Title

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Journal

Complex Psychiatry, 2(2)

ISSN

2673-3005

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Publication Date

2016

DOI

10.1159/000446778

Peer reviewed

Original Paper

Molecular Neuropsychiatry

Mol Neuropsychiatry 2016;2:107–114
DOI: 10.1159/000446778

Received: January 14, 2016
Accepted: May 11, 2016
Published online: June 24, 2016

The Planar Cell Polarity Transmembrane Protein Vangl2 Promotes Dendrite, Spine and Glutamatergic Synapse Formation in the Mammalian Forebrain

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Key Words
Dendrite · Forebrain · Planar cell polarity · Pyramidal neuron · Spine · Synapse · Vangl2 · Wnt

Abstract
The transmembrane protein Vangl2, a key regulator of the Wnt/planar cell polarity (PCP) pathway, is involved in dendrite arbor elaboration, dendritic spine formation and glutamatergic synapse formation in mammalian central nervous system neurons. Cultured forebrain neurons from Vangl2 knockout mice have simpler dendrite arbors, fewer total spines, less mature spines and fewer glutamatergic synapse inputs on their dendrites than control neurons. Neurons from mice heterozygous for a semidominant *Vangl2* mutation have similar but not identical phenotypes, and these phenotypes are also observed in Golgi-stained brain tissue from adult mutant mice. Given increasing evidence linking psychiatric pathophysiology to these subneuronal sites and structures, our findings underscore the relevance of core PCP proteins including Vangl2 to the underlying biology of major mental illnesses and their treatment.

Nathan D. Okerlund and Robert E. Stanley contributed equally.

Introduction

Dendrite, dendritic spine and excitatory (glutamatergic) synapse formation and plasticity are molecularly interrelated developmental prerequisites for proper brain function and behavior. Defects in spine and synapse formation and turnover are increasingly understood to be key contributors to neuropsychiatric disorders including autism, schizophrenia and major affective disorders [1–5].

Cell communication pathways with well-established roles in other aspects of development – including both the Wnt/β-catenin pathway [6–8] and the Wnt/planar cell polarity (PCP) pathway [9–11] – participate in these processes. The four-pass transmembrane protein Van Gogh-like 2 (Vangl2) is a key player in the PCP pathway and interacts with several proteins that influence synapse formation, including other PCP proteins such as Dishevelled (Dvl) [12] and Dapper-antagonist of catenin-1 (Dact1) [13] as well as the postsynaptic protein PSD95 [14–16]. Vangl2 also participates in signaling upstream of small GTPases [17, 18] regulating cytoskeletal dynamics crucial to dendrite and dendritic spine formation and plasticity [19–22]. *Looptail (Lp)* is a missense mutation in *Vangl2* [23] that causes semidominant phenotypes reflective of abnormal PCP including, in heterozygous animals, the curled or kinked tail from which the mutation gets its name, and in homozy-

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gous animals, craniorachischisis, a completely open neural tube and exposed brain [23–25]. Genetically engineered *null* mutations in Vangl2 cause similar phenotypes, but recessively and with lower penetrance in homozygous mutant animals [26, 27]. Using both allele types, we show here that Vangl2 functions during neural differentiation in dendrite arborization, spine formation, spine maturation and glutamatergic synapse formation.

Methods

Genetics

The *Vangl2*$^{\Delta}$ allele [26] is here referred to as *Vangl2KO* or *Vangl2*$^{-}$. The *Ltap*^{Lp} allele (Jackson Laboratory stock No. 000220) [23] is here referred to as *Vangl2*^{Lp} or *Lp*. All assays compared littermates of the designated experimental and control genotypes derived from *Vangl2*$^{-/+}$ or *Vangl2*$^{Lp/+}$ intercrosses.

Recombinant DNA

The mouse Vangl2 cDNA clone and expression plasmid has been described previously [28].

Primary Culture and Immunostaining

Dissociated neurons were obtained from embryos, fixed, transfected with pEGFP-C1 (Clontech) and immunostained for synaptic markers as previously described [29, 30]. Hippocampal cultures were used where possible because of the ease of producing populations of predominantly pyramidal neurons and prior validation as a model system relevant to dendrite, spine and synapse formation in the forebrain [31]. The borders of the hippocampus were not consistently identifiable in mutant homozygotes (both *Vangl2*$^{\Delta/\Delta}$ and *Vangl2*$^{Lp/Lp}$) due to craniorachischisis and neural precursor migration defects [27, 32]. Therefore, for these genotypes the entire forebrain (cortex and hippocampus) was prepared and compared to entire forebrain cultures of wild type (WT) and heterozygous littermates.

Visualization and Quantification

Cells were visualized on a Nikon CS1i upright spectral confocal at ×40 magnification or a custom-built spinning disc confocal microscope (Zeiss Axiovert 200M with Perkin-Elmer spinning disc and Melles Griot 43 series ion laser, Cascade 512B digital camera; Roper Scientific) at ×40 magnification. Golgi images were obtained on an Olympus IX51 compound inverted fluorescence microscope, also at ×40. Images were analyzed with ImageJ software (NIH). Sholl analysis, dendritic spine binning and spine and synapse quantification were performed as previously described [30].

Golgi Staining

Golgi-Cox silver staining was performed on 4-month-old littermates using the FD GolgiStain kit (FD Neurotechnologies) according to the manufacturer's instructions.

Statistics

All p values were calculated by unpaired parametric t tests (2-way comparison) or one-way ANOVA (≥3 comparisons) with Tukey's post hoc analysis using Graphpad Prism software. Each comparison entailed ≥8 neurons and ≥11 dendrites per condition derived from multiple independent experiments; all reported differences reflect a minimal $p \leq 0.05$ for experimental vs. control mice.

Results

Genetic Elimination of Vangl2 Reduces Dendrite Arbor and Spine Formation

On inspection, cultured *Vangl2*$^{-/-}$ forebrain pyramidal neurons had simpler dendrite arbors than controls (fig. 1a vs. b). Sholl analysis confirmed that mutant neurons had reduced numbers of dendrite branch crossings (fig. 1f). The maturation and density of dendritic spines were also affected (fig. 1a′ vs. b′): the density of spines along dendrites was lower in *Vangl2*$^{-/-}$ neurons compared to WT (fig. 1g). Moreover, *Vangl2*$^{-/-}$ neurons had an increased percentage of immature (i.e. filopodial) relative to mature (i.e. thin, mushroom or stub-shaped) spines (fig. 1h).

Recombinant expression of Vangl2 rescued the dendrite arbor phenotype in *Vangl2*$^{-/-}$ neurons (fig. 1c, f). It also rescued spine density and spine maturity (fig. 1a′, c′, g, h).

Vangl2 Overexpression and Heterozygosity Do Not Alter Dendrites or Spines

To investigate effects of other genetic manipulations expected to alter (but not eliminate) Vangl2 levels, we examined phenotypes in WT neurons recombinantly overexpressing Vangl2, and also in heterozygous *(Vangl2*$^{-/+}$*)* neurons. Neither of these genetic manipulations had any effect on dendrite complexity (fig. 1a vs. d, e; f), spine density (fig. 1a′ vs. d′, e′; g) or spine maturity (fig. 1a′ vs. d′, e′; h).

Vangl2$^{-/-}$ *Dendrites Have Fewer Glutamatergic Synaptic Contacts*

We quantified density of glutamatergic synapses along dendrites by visualization with antibodies specific for VGlut1 (presynaptic marker) and PSD95 (postsynaptic marker). *Vangl2*$^{-/-}$ neurons had reduced glutamatergic synapse density (fig. 1i vs. j; n). Recombinant expression of Vangl2 in *Vangl2*$^{-/-}$ neurons rescued glutamatergic synapse density (fig. 1j vs. k; n). Neither WT neurons recombinantly overexpressing Vangl2 (fig. 1l) nor *Vangl2*$^{-/+}$ neurons (fig. 1m) were significantly different from WT in this assay (fig. 1n). In contrast to this glutamatergic synapse phenotype, *Vangl2*$^{-/-}$ neurons had no significant reduction in inhibitory (GABAergic) synapse density on their dendrites measured similarly (data not shown).

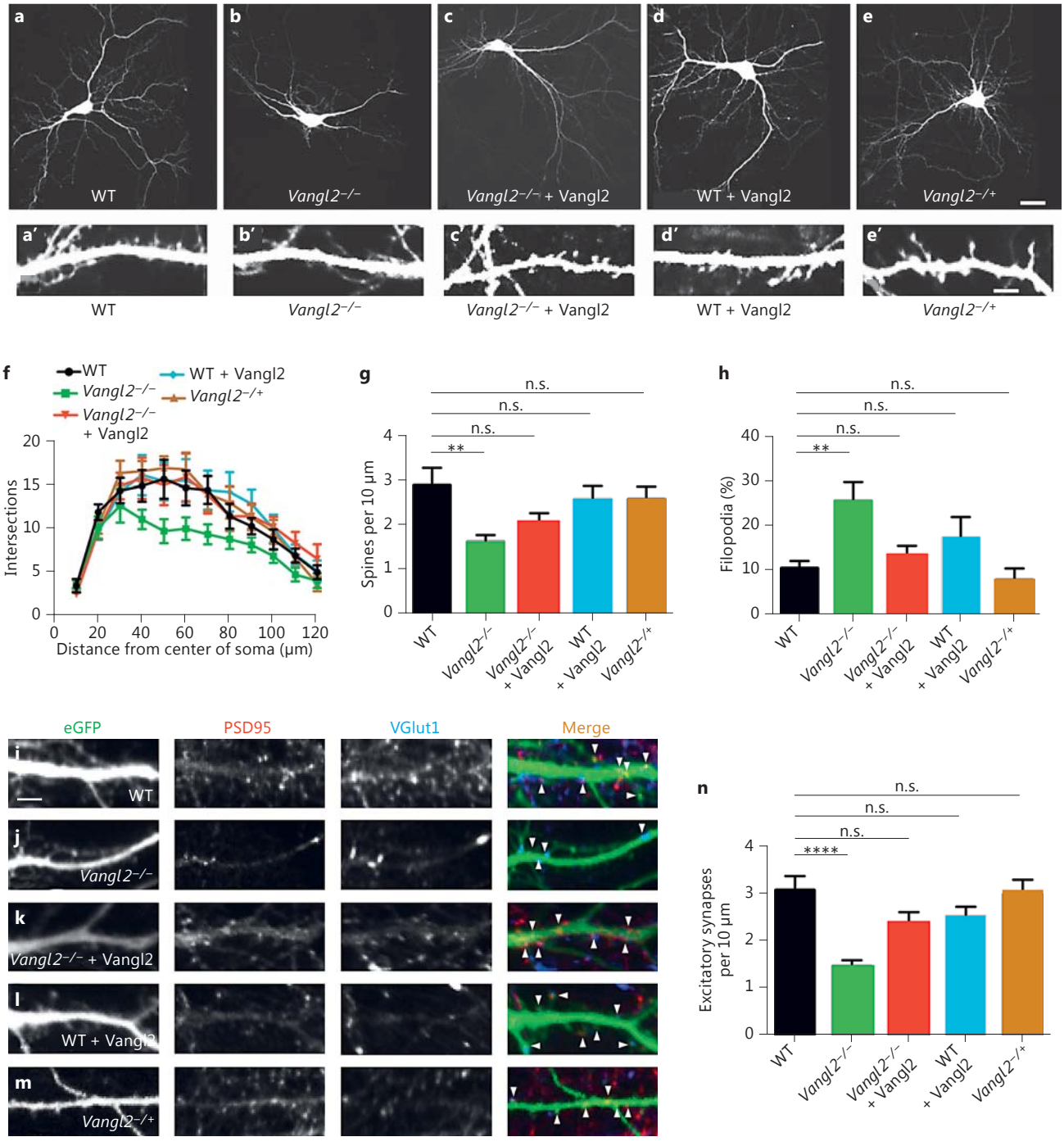
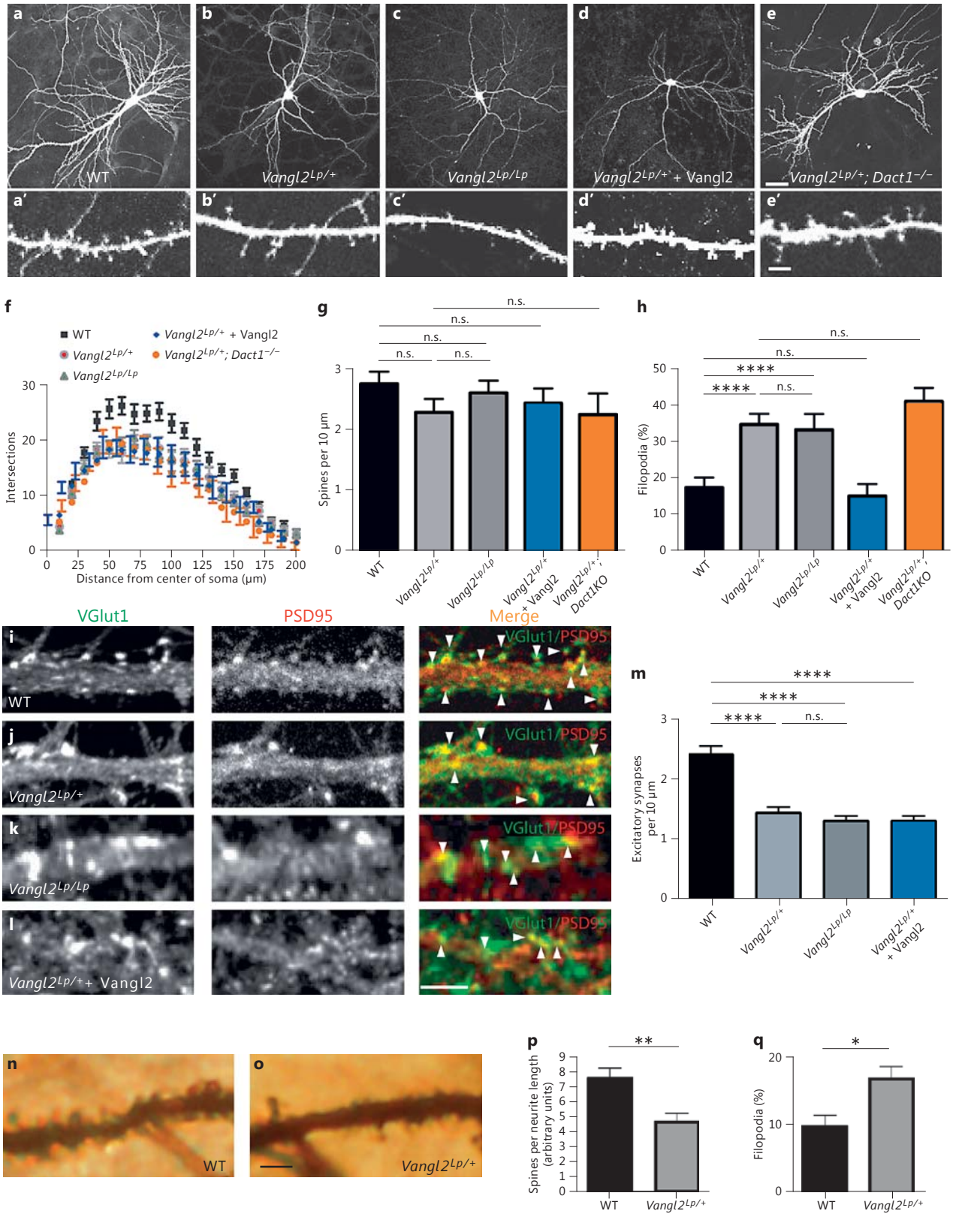


Fig. 1. Differentiation phenotypes in cultured *Vangl2KO* forebrain neurons. **a–e** EGFP-transfected cultured forebrain neurons from WT (**a**), *Vangl2*$^{-/-}$ (**b**), *Vangl2*$^{-/-}$ + Vangl2 (**c**), WT + Vangl2 (**d**) and *Vangl2*$^{-/+}$ (**e**). **a′–e′** Corresponding dendritic segments at a higher magnification. **f–h** *Vangl2*$^{-/-}$ neurons have simpler dendritic arbors as quantified by Sholl analysis (**f**), fewer dendritic spines (**g**) and less mature spines (**h**) than controls. **i–m** Immunostaining for glutamatergic synapse markers along dendrite segments from WT (**i**), *Vangl2*$^{-/-}$ (**j**), *Vangl2*$^{-/-}$ + Vangl2 (**k**), WT + Vangl2 (**l**) and *Vangl2*$^{-/+}$ (**m**) neurons. **n** Quantification. Scale bars = 30 μm (**a–e**); 5 μm (**a′–e′**, **i–m**). n.s. = $p > 0.05$, ** $p \leq 0.01$, **** $p \leq 0.0001$.



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Lp Causes Mixed Effects on Dendrite Arbors, Spines and Glutamatergic Synapses

As with *Vangl2*$^{-/-}$ neurons, upon visual inspection, *Vangl2*$^{Lp/+}$ neurons had simpler dendrite arbors than WT (fig. 2a, b). Interestingly, although homozygous *(Vangl2*$^{Lp/Lp}$*)* mice have more severe cell polarity phenotypes than heterozygous *(Vangl2*$^{Lp/+}$*)* mice in embryonic axis elongation, inner ear epithelia, and neural precursor proliferation and migration [26, 32], neurons from *Vangl2*$^{Lp/Lp}$ mice did not have a greater decrease in dendrite complexity than those from *Vangl2*$^{Lp/+}$ mice (fig. 2b, c). Sholl analysis revealed that *Lp* neurons, whether heterozygous or homozygous, had similar reductions in number of dendrite branch crossings (fig. 2f). Dendritic spines were also affected by the *Lp* mutation, not in terms of density (fig. 2a′, b′, c; g) but in terms of maturity (fig. 2h).

Similar to *Vangl2*$^{-/-}$ neurons, glutamatergic synapse density along dendrites of *Vangl2*$^{Lp/+}$ and *Vangl2*$^{Lp/Lp}$ neurons was reduced compared to WT (fig. 2i–k; m). As in *Vangl2*$^{-/-}$ neurons, GABAergic synapse density was unaffected (data not shown).

Vangl2 Overexpression Rescues Only Some Lp Neurodevelopmental Phenotypes

As stated above, neurons carrying the *Vangl2*^{Lp} allele, whether heterozygous or homozygous, displayed similar decreases in dendrite complexity, spine maturation and glutamatergic synapse density. Interestingly, only the spine maturation phenotype was rescued by recombinant expression of Vangl2: recombinant overexpression of Vangl2 did not rescue dendrite complexity (fig. 2e, f), nor did it rescue glutamatergic synapse density along dendrites in *Lp* mutant neurons (fig. 2l, m). In contrast, overexpression of WT Vangl2 did rescue spine maturity in these neurons (fig. 2b′, d′; h).

Elimination of Dact1 Does Not Rescue Lp Neurodevelopmental Phenotypes

We previously showed that genetic loss of the Wnt signal pathway scaffold protein Dact1 can rescue embryonic phenotypes in *Vangl2*$^{Lp/+}$mice [13]. Dact1 is expressed in differentiating forebrain neurons, and its loss causes reductions in dendrite arbor complexity, dendritic spine maturity and glutamatergic synapse formation [30] similar to the *Vangl2* mutant phenotypes reported here. Nonetheless, neurons from *Vangl2*$^{Lp/+}$; *Dact1*$^{-/-}$ mice had no rescue of dendrite complexity (fig. 2b vs. e; f) and no rescue of spine maturity (fig. 2b′ vs. e′; h) relative to *Vangl2*$^{Lp/+}$ neurons.

Golgi Staining Confirms Spine Reductions in the Vangl2 Mutant Forebrain

All the preceding assays were conducted using cultured forebrain neurons. To confirm that similar phenotypes occur in intact mammalian forebrain tissue, we analyzed the morphology of pyramidal neurons in the CA1 region of the hippocampus via Golgi-Cox staining on brains taken from adult (4- to 6-month-old) *Vangl2*$^{Lp/+}$ and littermate control mice. (The prenatal death of *Vangl2*$^{-/-}$ and *Vangl2*$^{Lp/Lp}$ mice precluded such analysis.) CA1 pyramidal neurons in *Vangl2*$^{Lp/+}$ mice had decreased spine density on apical dendrites compared to controls (fig. 2n–p). They also had an increased percentage of immature (filopodial) projections (fig. 2q).

Fig. 2. Differentiation phenotypes in *Vangl2*^{Lp} forebrain neurons. **a–e** EGFP-transfected cultured neurons from WT (**a**), *Vangl2*$^{Lp/+}$ (**b**), *Vangl2*$^{Lp/Lp}$ (**c**), *Vangl2*$^{Lp/+}$ + Vangl2 (**d**) and *Vangl2*$^{Lp/+}$; *Dact1*$^{-/-}$ (**e**). **a′–e′** Corresponding dendritic segments at a higher magnification. **f–h** Quantification. *Vangl2*^{Lp} neurons have simpler dendritic arbors (**f**), no reduction in total density of dendritic projections (spines + filopodia) (**g**), but a larger proportion of immature (filopodial) dendritic projections (**h**) than controls; only the last phenotype is rescued by recombinant expression of Vangl2 (blue bar; color refers to the online version only). **i–l** Immunostaining for glutamatergic synapse markers along dendrite segments from WT (**i**), *Vangl2*$^{Lp/+}$ (**j**), *Vangl2*$^{Lp/Lp}$ (**k**) and *Vangl2*$^{Lp/+}$ + Vangl2 (**l**) cultured neurons. **m** Quantification. **n**, **o** Segments of apical dendrite from a Golgi-stained pyramidal neuron in hippocampal CA1 of WT (**n**) and *Vangl2*$^{Lp/+}$ (**o**) littermates. Quantification of total spines (**p**) and immature (filopodial) spines (**q**). Scale bars = 30 μm (**a–e**); 5 μm (**a′–e′**, **i–l**, **n**, **o**). n.s. = $p > 0.05$, * $p \leq 0.05$; ** $p \leq 0.01$, **** $p \leq 0.0001$.

Discussion

Loss of Vangl2 function, whether via the semidominant *Lp* missense mutation or a targeted knockout, leads to decreased dendrite arbor complexity, spine maturity and glutamatergic synapse density without similar losses in GABAergic synapse density on forebrain pyramidal neuron dendrites. As dendritic spines are the specific subcellular site of glutamatergic synapses in pyramidal neurons, these data are consistent with previous findings that Vangl2 localizes to the postsynaptic compartment of glutamatergic synapses, where it interacts with PSD95, transsynaptic adhesion molecules and the PCP pathway protein Prickle2 [14–16]. Our genetic data corroborate previous reports of similar phenotypes following shRNA-mediated knockdown of Vangl2 in cultured neu-

rons [15, 33] and have allowed us to compare and contrast neurodevelopmental phenotypes induced by two molecularly distinct (engineered *null* vs. spontaneous missense) alleles at this locus.

Unlike the *KO (null)* allele, the *Lp* allele of *Vangl2* is not a simple loss of function: it causes dominant phenotypes to varying degrees in different biological contexts and exerts complex cell biological and biochemical effects on the encoded protein [12, 26, 27, 34]. Unlike homozygous *Vangl2KO* neurons, *Lp* neurons do not have reductions in spine density and their dendrite complexity and glutamatergic synapse phenotypes are not rescued by recombinant overexpression of Vangl2; this suggests that Vangl2 has a molecularly distinct role in spine formation and maturation compared to dendrite arborization and glutamatergic synapse formation. The differences in rescue of these *Lp* neurodevelopmental phenotypes cannot be explained by different temporal requirements for Vangl2 in these subneuronal compartments and processes, because recombinant expression of Vangl2 in the same manner rescues all four phenotypes (dendrite complexity, spine number, spine maturity and glutamatergic synapse density) in $Vangl2^{-/-}$ neurons.

Conclusions

PCP Pathway Proteins Play Important Divergent Roles in Neurons

Genetically altering Vangl2 function, whether by semidominant missense ($Vangl2^{Lp}$) or engineered knockout *(Vangl2KO)*, results in forebrain pyramidal neurons with simpler dendrite arbors, a larger proportion of immature spines and fewer glutamatergic synapses. Genetic disruption of other PCP genes in mammals, including Dvl1 [35] and Dact1 [30], causes similar phenotypes. Given the widespread neural expression of Vangl2 and several other PCP genes during prenatal development, postnatal development and in the mature brain, it is plausible that the neural functions of these molecules are similarly widespread and continuous over the lifespan. In prior work, we have demonstrated requirements for the Vangl2 partner Dact1 during dendrite, spine and synapse development in both pyramidal neurons [30] and interneurons of the cerebral cortex [36, 37]. However, mutations in *Vangl2* and *Dact1* that exhibit strong mutual rescue during gastrulation [13] do not exhibit similar reciprocal functional relationships during neurodevelopment, suggesting that the molecular mechanisms underlying these phenotypes differ. Consistent with other studies [15, 38, 39] our genetic work therefore suggests that although components of the PCP pathway play important roles in the nervous system, the molecular pathways by which they function in developing neurons differ substantially from the PCP pathway established in studies of basic embryonic development.

The Goldilocks Principle in Molecular Neuropsychiatry

Evidence increasingly supports that neurodevelopmental and neuroplastic processes regulating spine and glutamatergic synapses contribute to the pathogenesis of psychiatric conditions including autism, schizophrenia and major affective disorders [2–4, 40–43]. In line with this, several PCP proteins contributing to these processes have been implicated in psychiatric pathophysiology. For example, in mice, the elimination of Dvl1 reduces social behavior [44] and Prickle2 sequence variants associated with autism lead to dendrite and glutamatergic synapse phenotypes [45]. Our work with Vangl2 accords with these findings and underscores that at some genetic loci and in some biochemical pathways different molecular defects can lead to similar neural phenotypes. We refer to the general idea that similar neural and behavioral phenotypes can result from functionally different and even opposite molecular defects as the 'Goldilocks Principle' – i.e. either *too much* or *too little* can be deleterious in molecular neuropsychiatry. This is, in fact, a well-established theme for Wnt/PCP signaling in other developmental contexts [13, 46, 47]. A similar phenomenon has been observed for loci contributing to neuropsychiatry through entirely different mechanisms, including ion channel proteins such as KCNA2 for which both gain- and loss-of-function mutations cause epilepsy [48] and CACNA1C for which both a gain-of-function mutation and reduced expression variants are associated with bipolar disorder [49]. This is now also firmly established for several copy number variants, such as 7q11.23 and 22q11.2, that contribute to psychiatric susceptibility either when deleted or duplicated [3, 50].

Acknowledgments

N.D.O. was supported in part by Autism Speaks Predoctoral Fellowship 4654. R.E.S. was supported in part by supplement R01HD55300-S1 to B.N.R.C. This research was also supported by a Simons Foundation Autism Research Initiative Grant (B.N.R.C.). We thank all members of the Cheyette laboratory for advice and support, and Yingzi Yang (NHGRI) for providing the $Vangl2^{\Delta}$ allele.

Statement of Ethics

All procedures involving live animals were carried out in accordance with an IACUC-approved animal use protocol at the University of California, San Francisco.

Disclosure Statement

The authors have no conflicts of interest to report.

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