantly expressed in WT cultured hippocampal neurons. The WT human isoform 2 protein had no effect on spine density, spine maturity or glutamatergic synapse density when recombinantly expressed in WT neurons (Figures 5f–h, gray bar). Similarly, no isoform 2 cDNAs encoding Wnt/β-catenin pathway hypoactivating or neutral SNVs, whether found in our control samples (K89I and C389F) or our ASD samples (R154T, R249Q, T401M and P285T), had dominant effects on neurodevelopment (Figures 5f–h, blue, left and middle red bars). In contrast, all the Wnt/β-catenin pathway hyperactivating SNVs (A87T, Q169R, K188N, Q218H and R367W) had dominant neurodevelopmental effects—that is, they caused decreased spine density, increased immature spine percentage and decreased glutamatergic synapse density (Figures 5f–h, right red bars).

DISCUSSION

Several lines of evidence reported here support that altered Wnt/β-catenin signaling generates neurodevelopmental and behavioral phenotypes in Dixdc1KO mice and contributes to neurodisruptive effects of rare *DIXDC1* sequence variants in human psychiatric patients. First, rare *DIXDC1* missense SNVs found in psychiatric patients interfere with the protein's Wnt/β-catenin signaling function *in vitro*. Second, a subset of SNVs that hyperactivate the Wnt/β-catenin pathway cause dominant neurodevelopmental effects on spine maturity, spine density and synapse density, whereas SNVs that do not hyperactivate the pathway do not cause dominant effects. Third, Wnt/β-catenin signaling hypoactivity is associated with similar neurodevelopmental and gene-dose-sensitive behavioral phenotypes in a Dixdc1KO mouse model. Fourth, pharmacologically mimicking pathway activation via direct inhibition of GSK3, whether by the psychiatric drug lithium chloride or a selective small-molecule GSK3i, corrects neurodevelopmental and behavioral phenotypes in this animal model.

Recent genomic and pharmacological studies suggest that the pathophysiology of ASD, Scz, BD and other psychiatric conditions overlaps and involves formation, maintenance, removal and function of spines and glutamatergic synapses.[1,2,4,35–39] Our findings fit into this narrative, adding further evidence that abnormal spine and glutamatergic synapse density contributes to psychiatric pathogenesis. Our work provides a compelling case that Dixdc1 contributes to the formation, plasticity and/or maintenance of dendritic spines and glutamatergic synapses via facilitation of Wnt/β-catenin signal transduction within pyramidal neurons and that rare missense variants in *DIXDC1* contribute to psychiatric susceptibility by decreasing or increasing Wnt pathway activity in these cells. This is consistent with evidence from large-scale sequencing and other studies showing that loss-of-function mutations in either Wnt/β-catenin pathway inhibitors (e.g. CHD8, APC)[40,41] or activators (e.g. CTTNβ1, WNT1)[4,42,43] contribute to susceptibility for ASD, as well as identifying differentiating cortical pyramidal neurons as a likely locus of cellular pathology in this disorder.[44]

Nonetheless, we cannot rule out that additional biochemical mechanisms may contribute to these neurodevelopmental and behavioral outcomes. For example, a β-catenin-independent mechanism downstream of GSK3, such as one that regulates cytoskeletal proteins,[45] could also be important in mediating spine and synapse density. Dixdc1 isoforms have been implicated in other signaling cascades including LKB-MARK1/4,[46] PI3K-AKT/AP1,[47–49] CKD5-DISC1-Ndel1,[7] JNK[50,51] and actin binding.[31] Our studies have focused on the late neurally enriched, Wnt/β-catenin pathway active isoform of DIXDC1 for which we found the most compelling genetic evidence for association with psychiatric disorders (Figures 4b and c). It is plausible that genetic variants affecting more than one DIXDC1 isoform (Supplementary Tables 2 and 3) contribute to psychiatric susceptibility by disrupting multiple biological pathways.[7]

Several different biochemical mechanisms have been proposed to underlie the anxiolytic, antidepressant and mood-stabilizing properties of lithium, a drug whose systematic use in modern psychiatry began in the first half of the last century.[52] Lithium's best-validated mechanisms of action are inhibitory effects on IMP and INPP1, central phosphatases in the phosphoinositide pathway and on GSK3, the central kinase in the Wnt/β-catenin and AKT pathways.[53] Our data showing that loss of Dixdc1 in mice leads to impaired neuronal Wnt/β-catenin signal-transduction and gene-dose-sensitive behavioral phenotypes rectified by lithium or a selective GSK3i support that GSK3 inhibition is a major contributor to lithium's therapeutic action. Moreover, in this mouse model the correlation between GSK3, behavior, dendritic spine and glutamatergic synapse phenotypes supports the notion that spines and glutamatergic synapses are critical biological substrates underlying lithium-responsive psychiatric conditions.[54–56]

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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