

Regulation of *Scn1b* and *Scn2a* by BCL9 in cortical neurodevelopment and neuronal excitability

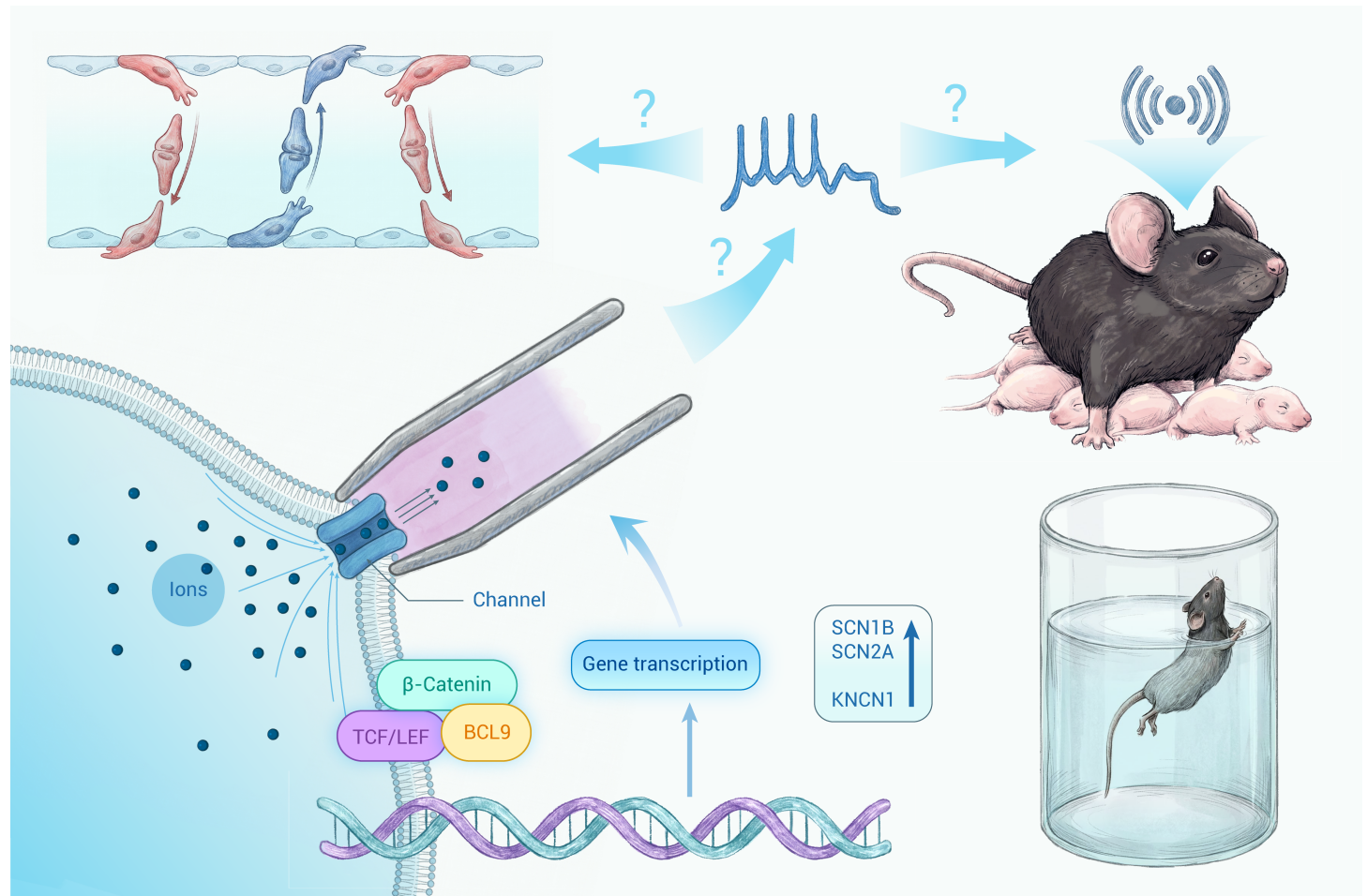
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GRAPHICAL ABSTRACT



PUBLIC SUMMARY

- BCL9 regulates cortical neurodevelopment and modulates sodium channel-dependent neuronal excitability.
- BCL9 controls *Scn1b/Scn2a* via transcription repression and post-translational coordination.
- BCL9 provides a prospective avenue for therapeutic intervention in neurodevelopmental disorders.

Regulation of *Scn1b* and *Scn2a* by BCL9 in cortical neurodevelopment and neuronal excitability

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The convergence of oncology and neurodevelopmental research has uncovered compelling links between genetic factors and mental health. Among these, B-cell chronic lymphocytic leukemia/lymphoma 9 (*BCL9*), located on human chromosome 1q21.1, has been identified as a key oncogene in precursor B-cell acute lymphoblastic leukemia (B-ALL). Subsequent studies have established that *Bcl9* promotes tumorigenesis through activation of the Wnt/ β -catenin signaling pathway. Emerging evidence from human genetic studies further implicates common variants of *Bcl9* in increased susceptibility to neurodevelopmental disorders, including schizophrenia and bipolar disorder. Despite growing interest in its role beyond cancer, the neurobiological functions of *Bcl9* remain poorly defined. In this study, we systematically investigated the role of *Bcl9* in neurodevelopment using mouse models. By employing in utero electroporation-mediated gene knockdown techniques and conditional knockout (cKO) mice, we demonstrate that *Bcl9* deficiency disrupts Wnt-dependent signal transduction and leads to upregulation of voltage-gated sodium channel (Nav) expression, thereby results in increased sodium current density but paradoxically reduced action potential firing. Based on our experimental findings, *Bcl9* and/or the *Bcl9* complexes may regulate the promoter region of the *Scn1b* gene and the area near the exons of *Scn2a*. Collectively, our findings establish *Bcl9* as a critical regulator of neuronal excitability and migration, providing a mechanistic framework that links ion channel dysregulation to the pathophysiology of neurodevelopmental disorders.

INTRODUCTION

The B-cell CLL/lymphoma 9 (*BCL9*) gene, initially identified for its role in precursor B-cell acute lymphoblastic leukemia, encodes a critical adaptor protein in the Wnt/ β -catenin signaling pathway.^{1,2} Whereas early investigations centered on *Bcl9*'s oncogenic potential in colorectal cancer and hematological malignancies,³⁻⁷ emerging evidence points to a broader role in developmental processes.^{8,9} Genetic ablation studies have illustrated that the simultaneous knockout of *Bcl9* and its paralog *Bcl9l* leads to embryonic lethality in murine models, highlighting its critical role in early development. Additionally, conditional knockout experiments reveal that *Bcl9* is essential for various organogenesis processes, notably impairing paired box protein (Pax6)-dependent ocular development.^{10,11} These findings suggest that *Bcl9* is not only integral to tumorigenesis but also plays a critical role in neural development, underscoring the need for further investigation into its multifaceted functions within neurodevelopmental pathways.

Voltage-gated sodium channels (VGSCs) are essential molecular components responsible for the initiation and propagation of action potentials in neurons, playing a critical role in maintaining normal nervous system function. *Scn1b* and *Scn2a* encode the $\beta 1$ auxiliary subunit and the principal α subunit Nav1.2 of sodium channels, respectively, both of which are vital for

regulating neuronal excitability, action potential generation, and neural circuit development. The $\beta 1$ subunit encoded by *Scn1b* not only modulates the electrophysiological properties and membrane localization of sodium channels but also influences axonal guidance and neural network formation through cell adhesion functions. The α subunit Nav1.2 encoded by *Scn2a* is abundantly expressed in the central nervous system and is directly involved in action potential initiation and conduction. Recent studies have increasingly linked *Scn1b* and *Scn2a* to a variety of neuropsychiatric disorders, including epilepsy, autism spectrum disorder (ASD), schizophrenia, and bipolar disorder,¹²⁻¹⁵ with mutations in these genes causing sodium channel dysfunction, neuronal hyperexcitability, and neural circuit disruption. Animal models and clinical genetic analyses have further validated their pivotal roles in disease pathogenesis. A deeper understanding of *Scn1b* and *Scn2a* functions not only sheds light on the molecular mechanisms underlying neurodevelopment and neuropsychiatric diseases but also provides a theoretical foundation and potential avenues for targeted sodium channel therapies.

Despite established associations between *Bcl9* and neurodevelopmental processes, direct experimental evidence defining the functional role of *Bcl9* in mammalian neural development remains scarce. Two critical knowledge gaps persist: (1) the precise spatiotemporal expression profile of *Bcl9* during key stages of neurodevelopment; and (2) how *Bcl9* deficiency impacts the maturation, structural integrity, and functional phenotypes of the central nervous system. To address these gaps, we generated *Bcl9*-deficient mouse models using in utero electroporation and gene knockout approaches, which we then utilized to investigate the aforementioned two key questions. In this study, we demonstrate enriched expression of BCL9 protein in embryonic neural tissues and characterize neurodevelopmental impairments following genetic perturbation. *Bcl9* deficiency disrupts Wnt signaling, leading to upregulated NAV expression and cortical neuron hyperexcitability, potentially through *Bcl9*'s regulation of *Scn1b* and *Scn2a* transcription via the promoter region of the *Scn1b* gene and the area near the exons of *Scn2a*. These findings establish *Bcl9* as a novel molecular regulator of neurodevelopment, offering new insights into the etiology of neuropsychiatric disorders and potential therapeutic targets for intervention.

MATERIALS AND METHODS

Mouse strains

Timed-pregnant C57BL/6 female mice were purchased from SLAC Laboratory Animal (Shanghai, China) for In Utero Electroporation. Mice were singly housed with food and water provided ad libitum and maintained on a 12 h light/12 h dark cycle. All animal experiments were performed according to the guidelines approved by the University Committee on Animal Care and Use of Shanghai Jiao Tong University, China. The Animal Protocol number is A2023190-001.

Bcl9 knockout (KO) mice were generated using transcription activator-like

effector nuclease (TALEN) technology (Cyagen Biosciences, Guangzhou, China). The mice *Bcl9* gene contains ten exons, with the ATG start codon in exon 2 and the TAA stop codon in exon 10. TALENs targeting exon 5 were designed, and TALEN mRNAs were microinjected into one-cell stage C57BL/6 embryos (Charles River, Canada). Offspring were screened for TALEN-induced deletions by PCR amplification of genomic DNA extracted from tail tips. A founder mouse harboring a 2-bp deletion (gggactccaaacg → gggac_ _caaacg) in *Bcl9* was identified, resulting in premature termination of BCL9 protein expression. This line was backcrossed with C57BL/6 mice (Charles River, Canada) to establish a stable KO colony. Littermate wild-type (WT) mice were used as controls.

In utero electroporation

Pregnant mice at embryonic day 13.5–15.5 (E13.5–E15.5) were anesthetized with isoflurane (4% induction, 1.5% during surgery). Plasmid DNA containing either control or *Bcl9* shRNA was injected into the lateral ventricles of embryos. For rescue experiments, the pCAGIG-m*Bcl9* plasmid was co-injected with shRNA plasmid. Electroporation was performed using an ECM830 electroporator (BTX, Harvard Apparatus, Holliston, MA, USA) with five 30 V pulses of 50 ms duration at 100 ms intervals. Following electroporation, the uterine horns were repositioned, and the abdominal wall and skin were sutured. Animals were placed in a warmed chamber for 1 h post-surgery recovery.

Ultrasonic vocalizations in p3 pups

Ultrasonic vocalizations (USVs) were assessed in postnatal day 3 (P3) mouse pups, as described previously with minor modifications (Hofer, Shair et al. 2002, Scattoni, Crawley et al. 2009). Briefly, lactating females were removed from the home cage and relocated to a separate, sound-attenuated room to eliminate maternal interference. Pups were individually transferred to the behavioral testing room and placed gently at the center of a standard test box. A condenser ultrasound microphone (CM16/CMPA, Avisoft Bioacoustics) was positioned vertically 15 cm above the test box floor to ensure optimal sound capture. Vocalizations were recorded continuously for 7 min using an UltraSoundGate 116Hb interface (Avisoft Bioacoustics, Berlin, Germany). Upon completion of recording, all pups were returned to their home cage and reunited with the lactating female. Recorded USVs were subsequently analyzed offline using Avisoft SASLab Pro software (Avisoft Bioacoustics).

Behavioral tests in knockout mice

Behavioral assessments were performed on 8–15-week-old knockout mice to evaluate multiple functional domains.

The open field test was performed as follows: prior to testing, mice were acclimated to the behavioral testing room for 30 minutes to reduce environmental stress. They were then placed individually in a novel open-field arena (diameter, 27.5 cm) and allowed to freely explore for a 20-minute session, with the total distance traveled recorded to evaluate locomotor activity.

Anxiety-like behavior was evaluated using an elevated plus maze, comprising two open arms and two closed arms (each 29 cm × 8 cm; closed arms enclosed by 17 cm-high walls). Prior to testing, mice were acclimated to the behavioral testing room for 30 minutes to minimize stress from environmental novelty. For the test, each mouse was placed at the center of the maze, facing a closed arm, and allowed to freely explore the apparatus for 5 minutes.

Social interaction was tested in a three-chamber apparatus. Mice were first allowed 10 minutes of free exploration. A novel 8–12-week-old mouse, housed in a wire mesh cup, was then placed in one lateral chamber, and the test mouse was reintroduced for 5 minutes; the time spent in each chamber was recorded.

Depressive-like behavior was examined via the forced swim test: mice were placed in a cylindrical container (diameter, 18 cm; height, 25 cm) filled with water at a constant temperature of 23 ± 2 °C. The mice were subjected to the test for 5 minutes, and their immobility time was quantified.

Real-time quantitative polymerase chain reaction

Total RNA was isolated from the mouse tissues using the SteadyPure Rapid RNA Extraction Kit (Accurate Biotechnology AG21023) following the

instructions. In reverse-transcription quantitative PCR (RT-qPCR), RNA was reverse transcribed into cDNA with the kit (Accurate Biotechnology AG11705). Real-time quantitative PCR was carried out with SYBR Green PCR Master Mix (Accurate Biotechnology AG11732). Relative mRNA expression levels were calculated using the $2^{-\Delta\Delta Ct}$ method and normalized to β -actin.

ChIP-qPCR

Samples were prepared from P3 mouse brain tissues subjected to IUE using an EZ-ChIP™ kit (17-371, Merck Millipore). Genomic DNA was sheared using a sonication to an average fragment size of 200–400 bp. ChIP-qPCR assays were performed on samples from control shRNA and *Bcl9* shRNA groups using an antibody against BCL9 (abclonal).

Computational docking

To explore potential structural compatibility between BCL9 and voltage-gated sodium channels, molecular docking analyses were performed using BCL9 and *Scn1b* or *Scn2a*. As experimentally determined structures were unavailable, three-dimensional protein models of BCL9, SCN1B or SCN2A were generated using AlphaFold3 (Abramson, Adler et al. 2024). Protein sequences for BCL9 (UniProt ID: Q67FY2), SCN1B (UniProt ID: A2APX8), and SCN2A (UniProt ID: B1AWN6) were retrieved from UniProt. The top-ranked AlphaFold3 models with high confidence scores were selected for subsequent docking analyses. The pore-forming α subunit of voltage-gated sodium channels consists of four homologous domains (DI–DIV). To reduce computational complexity and facilitate docking, domain III (residues 1200–1514 in SCN1B and 1199–1505 in SCN2A) was selected as a representative structural region for interaction analysis. Putative binding residues within domain III were predicted using PrankWeb (Jakubec, Skoda et al. 2022) and used to guide docking. Protein–protein docking was performed using HADDOCK2.4 (de Vries, van Dijk et al. 2010), with BCL9 defined as the ligand and SCN1B or SCN2A domain III as the receptor. Binding affinities of the docked complexes were further estimated using PRODIGY (Vangone and Bonvin 2015) to obtain predicted ΔG and K_d values. Interacting residues were analyzed using PDBsum (Xue, Rodrigues et al. 2016) and structural models of the complexes were visualized using PyMOL (version 2.5.5, Schrödinger, LLC).

Statistical analysis

Data are presented as mean ± SEM. Statistical analyses were performed with GraphPad Prism. Comparisons between two groups were made using unpaired two-tailed Student's t-test. For multiple group comparisons, one-way ANOVA followed by Tukey's post hoc test was applied. Statistical significance was defined as $P < 0.05$ (* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$).

RESULTS

Bcl9 regulates neuronal migration in the developing mouse brain

Bcl9, functioning as a Wnt coactivator or independent of canonical Wnt-signaling, modulates diverse biological processes, including cell proliferation and cell fate determination. However, its role in neuronal development remains unexplored. We primarily aimed to elucidate the functional contribution of *Bcl9* to neuronal development in the developing mouse brain. BCL9 protein is expressed in the embryonic day 13.5 (E13.5) (Figure S1A) and neonatal cortex (Figure S1B). Then we performed in utero electroporation (IUE) at E13.5–E15.5, delivering either control short hairpin RNA (shRNA) or *Bcl9*-targeting shRNA (*Bcl9* shRNA) into the lateral ventricles (Figure 1A). *Bcl9* shRNA effectively suppressed *Bcl9* expression in the developing mouse brain (P0), while control shRNA had no impact on BCL9 protein translation (Figure 1B). To assess the impact of *Bcl9* knockdown on cortical neuron migration, we analyzed GFP-labeled neurons in P0 postnatal day0 (P0), P3, and P5. Compared with control shRNA, *Bcl9* knockdown led to an accumulation of transfected neurons in the upper cortical plate (UCP) (Figures 1C–E & G). These data demonstrate that the migration of *Bcl9*-knockdown neurons from the inner to the outer cortical layers is accelerated during early life development. Further analysis using layer-specific markers demonstrated that *Bcl9* knockdown disrupted neuronal positioning within cortical layers IV/V (Ctip2-positive neurons) and II/III (Cux1-positive neurons) (Figures 1D, F & H). Notably, co-expression of *Bcl9* shRNA and mouse *Bcl9* cDNA (m*Bcl9*) rescued these migration defects (Figures 1D & H), confirming the specificity

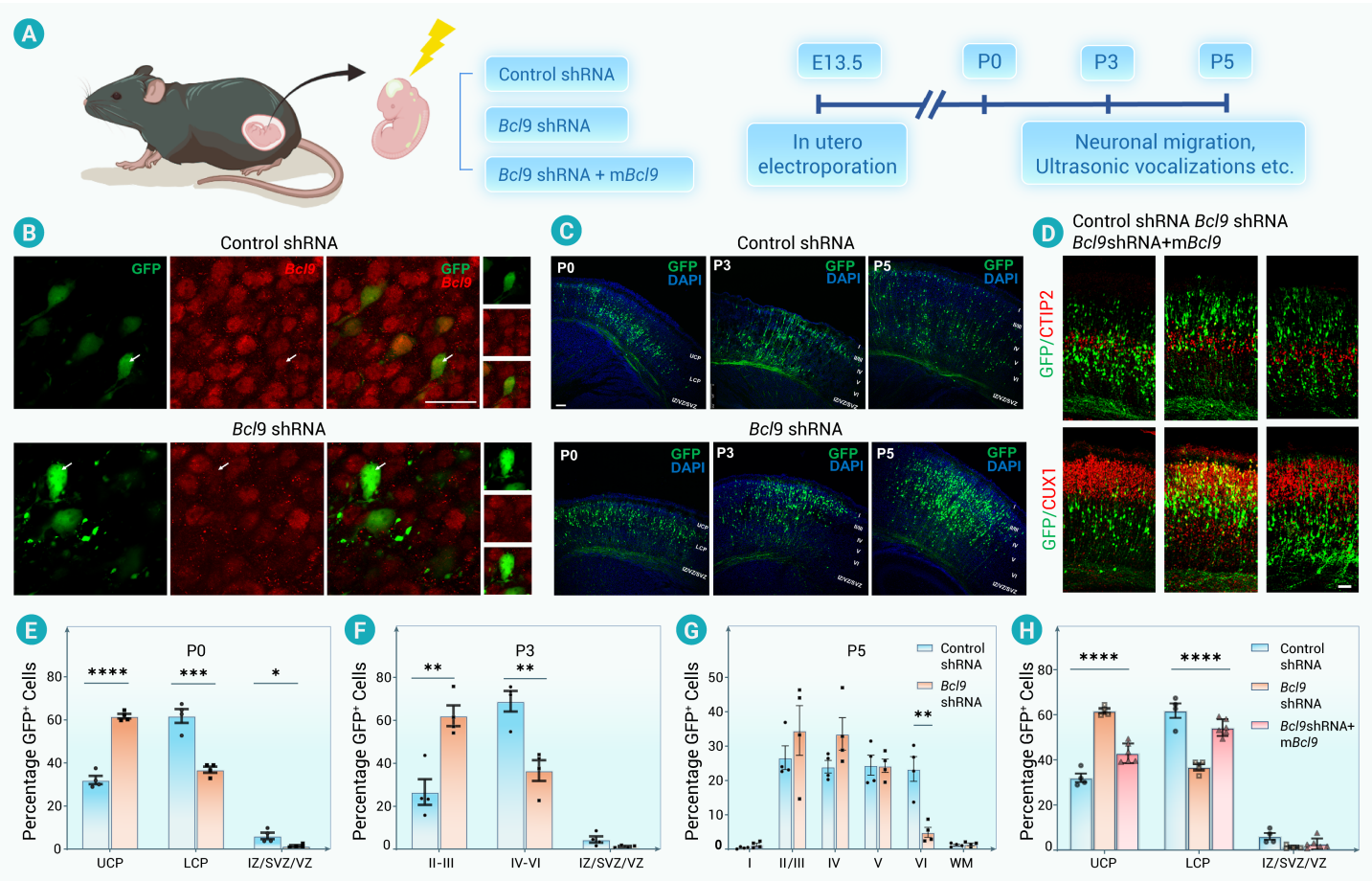


Figure 1. Knockdown of *Bcl9* in the developing cortex (A) Diagram of the in utero electroporation. (B) Migrating neurons were positively stained with the BCL9 antibody, whereas Bcl9 immunofluorescence was reduced in neurons electroporated with Bcl9 shRNA. Arrows indicate neurons transfected with control shRNA or Bcl9 shRNA. Scale bar: 20 μ m. (C) Coronal sections of P0, P3, and P5 mouse cortices showing the distribution of GFP-positive neurons across cortical layers ($n = 3$ per group). Scale bar: 100 μ m. (UCP: upper cortical plate; LCP: lower cortical plate; VZ: Ventricular Zone; SVZ: Subventricular Zone; IZ: Intermediate Zone) (D) Representative coronal sections at P0 immunostained for CUX1 or CTIP2. Expression of mouse *mBcl9* partially rescued the abnormal migration induced by Bcl9 shRNA. Scale bar: 100 μ m. (E–H) Quantification of the percentage of GFP⁺ cells corresponding to panels C and D. For pairwise comparisons between two groups, statistical significance was determined by unpaired two-tailed Student's *t*-test. For comparisons among three or more groups, statistical significance was assessed by one-way analysis of variance (ANOVA) followed by Tukey's post hoc test for multiple pairwise comparisons. $P < 0.05$ was considered statistically significant. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

of the observed phenotype.

Furthermore, fluorescence micro-optical sectioning tomography (fMOST) was employed to obtain high-resolution, whole-brain datasets of transfected neurons and their projections, accompanied by detailed anatomical annotation (Supplementary Video. fMOST imaging of GFP-labeled neurons in *Bcl9* knockdown vs. control cortex).

Knockdown of *Bcl9* increases ultrasonic vocalization in pups

We next investigated the contribution of neuronal migration to the behavioral phenotypes in mice. Ultrasonic vocalizations (USVs) emitted by newborn mice represent an early form of communication between pups and their mothers, and are widely used as a behavioral indicator in mouse models of neurodevelopmental disorders.²⁴ To determine whether *Bcl9* knockdown influences the form of USVs, we recorded the vocalization patterns of P3 pups upon isolation from their mother and littermates. Those receiving *Bcl9* shRNA exhibited a significantly elevated vocalization rate at P3, while pups injected with control shRNA displayed no significant difference from their WT littermates (Figures 2A–B). In contrast, the duration and peak frequency of USVs remained unchanged between *Bcl9* knockdown and WT pups (Figures 2C–D).

Adult *Bcl9* knockout mice exhibit behavioral deficits

To investigate the functional consequences of *Bcl9* loss on adult mouse behavior, we established a *Bcl9* knockout (*Bcl9*^{−/−}) model using TALEN-mediated genome editing, wherein exon 5 of the *Bcl9* gene was specifically targeted to abrogate endogenous BCL9 protein expression. Phenotypic char-

acterization revealed that *Bcl9*^{−/−} mice had significantly lower body weight (Figures 2E–F) and reduced brain size (Figures 2G–H) relative to their WT littermates, suggesting that *Bcl9* plays a critical role in regulating postnatal growth and brain development. Accordingly, behavioral analyses were performed on *Bcl9*^{−/−} mice to assess the functional consequences of *Bcl9* deficiency. In the forced swim test, *Bcl9*^{−/−} mice exhibited a significantly longer immobility time compared to WT mice (Figure 2I), indicating increased depressive-like behavior. In contrast, no significant differences were observed between *Bcl9*^{−/−} and WT mice in the elevated plus maze, the open-field test, three-chamber sociability tests. (Figures 2J–N). Collectively, these data demonstrate that *Bcl9* deficiency induces selective behavioral deficits in adult mice, primarily manifesting as enhanced depressive-like behavior.

To investigate whether *Bcl9* knockdown or knockout affects the Wnt signaling pathway in the central nervous system, we analyzed the expression of Cyclin D1 and cMyc, well-characterized target genes in the canonical Wnt signaling pathway, in cortical tissues of *Bcl9*-shRNA mice. Cyclin D1 was significantly downregulated in cortical tissue in *Bcl9*-shRNA mice (Figure 2O – P & S3A).

Bcl9 deficiency uncouples sodium channel upregulation from neuronal firing

To investigate the effect of *Bcl9* knockdown on electrophysiological properties, we performed whole-cell patch-clamp recording in P3 migrating neurons after IUE (Figure 3A). *Bcl9* knockdown significantly increased voltage-dependent transient inward current (Figure 3B) and did not have an effect on sustained outward current (Figure S2B), input resistance (Figure S2D), or

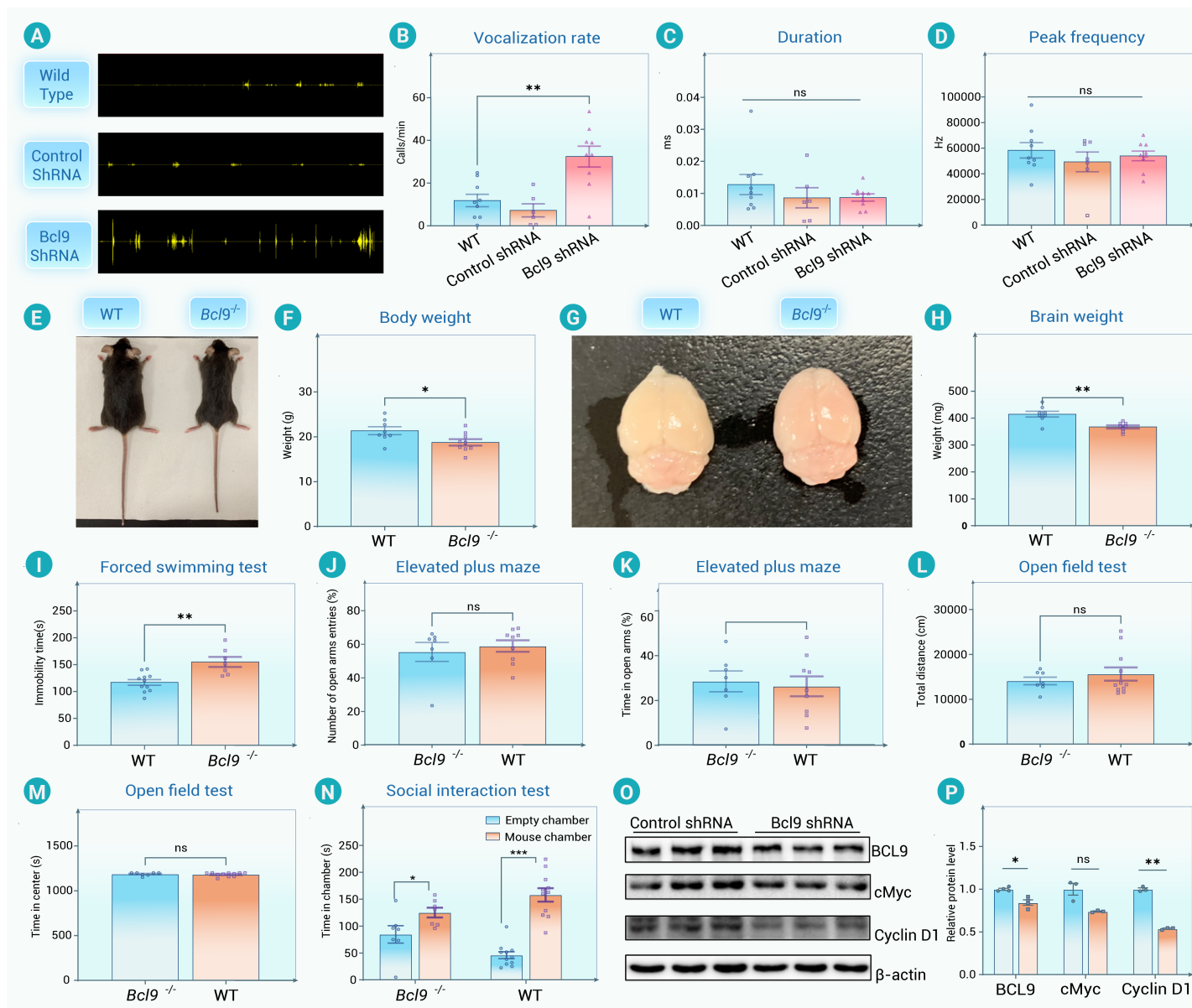


Figure 2. Developmental and behavioral phenotypes in *Bcl9* knockdown and knockout mice (A) Schematic representation of experimental groups: control shRNA, *Bcl9* shRNA, or naïve littermates (WT), showing ultrasonic vocalizations (USVs) recorded over a 7-min period. (B) *Bcl9* knockdown increased the vocalization rate compared with control shRNA and naïve littermates (WT: $n = 12$; control shRNA: $n = 8$; *Bcl9* shRNA: $n = 10$) during isolation from the dam. (C-D) The duration (C) and peak frequency (D) of these vocalizations in the *Bcl9* knockdown pups were indistinguishable from wild-type animals. (E) Representative images of 8-week-old male WT and *Bcl9*^{-/-} (KO) mice. (F) KO mice weighed significantly less than WT mice at 8 weeks (WT: $n = 7$; KO: $n = 9$; mean $\pm$ SEM; $p < 0.05$). (G) Representative images of brains from 8-week-old male WT and KO mice. (H) KO mouse brains weighed significantly less than WT brains at 8 weeks (WT: $n = 7$, male; KO: $n = 9$, male; mean $\pm$ SEM; $p < 0.01$). (I) Adult KO mice displayed increased immobility time compared with WT mice in the forced swim test (WT: $n = 11$; KO: $n = 7$; mean $\pm$ SEM; $p < 0.01$). (J-N) Negative behavioral tests in *Bcl9* KO mice. No significant differences were detected between WT and KO mice in the elevated plus maze, open field test, or three-chamber social interaction test (WT: $n = 11$; KO: $n = 7$; mean $\pm$ SEM). (O) Western blot showed downregulation of Wnt target genes, such as cMyc and Cyclin D1 in P3 brain of *Bcl9* knockdown pups (*Bcl9*, $n = 4$; cMyc, Cyclin D1, $n = 3$).

resting membrane potential (Figure 3C). In addition, the expressions of sodium channel proteins $Na_v1.1$ and $Na_v1.2$ were upregulated in *Bcl9*-shRNA mice (Figure 3D). Kinetic analysis showed *Bcl9* shRNA migrating neurons had a twofold faster rise time than control (Figure 3E). The effect of *Bcl9* knockout on electrophysiological properties was also assessed. Concurrently, *Bcl9* KO displayed a decreased number of APs firing in a range of stimulating currents (Figure S2F). These results suggested that loss of BCL9 function decreases the sodium channel-dependent neuronal excitability in adult mice. In addition, the expressions of sodium channel proteins $Nav1.1$ and $Nav1.2$ were upregulated in the *Bcl9* KO brain (Figure S2G). *Bcl9* knockdown reduces action potential firing and alters firing patterns in cortical neurons, with fewer tonic and phasic firings observed (Figures 3F & G), decreased spontaneous neuronal activity in *Bcl9* knockout mice (Figure 3H), which is confirmed by significantly lower firing frequencies (Figures 3I-J). Collectively, our findings demonstrate that enhanced expression of voltage-gated sodium channels

($Nav1.1/Nav1.2$) and the resulting increase in transient inward currents do not necessarily translate to enhanced neuronal excitability. Instead, the upregulation of *Kcnc1*-encoded $Kv3.1$ potassium channels (Figure S3) acts as a compensatory brake on excitability, effectively counterbalancing the pro-excitatory effects of elevated sodium currents. This reveals a critical principle in neuronal physiology: the intrinsic excitability of a neuron is not simply determined by the magnitude of inward currents, but rather by the dynamic interplay and temporal coordination between opposing ionic conductances. Our data underscore the importance of considering multi-ionic mechanisms when interpreting electrophysiological phenotypes in gene knockout models.

***Bcl9* is associated with sodium channel regulators**

Given its role as a transcriptional co-activator, we next explored how *Bcl9* regulates sodium channel expression. We performed Kyoto Encyclopedia of Genes and Genomes (KEGG) and Gene Ontology (GO) enrichment analysis on

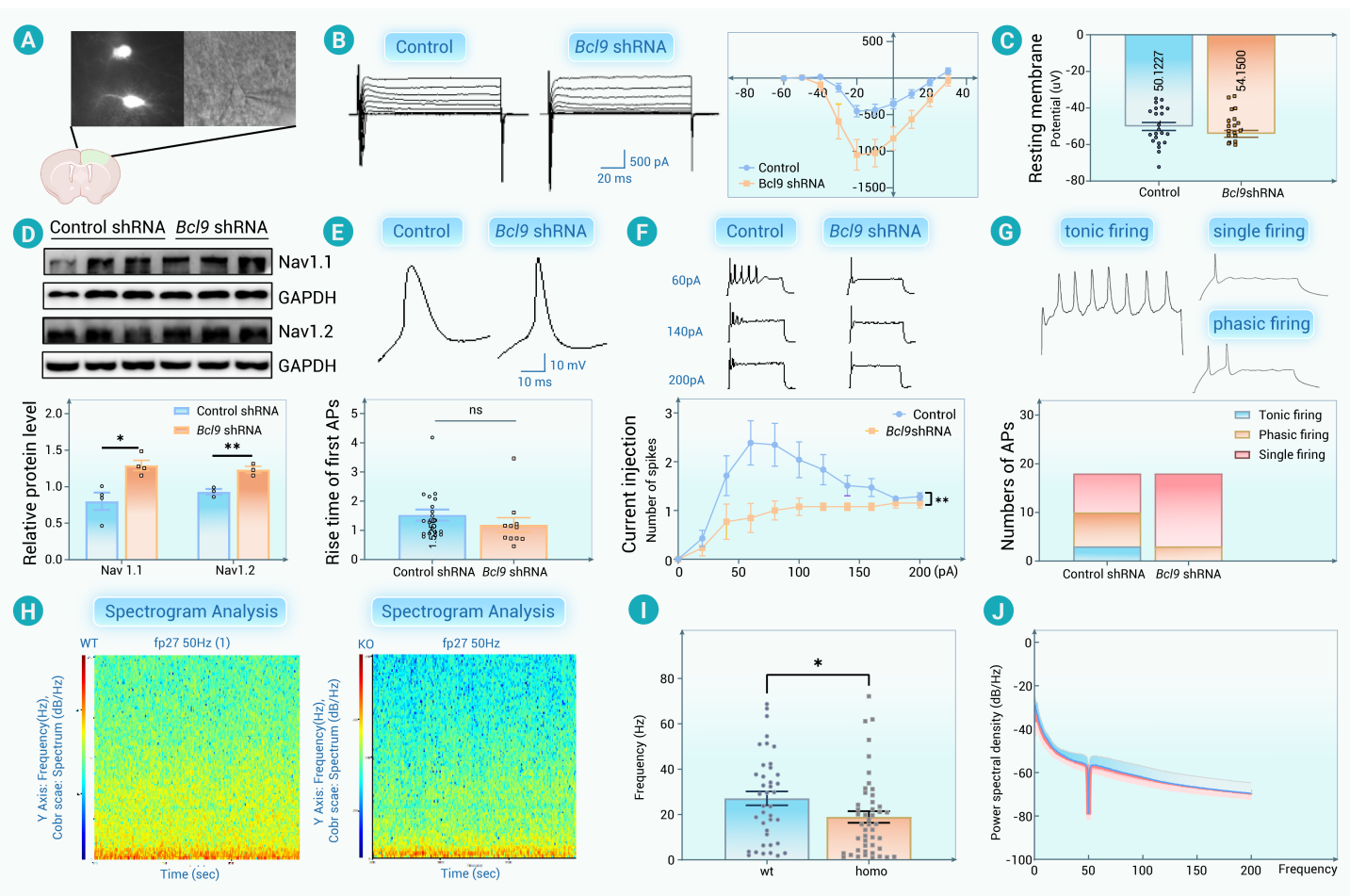


Figure 3. Electrophysiological properties of neurons in control and *Bcl9*-deficient mice (A) Representative images showing a brain slice and the patch-clamp recording configuration targeting neurons in a specific brain region (schematic below). The left panel shows a fluorescence image indicating recorded cells, and the right panel shows the corresponding brightfield image. (B) Left: Representative traces of whole-cell patch-clamp recordings from wild-type (WT) and *Bcl9* knockdown neurons in response to a series of voltage steps. Right: Quantification of inward currents (pA) during voltage-clamp steps in control and *Bcl9* shRNA conditions (mean $\pm$ SEM). (C) Scatter and bar plots of resting membrane potential (mV) in the control (blue) and *Bcl9* shRNA (orange) groups. Each dot represents a recorded cell; bars indicate mean $\pm$ SEM, with sample sizes shown. (D) Western blot analysis of voltage-gated sodium channel subunits Nav1.1 and Nav1.2 protein levels in cortical tissue. (E) Representative traces of the AP recorded from control shRNA and *Bcl9* shRNA-treated cortical neurons (top). Quantification of the rise time of the AP (bottom). (F) Current-clamp mode in control shRNA and *Bcl9* shRNA conditions, following depolarizing current injections of 60 pA, 140 pA, and 200 pA. The traces show reduced spike frequency in *Bcl9* knockdown neurons compared to controls (top), the graph below quantifies the number of APs elicited at increasing current steps (bottom). (G) Schematic examples of different neuronal firing patterns (tonic, phasic, and single firing) (top). Middle and right: Pie charts summarizing the proportions of each firing pattern in the two experimental groups (total cell numbers indicated). (H) Representative heatmaps of spontaneous synaptic events recorded under the two conditions, illustrating the temporal dynamics of neuronal activity. (I) Scatter and bar plots comparing the frequency (Hz) of spontaneous synaptic events between WT and homozygous knockout (KO) neurons. Each point represents a recorded cell; bars indicate mean $\pm$ SEM. Asterisks denote statistical significance ($p < 0.05$). (J-L) Averaged power spectral density (dB/Hz) plots across frequency for the two groups (lines $\pm$ shaded areas indicate SEM).

proteins identified by qualitative mass spectrometry. In the KEGG, enriched terms included protein transport, intracellular trafficking, mitochondrial aerobic respiration, ATP biosynthesis, mRNA splicing, and chromatin remodeling, suggesting active roles in energy metabolism and transcriptional regulation (Figure 4A). Notably, processes related to nervous system development and neurotransmitter secretion were also enriched, indicating relevance to neurodevelopmental pathways (Figure 4A). GO Cellular Component analysis showed that the proteins were predominantly localized to the cytoplasm and cytosol, with additional enrichment in the nucleus, mitochondria, dendritic spines, and glutamatergic synapses (Figure 4B). Molecular Function analysis further revealed that most proteins were involved in protein binding, RNA binding, ATP binding, and enzyme interactions (Figure 4B). Together, these findings suggest that the identified proteome is functionally diverse, participating in key cellular pathways linked to neuronal regulation, gene expression, and intracellular signaling.

PPI network visualizes interactions among identified proteins, with nodes as detected proteins and edges as their physical/functional links, clustered by color - coded functional modules (Figure 4C). Most proteins do not directly constitute sodium channels, but are closely related to the functions of SCN1B ($\beta 1$ subunit) and SCN2A (α subunit) through mechanisms such as cytoskeletal anchoring, phosphorylation regulation, folding assistance, or synaptic

coordination. Among them, ANK3, the CAMKII family, HSP90, and the ACTB/ β -tubulin family have the most well-established associations and are key regulatory factors for the normal functions of sodium channels (localization, activity, and stability).

BCL9 modulates sodium channel expression through *Scn1b* and *Scn2a* transcription

In order to determine whether BCL9 protein or its complexes associate with chromatin of *Scn1* and *Scn2*, Chromatin immunoprecipitation (ChIP) experiments were performed using anti-BCL9 antibodies (Figures 4D-G). ChIP-PCR results demonstrated that *Bcl9* knockdown in the cortex led to a significant increase in fluorescence intensity at the promoter region of *Scn1b* and the area near exons of *Scn2a* compared with the control group (Figures 4D-E). Based on the ChIP-seq results, we found that *Bcl9* knockdown affected the binding to the promoter region of the *Scn1b* gene (Figure 4F) and the area near the exons of *Scn2a* (Figure 4G), indicating that BCL9 protein may be involved in processes such as gene expression regulation by modulating these regions.

Molecular docking indicates absence of BCL9 protein interaction with Nav1.1 and Nav1.2 sodium channel proteins

Bcl9 is widely recognized as a transcriptional co-factor that does not

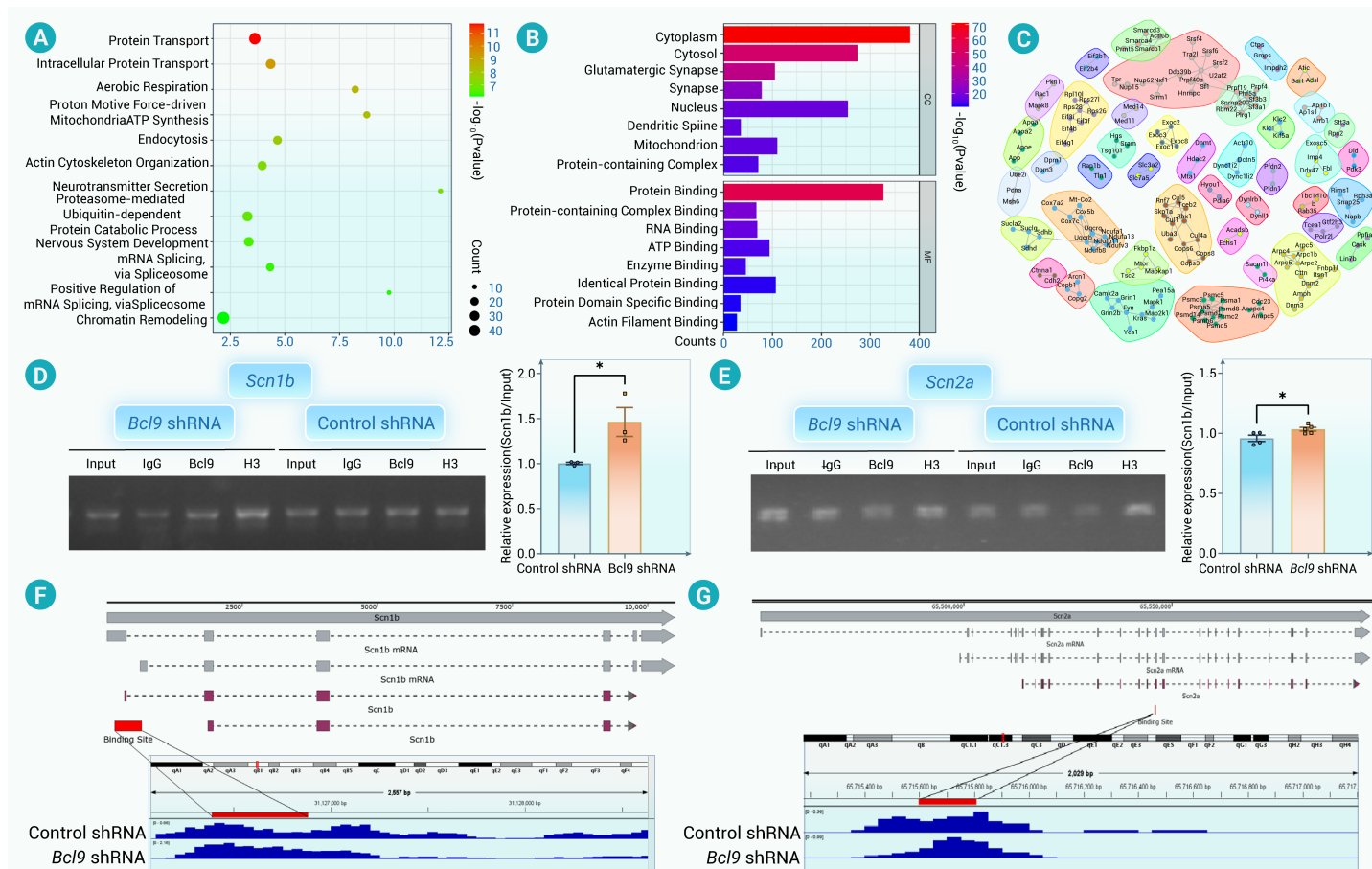


Figure 4. BCL9 protein complexes associate with *Scn1b* and *Scn2a* (A) KEGG pathway analysis, (B) GO analysis of 'Cellular Component' and 'Molecular Function', and (C) protein-protein interaction (PPI) network of differentially expressed proteins in brain tissues from *Bcl9* knockdown and WT mice. (D–E) ChIP-PCR showing significant enrichment of BCL9 protein binding near the promoter regions of *Scn1b* and *Scn2a* in *Bcl9* knockdown mice (*Bcl9* shRNA: *n* = 6; WT: *n* = 4). (F–G) ChIP-seq analysis identifying BCL9–*Scn1b* (F) and BCL9–*Scn2a* (G) co-enriched chromatin sequences (*Bcl9* shRNA: *n* = 3; WT: *n* = 3).

directly bind DNA. However, our molecular docking analyses uncovered specific amino acid-mediated interactions between BCL9 protein and the promoter regions of *Scn1b* and *Scn2a*, with docking scores of -124.57 and -108.84, respectively. Structural dissection revealed that BCL9 protein formed robust polar interactions with *Scn1b* promoter DNA via seven distinct residues: N499, M500, Q503, K519, E522, Y525, and R529. In contrast, BCL9 engaged *Scn2a* promoter DNA through three residues (H244, V391, I261). Collectively, these findings suggest that BCL9 protein may exert more stringent transcriptional control over *Scn1b* than *Scn2a* (Figure 5).

We next sought to examine whether it is involved in direct interaction with SCN1B and SCN2A proteins. To address this question, we performed molecular docking analysis using the top predicted protein models (Figures S4A–C). The docking scores were positive for both the BCL9–SCN1B (435.7 ± 5.1) and BCL9–SCN2A (535.6 ± 10.2) complexes (Figure S4D). Positive docking scores typically indicate weak or non-specific complex formation. Importantly, interaction analysis of the docked complexes further revealed that no active-site residues of either SCN1B or SCN2A were involved in binding with BCL9 protein (Figures S4E–F).

DISCUSSION

Our findings identify *Bcl9* as a negative regulator of voltage-gated Na⁺ channel expression that modulates neuronal excitability and drives abnormal behaviors in mice, and further reveal associations between *Bcl9*, brain structural variation, and psychotic-like phenotypes.

Bcl9 modulates sodium channel-dependent neuronal excitability

We identify *Bcl9* as an upstream regulator of neuronal excitability that operates through convergent mechanisms. BCL9 protein represses *Scn1b* and *Scn2a* expression, increasing sodium current amplitude, yet overall firing

output is reduced. Our electrophysiological and biochemical analyses point to a compensatory increase in the *Kcnk1*-encoded Kv3.1 potassium channel as the primary mechanism underlying this dissociation. The robust rise in Kv3.1 protein levels (Figure S3), together with its established function in accelerating repolarization and restricting high-frequency firing, supports a homeostatic adjustment that curtails excitability despite augmented sodium conductance. Other contributing factors—such as altered sodium channel gating or redistribution along the axon initial segment—remain possible but are not supported by current evidence. In parallel, *Bcl9*'s Wnt-dependent functions shape cortical development, layer formation, and growth. The convergence of altered neuronal excitability and disrupted circuit architecture manifests in early-life communication deficits and selective depressive-like behaviors in adulthood, positioning *Bcl9* as a central integrator linking ion-channel homeostasis, developmental patterning, and neuropsychiatric phenotypes relevant to neurodevelopmental disorders. Combined with chromatin immunoprecipitation (ChIP) assay results, we found that BCL9 protein may regulate the expression of *Scn1b* and *Scn2a*, which are essential for action potential initiation and neuronal excitability, and their dysfunction drives neurological diseases, such as epilepsy, ASD, and developmental related encephalopathy.^{12–15}

We observed consistent findings in both Western blot and chromatin immunoprecipitation-quantitative PCR assays: knockdown of *Bcl9* led to upregulated expression of sodium channel-associated proteins and their corresponding genes; although our ChIP-seq data indicated BCL9 protein binding to the promoter region of the *Scn1b* gene and the area near the exons of *Scn2a*. In the context of *Bcl9*-mediated regulation of *Scn1b/Scn2a*, two key explanations can be proposed: *Bcl9* functions as a transcriptional repressor of *Scn1b/Scn2a*—instead of recruiting transcriptional activation complexes when binding to the promoter regions of these target genes, BCL9 protein

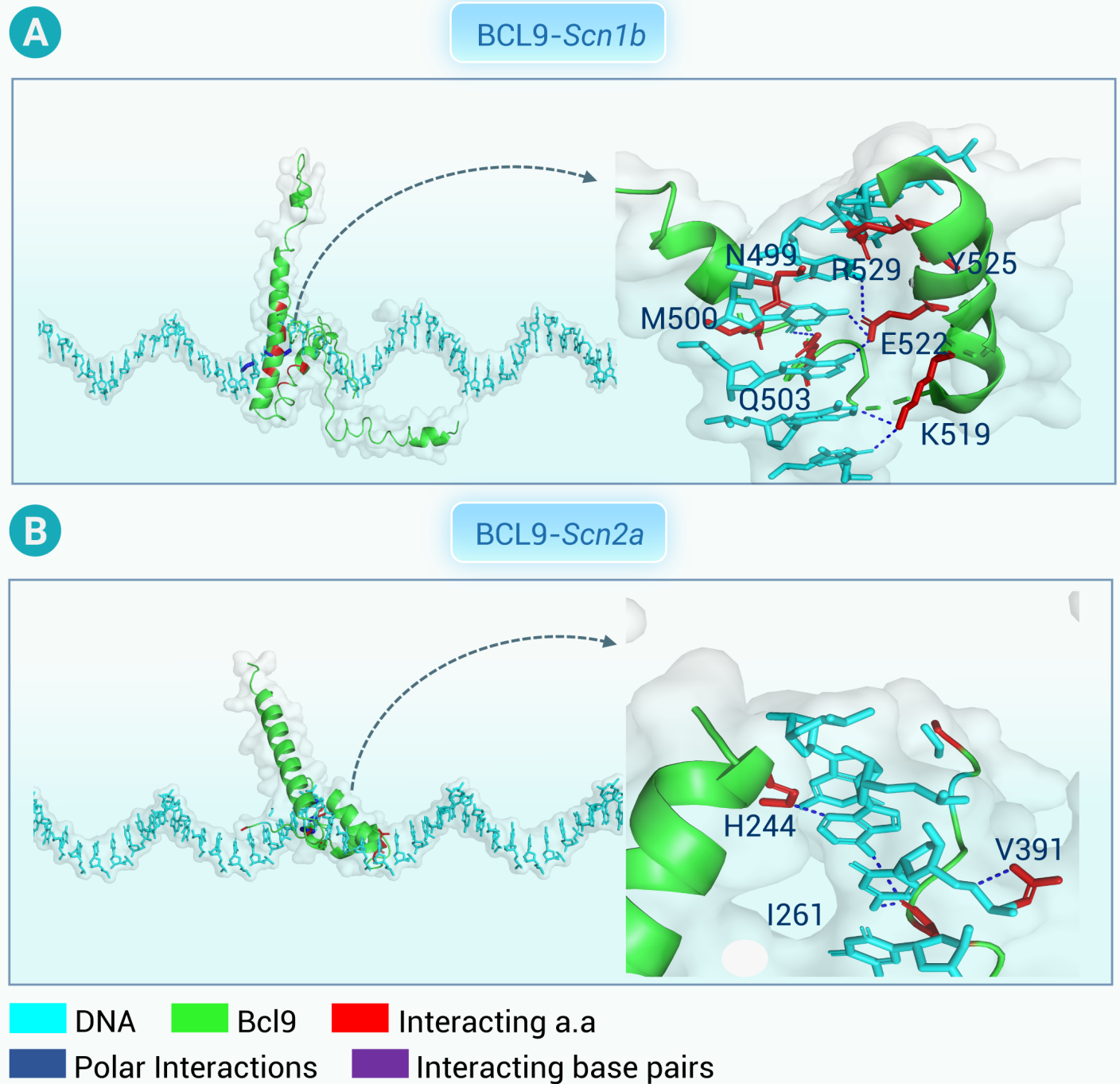


Figure 5. Molecular docking of BCL9 protein with *Scn1b* and *Scn2a* DNA Docking results of the BCL9 protein with the promoter DNA regions of *Scn1b* (A) and *Scn2a* (B). Key interacting amino acid residues are labeled in the zoomed view in the right panel.

represses transcription either by occupying the core promoter regions to prevent the binding of RNA polymerase II or transcriptional activators, or by recruiting transcriptional repression complexes to induce chromatin condensation into a "silent" state and suppress transcriptional initiation, such that *Bcl9* knockdown (KD) relieves this inhibitory effect and thus leads to increased mRNA or protein expression levels of *Scn1b/Scn2a*; alternatively, this observation may be attributed to an indirect regulatory mechanism, in which *Bcl9* does not modulate *Scn1b/Scn2a* directly but instead inhibits the expression or activity of a certain ProteinX that acts as a transcriptional activator of *Scn1b/Scn2a*. The suppression on ProteinX is abolished, allowing ProteinX to bind abundantly to the promoters of *Scn1b/Scn2a* and enhance their transcription, with the BCL9-promoter binding detected by ChIP assays possibly representing indirect binding formed via complexation with ProteinX. We anticipate that subsequent experiments will enable us to clarify whether

there are proteins that interact with BCL9 and modulate the expression of *Scn1b/Scn2a*.

To resolve whether *Bcl9* directly modulates VGSCs (Voltage-Gated Sodium Channels), we performed molecular docking—a critical step to distinguish direct from indirect control. These results reinforce our model: BCL9 protein does not directly regulate SCN1B/SCN2A via active-site binding. Its effects on excitability are indirect—mediated by transcriptional control of *Scn1b/Scn2a* and post-translational coordination via associated proteins. This aligns with the role of BCL9 protein as a transcriptional cofactor and helps resolve the "excitability paradox: without direct active-site regulation, electrophysiological changes likely reflect transcriptional upregulation of *Scn1b/Scn2a* and compensatory mechanisms. Interestingly, we observed dissociation between the augmented transient sodium current and the reduction in action potential output. Although increased sodium conductance is typically expected to

enhance neuronal excitability, our recordings revealed a concomitant and marked upregulation of Kv3.1. Kv3.1 channels mediate fast-activating, fast-inactivating potassium currents that promote rapid repolarization and thereby impose a brake on spike initiation and high-frequency firing. Among the candidate mechanisms that could account for this excitability paradox, Kv3.1 upregulation is most strongly supported by our data for two reasons: 1) the sustained outward current (Figure S2B) and Kir2.1 expression (Figure S2E) are unchanged, this may indicate that the compensatory response is selective for Kv3.1 rather than reflecting a broad enhancement of potassium conductances; 2) the biophysical properties of Kv3.1—high activation threshold, rapid activation–deactivation kinetics, and an ability to accelerate repolarization—are suited to counterbalance increased sodium influx. On this basis, we interpret Kv3.1-mediated compensation as the most parsimonious explanation for the observed phenotype, while acknowledging that definitive resolution will require targeted electrophysiological and computational analyses in future work.

***Bcl9* as a convergent regulator of cortical development and neuropsychiatric behaviors**

Bcl9's role in canonical Wnt signaling and oncogenesis has been well established.^{3-5,24} Beyond tumor biology, *BCL9* influences diverse developmental processes: it regulates enamel formation,¹¹ contributes to cardiac development in vertebrates,²⁵ and guides myogenic progenitor differentiation during muscle regeneration.²⁶ Li et al. and Yuan et al.^{8,27} have shown that *BCL9* is associated with schizophrenia, as well as bipolar and major depression in the Chinese Han population. Xu et al.¹⁰ found a significant association between the *BCL9* gene and the negative symptoms of SCZ. However, the role of *BCL9* in the central nervous system and its functional validation by biological experiments remain largely unexplored. Our findings reveal that *Bcl9* knockdown during embryonic development causes abnormal cortical neuronal migration. Neuronal migration defects are frequently associated with long-term behavioral abnormalities in neurodevelopmental disorders. Peñagarikano et al.²⁸ reported that a candidate gene for autism spectrum disorder causes abnormalities in neuronal migration, which are associated with impaired neuronal network function and the onset of seizures. Prem et al.²⁹ reported that organoids derived from ASD patients display impaired neurite outgrowth and migration in neural precursor cells (NPCs). To date, direct evidence connecting abnormal neuronal migration to psychiatric disorders remains scarce, and further studies are needed to complete this mechanistic loop.

In the present study, we used complementary mouse models to investigate the role of *Bcl9* in psychiatric disorder models—related behaviors, including in utero electroporation (IUE)—mediated knockdown of *Bcl9* in the developing cortex and a global *Bcl9* knockout mouse line. In the IUE model, we used Ultrasonic vocalizations (USVs) to test. USVs are increasingly used to study psychiatric and neurological disorders, especially NDDs and ASD, in which communication and social deficits are prominent. Mouse models of these disorders show strain-dependent alterations in USVs, including changes in call number, duration, frequency, and call types.^{17,30-32} Our data demonstrate that *Bcl9* knockdown in P3 mouse pups leads to an increase in the USV rate. These results suggest that *Bcl9* deficiency disrupts neural circuits involved in early communicative behavior, supporting a role for *Bcl9* in central nervous system function.^{17,33} In adult *Bcl9* knockout (*Bcl9*^{-/-}) mice, we observed two key phenotypes: broad developmental impairments, including reduced body weight and smaller brain size, as well as selective depressive-like behavioral alterations. The two models presented herein suggest that the role of *Bcl9* is not confined to a single psychiatric disorder but may represent a shared mechanism across multiple psychiatric conditions.

Notably, symptoms related to depression, anxiety, and social dysfunction are highly heterogeneous, encompassing anhedonia, cognitive impairment, psychomotor retardation, sleep disturbances, and other characteristic features. The behavioral assays used in the present study—including the forced swim test, three-chamber social interaction test, open field test, and elevated plus maze—capture only limited facets of these multidimensional symptom domains. Thus, further studies integrating clinical evidence with more comprehensive experimental approaches will be necessary to clarify the specific contribution of *BCL9* deficiency to distinct psychiatric phenotypes.

types.

CONCLUSIONS

Our findings identify *Bcl9* as a critical upstream regulator of neuronal excitability that operates through multi-layered mechanisms. By repressing the expression of *Scn1b* and *Scn2a*, loss of *Bcl9* leads to enhanced sodium currents; however, action potential firing is paradoxically reduced. This effect may be due to a compensatory upregulation of *Kcnc1*-encoded Kv3.1 potassium channels, which accelerate membrane repolarization and suppress high-frequency firing, thereby preventing pathological hyperexcitability despite elevated inward conductance. In parallel, the Wnt-dependent functions of *Bcl9* contribute to cortical development, layer formation, and brain growth. The convergence of altered neuronal excitability and disrupted circuit architecture manifests as early-life communication deficits and selective depressive-like behaviors in adulthood, positioning *Bcl9* as a central integrator linking ion-channel homeostasis, developmental patterning, and neuropsychiatric phenotypes relevant to neurodevelopmental disorders.

LIMITATIONS AND FUTURE DIRECTIONS

We acknowledge that the current study is constrained by the use of whole-cell current measurements. To address these limitations, we plan to perform the following experiments in future work:

(i) Directly record *Kcnc1*-mediated (Kv3.1) potassium currents in *Bcl9*-deficient neurons to quantify the magnitude of this compensatory outward conductance;

(ii) Conduct detailed kinetic analyses of sodium and potassium channel gating to elucidate how the interplay between enhanced inward currents and compensatory outward currents sculpts action potential waveforms;

(iii) Develop conductance-based computational models constrained by our experimental data to validate whether the observed firing phenotypes can be recapitulated by altered Na⁺ and K⁺ conductances;

(iv) Investigate the spatial distribution of Nav and Kv3.1 channels at the axon initial segment using high-resolution imaging techniques.

To gain deeper insights into the function of *Bcl9* within the nervous system, we will additionally investigate the molecular pathways underlying *Bcl9*-mediated regulation of neural elements (e.g., synapses), drawing upon our available mass spectrometry datasets and related experimental information.

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AUTHOR CONTRIBUTIONS

YK, YZ, and BY carried out the experiments, performed statistical analyses, and wrote the manuscript. ZW, DM, XZ, YL, and CL participated in the completion of part of the experiments and data statistics. GH, EIK, SS, TT, DA, and PF helped design the experiments, reviewed the statistical analyses, and revised the manuscript. The molecular docking analyses were performed by A. Khan. ZL and WL conceived the project, designed the experiments, reviewed the statistical analyses, and wrote the manuscript. All authors read and approved the final manuscript.

DECLARATION OF INTERESTS

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

ETHICAL STATEMENT AND PATIENT CONSENT

All animal experiments were performed according to the guidelines approved by the University Committee on Animal Care and Use of Shanghai Jiao Tong University, China. The Animal Protocol number is A2023190-001.

DATA AND CODE AVAILABILITY

Data are available from the corresponding author upon reasonable request.