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LETTER TO THE EDITOR

GABA Signaling Pathway-associated Gene *PLCL1* Rare Variants May be Associated with Autism Spectrum Disorders

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Dear Editor,

Autism spectrum disorders (ASDs) are neurodevelopmental disorders with phenotypic and genetic heterogeneity, and are among the most heritable of neurodevelopmental disorders [1]. Rare single nucleotide variants (SNVs) of genes and/or rare copy number variants (CNVs) involving gene variants and/or genomic imbalances play an important role in ASD, but their molecular pathogenic mechanisms remain indistinct [2]. Over the decades, genetic and neurobiological studies mainly involving severe ASD comorbid with intellectual disability (ID) or developmental delay (DD) have indicated that loss of function affects neural development [3].

GABAergic signaling and impaired $GABA_A$ receptor function may contribute to the pathogenesis of multiple neuropsychiatric disorders including ASD [4]. Phospholipase C-like 1 (PLCL1), also named phospholipase C (PLC)-related inactive protein type 1 (PRIP-1), plays a central role in controlling $GABA_A$ receptor phospho-dependent modulation, and therefore, the efficacy of synaptic inhibition mediated by these receptors [5]. Several studies have focused on the mouse PLCL1 loss-of-function models. The *Plcl1*$^{-/-}$ mice exhibit altered behaviors including frequent ambulation and impaired motor coordination [6], or show an epileptic phenotype [7]. While in double knockout of *Plcl1* and *Plcl2* (compensatory to *Plcl1* function) mice, orofacial movements are affected; including reduced vertical jaw movements, tongue protrusions and movements of the head and vibrissae [8]. In humans, a pediatric specific Phenome-Wide Association Study revealed that one single nucleotide polymorphism (rs1595825) in *PLCL1* intron 1 is associated with developmental delays of speech and language [9]. Despite the relevant studies on animal models and human, the role of *PLCL1* in neural development, particularly, whether ASD patients have rare *PLCL1* variants, and whether these variants have harmful effects that may be associated with the development of ASD remains unknown.

To identify rare variants of the *PLCL1* gene in ASD, we first screened 437 American patients with ASD using array comparative genomic hybridization (array CGH), and identified 35 pathogenic CNVs (supplementary materials and methods). Among them, a 542,605 bp microdeletion (542 kb deletion) located in chromosome 2q33.1 was

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identified in one of the 437 patients (Table S1), which covers the exons 2–6 of the *PLCL1* gene, *LOC101927619* (long intragenic non-protein coding RNA 1923), exons 3 and 4 of *LOC105373831,* and the whole length of *LOC105373830* (two uncharacterized non-coding RNA genes). Neither of the patient's parents carried the same variant, and no similar loss was found in 8,045 non-neurological/psychiatric patients [the database of Genetic Diagnostics Lab of Boston Children's Hospital (BCH-GDL database)]. Therefore, we conclude that the 542 kb deletion found in ASD is rare and *de novo*.

We further searched several publicly available databases of CNVs or SNVs detected/submitted by clinical genetic diagnosis laboratories. In the DECIPHER database (website: decipher.Sanger.ac.uk), we found approximately 45 genomic deletions and duplications that included an overlapped region similar to the 542 kb deletion. Of them, 6 relevant smaller microdeletions were selected to compare with the 542 kb deletion (Table S1). Notably, all 6 deletions (range 2.54–6.94 Mb) were associated with DD, but were not seen in normal controls. Furthermore, the *PLCL1* deletion was the only gene shared within all 7 cases (Table S1). Most interestingly, their phenotypes were comorbid within all 7 cases: 6/7 had ID and 4/7 had delayed speech and language development, which were frequently observed in patients with ASD/ID/DD; while the 542 kb deletion only involved one coding gene, *PLCL1*. Therefore, these results strongly suggest that the 542 kb deletion/*PLCL1* gene deletion is likely a pathogenic variant associated with ASD/DD/ID. We further performed functional studies of *PLCL1* in mice.

Moreover, we screened 455 Han Chinese patients with ASD, and found four missense variants of *PLCL1* in coding sequence (c.82 G>A, c.311 A>G, c.434 G>A, and c.2922 G>T) and two non-coding region variants in the upstream regulatory region of *PLCL1* (c.−636 C>A and c.−136_−134 delGCC) by Sanger sequencing (Fig. 1A, Table S2). All the variants were heterozygous (Fig. S1) and existed in unrelated individual samples. Moreover, the variants were not present in 576 control DNA samples or in the dbSNP, Ensembl, and ExAC databases, thus we considered they may be rare variants that only present in ASD patients.

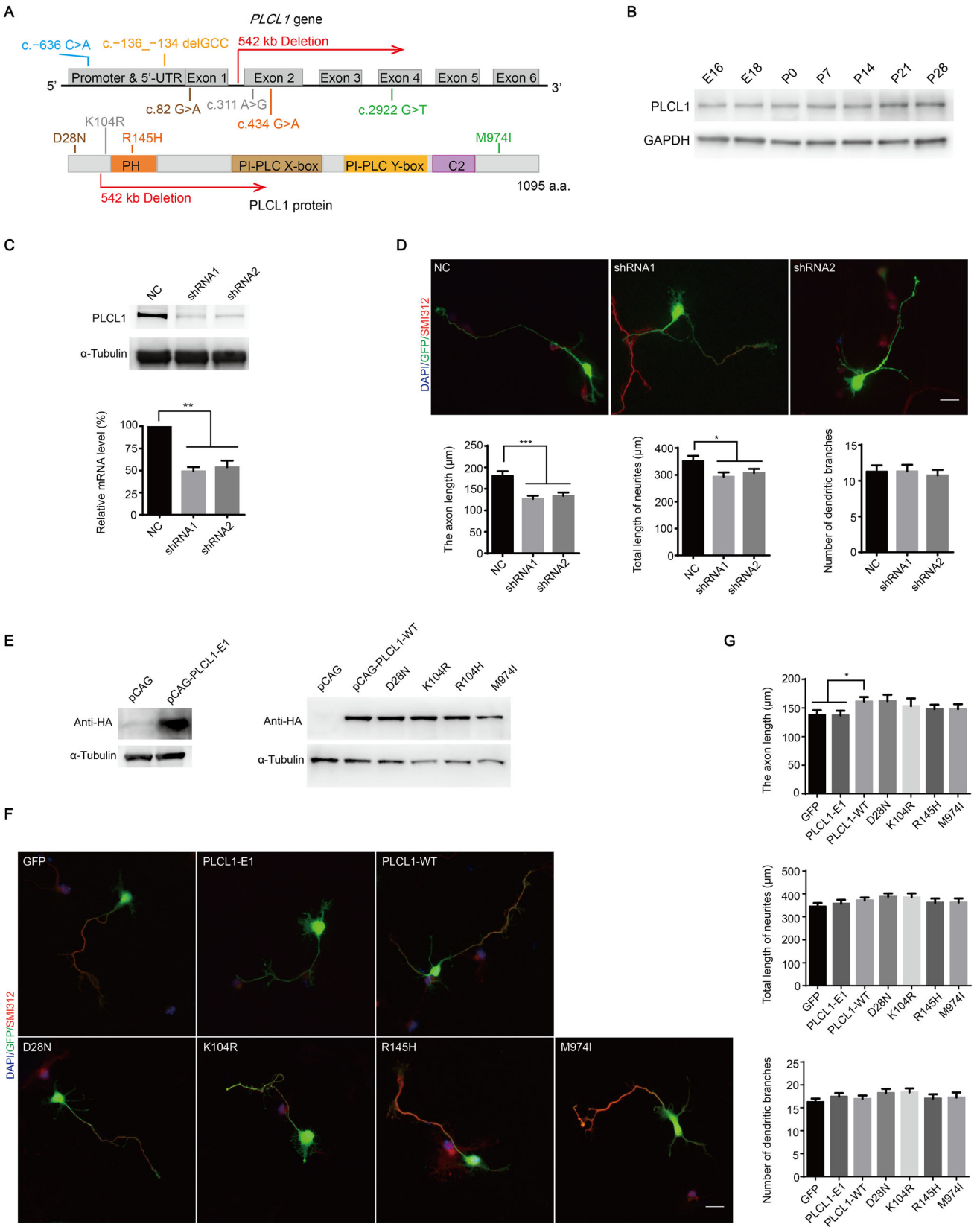
By genetic/genomic screening, it seemed that the *PLCL1* deletion and variants were not uncommon in ASD/DD/ID, and potential detrimental effects (Tables S1, S2; Fig. S2) of the above *PLCL1* variants may exist, so we subsequently investigated the role of *PLCL1* in neural development, particularly whether these rare *PLCL1* variants have loss- or gain-of-function effects.

First, we explored *Plcl1* gene expression in the early stage of mouse cerebral cortex. We examined PLCL1 protein levels in embryonic (E) and postnatal (P) cortex of mouse brains and found that PLCL1 was strongly expressed from E16 to P28 (Fig. 1B). Furthermore, human PLCL1 protein has been reported to be distributed specifically in the brain and expressed abundantly in the cerebral cortex and hippocampus [10]. Taken together, the results suggest that PLCL1 may play an important role in brain development and normal neuronal function.

The formation of protrusions and the abnormal elongation of axons and dendrites may be the structural basis for the pathogenesis of ASD [11]. To elucidate the role of PLCL1 in the growth of neuronal axons and dendrites, we specifically knocked down *Plcl1* in mouse primary cortical neurons with short hairpin RNAs (shRNAs) (Fig.1C), and found that the length of axons and dendrites were significantly decreased compared with control neurons that were transfected with nonspecific control shRNAs (NC), while the number of dendritic branches was not affected (Fig. 1D).

Since the encoded peptide chain of exon 1 of *PLCL1* does not locate in the coding region of any domain of *PLCL1* (Fig. 1A), we speculate that the deletion of exons 2–6 of the *PLCL1* gene found in ASD patients may cause loss-of-function of PLCL1 protein and affect the proper neurite development, similar to the effect of reducing PLCL1 protein. To explore whether the deletion of exons 2–6 and the four missense variants of *PLCL1* detected in ASD patients might affect the normal function of PLCL1 protein, we transfected the plasmids only expressing GFP

Fig. 1 The dosage of PLCL1 is crucial for neurite outgrowth of primary cultured mouse cortical neurons, and the deletion of *PLCL1* exons 2–6 affects the promotion of neuronal growth, while *PLCL1* missense variants have no effect. **A** The location of *PLCL1* rare variants in studied ASD patients on the *PLCL1* gene and coding protein. Colored bars represent the PLCL1 protein functional domains. **B** The PLCL1 expression pattern in mouse cerebral cortex during the embryonic and postnatal period. **C** The efficiency of endogenous *Plcl1* knockdown was verified using shRNA packaged in lentivirus by Western blot (top) or using pFUGW-H1-shRNA plasmids by qRT-PCR (bottom) in primary cultured mouse cortical neurons. **D** *Plcl1* knockdown affects mouse neuronal growth. Upper panel shows the specific cellular morphology of neurons transfected with pFUGW-H1 vector (NC, as a control), *Plcl1* shRNA1 and shRNA2. All neurons were co-labeled with DAPI (to identify nuclei), GFP (to identify overall neuronal morphology) and SMI 312 (an axonal marker). Scale bar, 20 μm. Bottom panel shows the statistical results of the axon length, total length of neurites, and the number of dendritic branches of mouse neurons with *Plcl1* knockdown. Approximately 90 cells from three independent experiments were counted and the *P*-value was determined by one-way ANOVA. $*P < 0.05$, $**P < 0.01$, $***P < 0.001$. **E** Western blot analysis of ectopic expression of PLCL1-WT, PLCL1-E1, and four missense variants in HEK-293T cells. **F** The morphology of neurons overexpressing the CAG vector (GFP, as a control), PLCL1-WT, PLCL1-E1, and four missense variants. Scale bar, 20 μm. **G** The axon length (upper), total length of neurites (middle), and the number of dendritic branches (bottom) were measured and analyzed as described above. Error bars, ± SEM.


A
PLCL1 gene
c.−636 C>A
c.−136_−134 delGCC
542 kb Deletion
Promoter & 5'-UTR
Exon 1
Exon 2
Exon 3
Exon 4
Exon 5
Exon 6
5'
3'
c.82 G>A
c.311 A>G
c.434 G>A
c.2922 G>T
K104R
D28N
R145H
M974I
PH
PI-PLC X-box
PI-PLC Y-box
C2
1095 a.a.
542 kb Deletion
PLCL1 protein
B
E16
E18
P0
P7
P14
P21
P28
PLCL1
GAPDH
C
NC
shRNA1
shRNA2
PLCL1
α-Tubulin
Relative mRNA level (%)
D
DAPI/GFP/SMI312
The axon length (μm)
Total length of neurites (μm)
Number of dendritic branches
E
pCAG
pCAG-PLCL1-E1
pCAG-PLCL1-WT
D28N
K104R
R104H
M974I
Anti-HA
α-Tubulin
F
GFP
PLCL1-E1
PLCL1-WT
D28N
K104R
R145H
M974I
G
The axon length (μm)
Total length of neurites (μm)
Number of dendritic branches

(as a control), or PLCL1-wild-type (WT), or PLCL1-E1 (the recombinant plasmid only including exon 1), or the four missense variants (Fig. 1E) into mouse primary cortical neurons, considering the PLCL1 is highly conserved between human and mouse (approximately 94.4% according to protein peptide alignment). We found that overexpression of PLCL1-WT significantly increased the axon length compared with control neurons expressing GFP alone (Fig. 1F, G), while the total length of neurites and the branching number of neuronal dendrites did not change. Overexpression of PLCL1-E1 had no effect on the neuronal growth, which resembled that of control (Fig. 1F, G). The result confirmed that PLCL1-E1 lacked the normal function of PLCL1-WT. In addition, the effect of overexpression of the four missense variants in mouse neurons did not significantly differ from that of PLCL1-WT (Fig. 1F, G), indicating that the four variants did not affect the normal function of PLCL1 in neuronal growth. Taken together, the PLCL1 dosage is critical for proper neurite and axonal outgrowth, and the deletion of *PLCL1* exons 2–6 is a loss-of-function mutant, while the four missense variants are not.

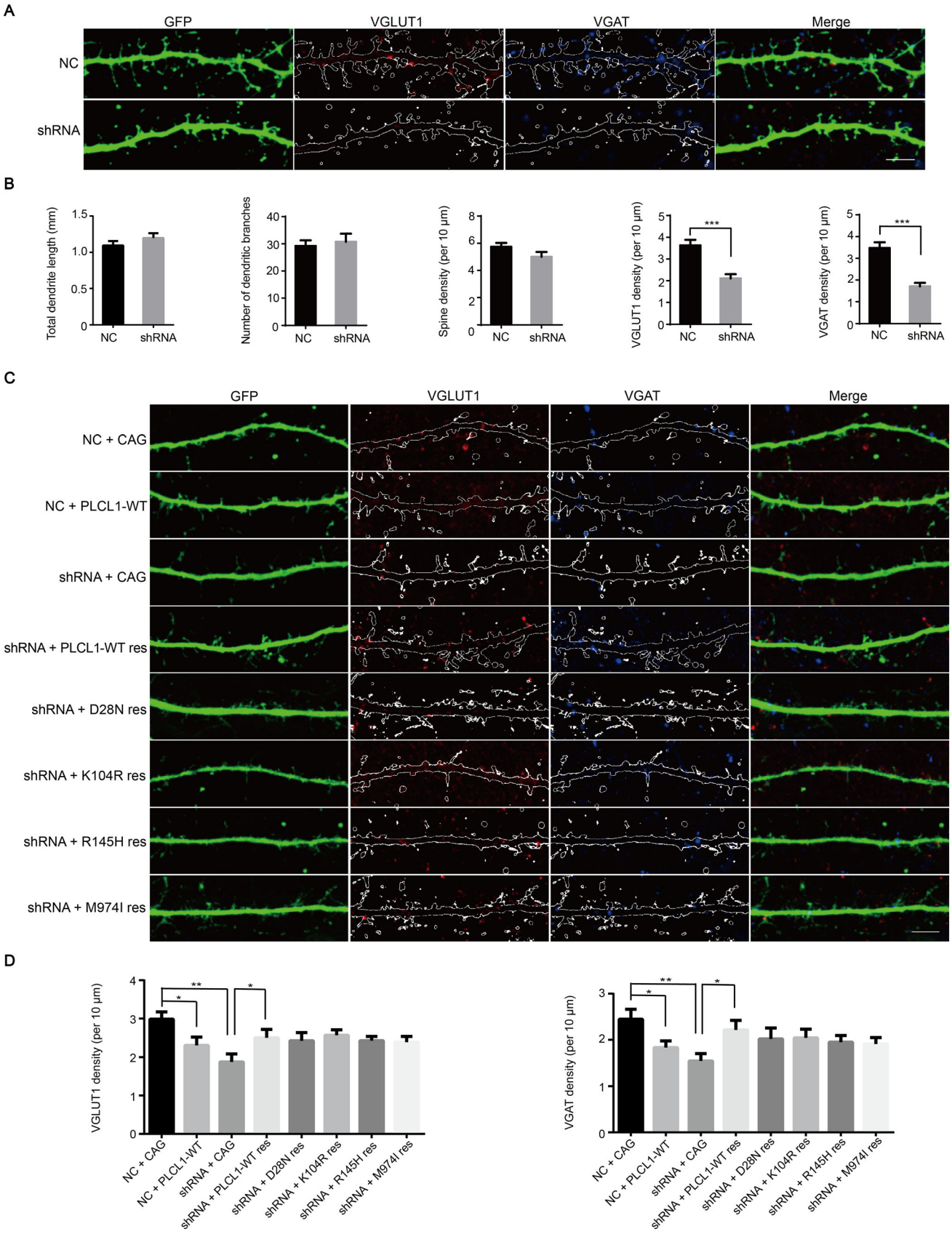
Dendritic spine abnormalities have been reported to be strongly associated with various neurological and neuropsychiatric disorders [12], and dysfunction of the GABAergic pathway in early development can lead to severe excitatory/inhibitory (E/I) imbalance in the neural circuitry, which may be the cause of behavioral defects in ASD patients [13]. Vesicular glutamate transporter 1 (VGLUT1) and vesicular GABA transporter (VGAT) are excitatory and inhibitory markers of synaptic transmission. To further explore the role of PLCL1 in synaptic development, we examined the function of PLCL1 in spine formation, dendrite development, and excitatory and inhibitory synapse formation by immunostaining in mouse primary cortical neurons. We found that *Plcl1* knockdown with shRNA resulted in a decrease in both VGLUT1 and VGAT density on dendrites and spines compared with the control, but not for the spine density, length of dendrites, and brunching number (Fig. 2A, B). Thus, *Plcl1* knockdown altered the excitatory and inhibitory synapse formation. Furthermore, PLCL1-WT successfully rescued the suppression of both VGLUT1 and VGAT density in *Plcl1* knockdown mouse neurons (Fig. 2C, D). The four missense variants had no significantly different effect compared with PLCL1-WT, indicating that they did not affect the normal function of PLCL1 in excitatory and inhibitory synapse formation (Fig. 2C, D).

In addition to PLCL1 depletion, overexpression of PLCL1 also reduced VGLUT1 and VGAT density (Fig. 2C, D), while the total dendrite length, branch number, and spine density were unaffected (Fig. S3). Thus, the dosage of PLCL1 is crucial for glutamatergic and GABAergic synapse development. We speculated that the variant with deletion of exons 2–6, a loss-of-function mutant as we mentioned above, may affect glutamatergic and GABAergic synapse development as well.

Fig. 2 PLCL1 is crucial in neuronal excitability and its dosage imbalance affects glutamatergic and GABAergic synapse development *in vitro*. **A** Decreased VGLUT1 and VGAT density caused by *Plcl1* knockdown. Morphology of the VGLUT1 and VGAT density of neurons transfected with *Plcl1* shRNA and control NC vector. The skeleton represents dendrite segments and dendritic spines. Scale bar, 5 μm. **B** Statistical analysis of the total dendrite length, branching number, spine density (per 10 μm), VGLUT1 density (red puncta number per 10 μm), and VGAT density (blue puncta number per 10 μm) between NC and shRNA groups. Forty neurons from three independent experiments were measured and counted. *P*-value is determined by unpaired *t*-test. ***P <0.001. **C** PLCL1-WT and mutant variants of PLCL1 rescues (res) the phenotype of decreased VGLUT1 and VGAT density caused by *Plcl1* knockdown. The specific morphology of neurons in each condition expressing the indicated vectors. All neurons were co-labeled with VGLUT1, VGAT, and GFP. Scale bar, 5 μm. **D** Statistical results for the VGLUT1 density and VGAT density between each group. Approximately 30 cells from three independent experiments were randomly selected and counted. *P*-value was determined by one-way ANOVA. Error bars, ± SEM. *P <0.05; **P <0.01.

According to the predicted transcription factors that bind to the *PLCL1* promoter (Table S2, Fig. S4A), we selected four relatively high-scoring transcription factors E2F4, E2F6, SP1, and GATA1 to assess the effects of the two promoter variants c.−636 C>A and c.−136_−134 delGCC on *PLCL1* transcription activity and protein expression. We ectopically expressed E2F4, E2F6, SP1, and GATA1 (Fig. S4B), and co-transfected the expression plasmids with wild-type *PLCL1* promoter (pGL3-WT, c.−1 to c.−901), mutant type c.−636 C>A (pGL3−636), c.−136_−134 delGCC (pGL3−136), and the empty vector into HEK-293T cells, and used promoter luciferase assays to determine the transcription activity of the promoters. We found that the activation of pGL3−136 by ectopic expression of GATA1 was significantly lower than that of pGL3-WT (Fig. S4C), while GATA1 had a similar strong activation effect on pGL3-WT and pGL3−636. E2F4, E2F6, and SP1 had similar effects on pGL3-WT and the two mutants (Fig. S4D). Therefore, we speculated that the mutant-type pGL3−136 may affect the transcriptional activation of *PLCL1* promoter by GATA1.

Consistent with the luciferase assay results, overexpression of E2F4, E2F6, and SP1 had no significant effect on PLCL1 expression in HEK-293T cells, while overexpression of GATA1 up-regulated PLCL1 protein (Fig. S4E). Moreover, GATA1-induced PLCL1 protein expression was weakened after ectopic *GATA1* knockdown (Fig. S4F). Therefore, we further verified that GATA1 has a positive regulatory effect on the expression of PLCL1 protein. Taken together, the rare variant c.−136_−134 delGCC of the *PLCL1* promoter in ASD patients suppressed the


A
GFP
VGLUT1
VGAT
Merge
NC
shRNA
B
Total dendrite length (mm)
Number of dendritic branches
Spine density (per 10 μm)
VGLUT1 density (per 10 μm)
VGAT density (per 10 μm)
***
NC
shRNA
C
GFP
VGLUT1
VGAT
Merge
NC + CAG
NC + PLCL1-WT
shRNA + CAG
shRNA + PLCL1-WT res
shRNA + D28N res
shRNA + K104R res
shRNA + R145H res
shRNA + M974I res
D
VGLUT1 density (per 10 μm)
VGAT density (per 10 μm)
**
*
NC + CAG
NC + PLCL1-WT
shRNA + CAG
shRNA + PLCL1-WT res
shRNA + D28N res
shRNA + K104R res
shRNA + R145H res
shRNA + M974I res

transcription-activating effect of GATA1 on *PLCL1*, and subsequently decreased PLCL1 expression. As abnormal expression of PLCL1 leads to abnormal neurite outgrowth and glutamatergic and GABAergic synapse development, we believe that the variant may be related to the pathogenesis of ASD.

In this study, we identified one *de novo PLCL1* variant, exons 2–6 deletion (within the 542 kb genomic deletion located on chromosome 2q33.1) in American ASD cohorts, and one *PLCL1* promoter rare variant c.−136_−134 delGCC in Chinese ASD patients, which may relate to the onset of ASD. A 778 kb duplication including *PLCL1* exons 2–6 was reported in one patient diagnosed with agenesis of the corpus callosum with ASD, DD, and seizures [14], which corroborated our observation that overexpression of PLCL1 reduced VGLUT1 and VGAT density, implying that the 542 kb deletion is likely related to neuronal development. Furthermore, whether the absence of three non-coding RNAs in this 542 kb region is involved in the pathogenesis of ASD warrants further research.

The four missense variants behaved similarly to PLCL1-WT when overexpressed in regard to neurite and axon outgrowth, and all could rescue the decreased VGLUT1 and VGAT density, that was caused by *Plcl1* knockdown. However, we cannot exclude that they may affect other functions of PLCL1, such as its function in the inositol 1,4,5-trisphosphate [Ins(1,4,5)P_3]-mediated Ca^{2+} signaling pathway, which was not assessed by this study. According to a "bilineal two-hit model" [15], that is, even rare SNVs are not a direct cause of ASD, they can increase the risk of ASD, thus the possibility that variants existing on other genes may be involved in the pathogenesis of ASD in cooperation with *PLCL1* variants should not be excluded.

In summary, we report and describe rare variants of the *PLCL1* gene in American and Chinese cohorts with ASD, and provided preliminary clues for further elucidating the role of PLCL1 in neuron development. Further research is needed to identify other essential genes which are required either independently or collaboratively with *PLCL1* for the phosphorylation or clustering of $GABA_A$ receptors, and to better understand the role of PLCL1 in GABAergic signaling as well as in the pathogenesis of central nervous system diseases including ASD.

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Conflict of interest The authors declare no conflict of interest in this work.

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