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Neuronal guidance signaling in health and neurological diseases

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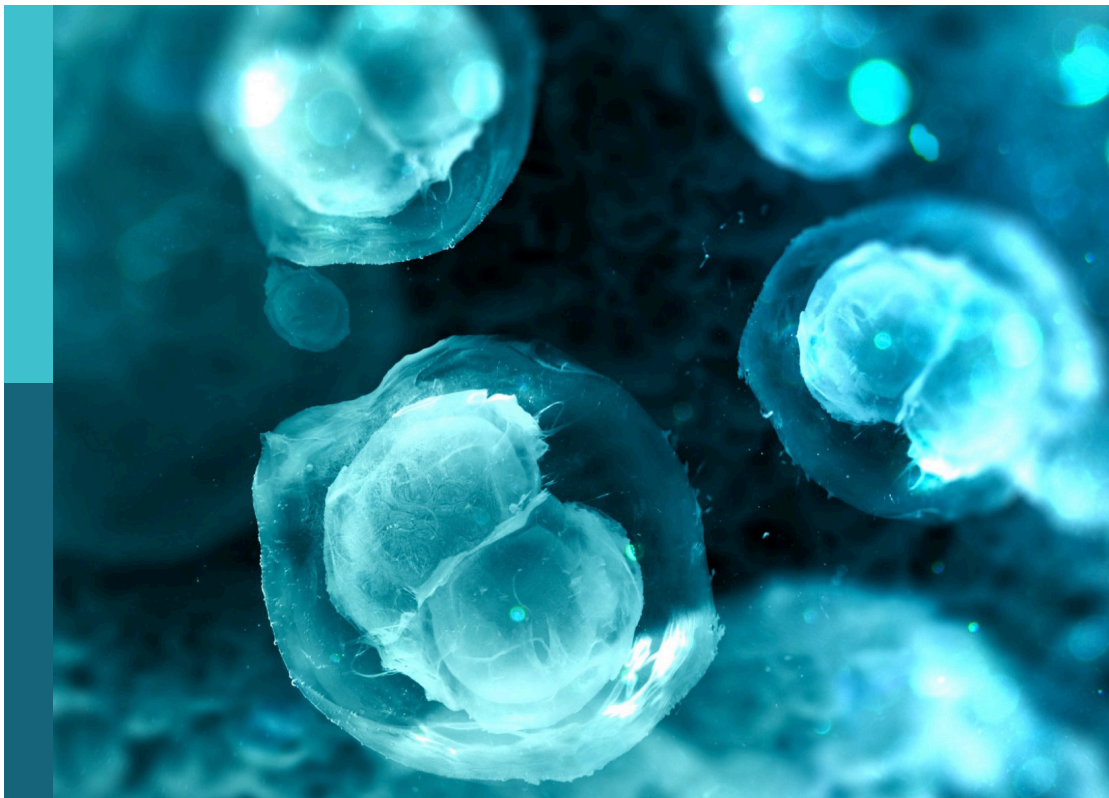

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Neuronal guidance signaling in health and neurological diseases

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Table of contents

04 Editorial: Neuronal guidance signaling in health and neurological diseases
Satoru Yamagishi, Masaki Hiramoto and Junichi Yuasa-Kawada

07 Netrin-1 stimulated axon growth requires the polyglutamylase TTLL1
Kyle R. Northington, Jasmynn Calderon and Emily A. Bates

22 GPR37 and its neuroprotective mechanisms: bridging osteocalcin signaling and brain function
Xuepeng Bian, Yangping Wang, Weijie Zhang, Changlin Ye and Jingjing Li

35 Implications of draxin in neurological disorders
Yohei Shinmyo

40 Arx revisited: involved in the development of GABAergic interneurons
Akio Tsuboi and Seiich Yoshihara

48 Slit-Robo signaling supports motor neuron avoidance of the spinal cord midline through DCC antagonism and other mechanisms
Kelsey R. Nickerson, Ferass M. Sammoura, Yonghong Zhou and Alexander Jaworski

60 Osteocalcin and GPR158: linking bone and brain function
Jingjing Li, Shujie Lou and Xuepeng Bian

74 Roles of LRRK2 and its orthologs in protecting against neurodegeneration and neurodevelopmental defects
An Phu Tran Nguyen, Linh Thi Nhat Nguyen, Bailey A. Stokke and Christopher C. Quinn

82 Emerging roles for E3 ubiquitin ligases in neural development and disease
Maya Hale and Greg J. Bashaw

98 Neuronal guidance behaviours: the primary cilium perspective
Melody Atkins, Coralie Fassier and Xavier Nicol

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Editorial: Neuronal guidance signaling in health and neurological diseases

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Editorial on the Research Topic
Neuronal guidance signaling in health and neurological diseases

Neuronal guidance signaling represents a cornerstone found in neuroscience, vital for the precise establishment of neural circuits during development. Neurons navigate complex environments, extending axons to target locations and forming synaptic connections through the interpretation of diverse extracellular cues. Central to this intricate process are neuronal guidance genes, encoding proteins that act as cues, receptors, or intracellular signaling effectors. These molecular players ensure accurate neural wiring, synapse formation, and ongoing neural maintenance throughout life (Yamagishi et al., 2021; Yuasa-Kawada et al., 2023). Disruption or dysregulation in these signaling pathways underlies many developmental, neuropsychiatric, and neurodegenerative disorders (Yuasa-Kawada et al., 2026). This Research Topic compiles original research and insightful reviews aimed at exploring both novel and classical mechanisms underlying neuronal guidance signaling, highlighting significant progress and identifying critical areas for future exploration to open new avenues toward developing clinical applications.

One of key signaling molecule extensively studied is Draxin, an axon guidance protein essential for the development of forebrain commissures. Shinmyo reviews the role of draxin, emphasizing its involvement in neurological disorders such as autism spectrum disorder (ASD). Draxin knockout mice display significant structural anomalies, notably in the corpus callosum, hippocampal commissure, and thalamocortical projections. Interestingly, the deletion of draxin gene was identified in ASD model BTBR/J mice, suggesting that draxin deletion is a genetic factor for ASD-like characteristics in the mice. Genetic manipulations further support draxin's essential function in establishing neural circuitry and highlight its potential role as a genetic determinant of ASD-related neuroanatomical changes.

Moving from axon guidance proteins to transcriptional regulation, Tsuboi and Yoshihara reviewed the Aristaless-related homeobox (Arx) gene. Arx mutations have been linked with a variety of neurological disorders, including intellectual disability and epilepsy. Their review emphasizes the pivotal role of Arx in the development and migration of GABAergic interneurons, especially within the cerebral cortex and olfactory bulb. By employing conditional knockout strategies, recent findings have identified Arx as a

crucial regulator of interneuron progenitor differentiation, highlighting its importance in neurodevelopmental disorders and cortical interneuron specification.

Nickerson et al. provide new insights into the complexities of axon guidance at the spinal cord midline through their study on Slit-Robo signaling. Utilizing genetic mouse models, they uncover that Robo receptors counteract DCC-mediated attraction to Netrin-1, preventing motor neurons and their axons from aberrant midline crossing. Their work reveals a sophisticated interplay between Slit and netrin signaling pathways, demonstrating how Slit proteins convert netrin's attractive cues into repulsive signals, a mechanism critical for precise motor circuit formation.

Complementing this, Northington et al. focus on the molecular interplay downstream of netrin-1 signaling, specifically how microtubule modifications mediate guidance responses. Their findings indicate that the polyglutamylase enzyme TTLL1 is required for netrin-1-induced axon growth, highlighting a previously underappreciated layer of complexity involving post-translational modifications of microtubules in guidance signaling. This discovery not only deepens our understanding of cytoskeletal dynamics in axon guidance but also offers new avenues for exploring therapeutic targets for conditions involving disrupted axonal pathfinding.

Exploring further downstream signaling mechanisms, Hale and Bashaw discuss the emergent roles of E3 ubiquitin ligases in neural development. Their review covers how ubiquitination, mediated by specific E3 ligases, regulates protein localization, degradation, and signaling. By examining ligase families such as RING and HECT, they elucidate their roles in neural specification, axon guidance, and dendrite morphogenesis. Critically, these ligases are linked to various neurodevelopmental disorders, emphasizing their potential as therapeutic targets to manage conditions like autism spectrum disorder, Angelman syndrome, and intellectual disability.

A novel intersection between bone-derived hormones and neural signaling is addressed by Bian et al., who discuss osteocalcin (OCN) and its receptor GPR37. Osteocalcin, traditionally associated with bone metabolism, has been recognized as an endocrine regulator influencing cognitive function and mood. GPR37, prominently expressed in the brain, mediates osteocalcin signaling, affecting neuronal migration, proliferation, and differentiation. This receptor pathway has potent neuroprotective effects, implicating it in neurodegenerative diseases like Parkinson's disease, and offering a unique perspective on the bone-brain axis in neurological health.

In parallel, Li et al. expand on osteocalcin signaling, examining GPR158, another key receptor involved in CNS functions. They propose that GPR158 plays a critical role in synaptic plasticity and cognition, influencing stress responses and metabolic regulation. The receptor is intricately linked with neurodegenerative diseases, suggesting that further exploration could yield valuable therapeutic interventions targeting cognitive and mood disorders through modulation of the bone-brain endocrine axis.

Further enriching this Research Topic, Atkins et al. address the novel perspective of primary cilia in neuronal guidance. Traditionally considered vestigial, primary cilia are now recognized as critical signaling antennas that concentrate neuronal guidance receptors. This review emphasizes the necessity of future investigations into ciliary signaling pathways, given their emerging relevance in neurological diseases including ciliopathies, neurodevelopmental, and neurodegenerative disorders.

Finally, Nguyen et al. provide critical perspectives on the role of LRRK2, leucine-rich repeat kinase 2, widely known for its association with Parkinson's disease. They underscore recent findings suggesting that LRRK2 and its orthologs not only prevent neurodegeneration but also safeguard against developmental defects, notably influencing axon guidance. Their discussion highlights autophagy regulation as a key pathway, indicating how disruptions in LRRK2 functions can simultaneously lead to neurodevelopmental abnormalities and later-life neurodegenerative conditions.

Collectively, these contributions underscore how neuronal guidance signaling extends beyond developmental contexts, implicating diverse signaling pathways and molecular mechanisms in lifelong neural health and disease. Advancements in understanding these intricate interactions not only enrich basic neuroscience but also pave the way for developing innovative therapeutic approaches to neurodevelopmental and neurodegenerative diseases. As this Research Topic demonstrates, exploring neuronal guidance signaling continues to reveal unexpected molecular players and mechanisms that could revolutionize our approach to neurological healthcare. This editorial emphasizes the interconnectedness of neuronal signaling pathways, urging continued collaborative exploration across disciplinary boundaries to fully uncover the complexities of brain development and pathology.

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Netrin-1 stimulated axon growth requires the polyglutamylase TTLL1

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Introduction: In the developing brain, neurons extend an axonal process through a complex and changing environment to form synaptic connections with the correct targets in response to extracellular cues. Microtubule and actin filaments provide mechanical support and drive axon growth in the correct direction. The axonal cytoskeleton responds to extracellular guidance cues. Netrin-1 is a multifunctional guidance cue that can induce alternate responses based on the bound receptor. The mechanism by which actin responds to Netrin-1 is well described. However, how Netrin-1 influences the microtubule cytoskeleton is less understood. Appropriate microtubule function is required for axon pathfinding, as mutations in tubulin phenocopy axon crossing defects of Netrin-1 and DCC mutants. Microtubule stabilization is required for attractive guidance cue response. The C-terminal tails of microtubules can be post-translationally modified. Post-translational modifications (PTMs) help control the microtubule cytoskeleton.

Methods: We measured polyglutamylation in cultured primary mouse cortical neurons before and after Netrin-1 stimulation. We used immunohistochemistry to measure how Netrin-1 stimulation alters microtubule-associated protein localization. Next, we manipulated TTLL1 to determine if Netrin-1-induced axon growth and MAP localization depend on polyglutamylation levels.

Results: In this study, we investigated if Netrin-1 signaling alters microtubule PTMs in the axon. We found that microtubule polyglutamylation increases after Netrin-1 stimulation. This change in polyglutamylation is necessary for Netrin-1-induced axonal growth rate increases. We next determined that MAP1B and DCX localization changes in response to Netrin-1. These proteins can both stabilize the microtubule cytoskeleton and may be responsible for Netrin-1-induced growth response in neurons. The changes in DCX and MAP1B depend on TTLL1, a protein responsible for microtubule polyglutamylation.

KEYWORDS

microtubule polyglutamylation, microtubule-associated protein 1B, DCX = doublecortin, Netrin-1, axon growth and guidance, tubulin (microtubules), TTLL1

Introduction

Axon pathfinding is a vital process that paves the way for neuronal circuit formation during brain development. Failure in axon pathfinding can lead to connectivity defects and age-related neurodegeneration (Buscaglia et al., 2021; Wegiel et al., 2018; Rachel et al., 2000; Livesey and Hunt, 1997). Netrin-1 is a well-established guidance cue that controls a bundle of

axons crossing the midline of the brain called the corpus callosum and commissures (Yung et al., 2015; Bin et al., 2015). Netrin-1 knockout mice fail to form commissures or the corpus callosum (Fothergill et al., 2014). Netrin-1 knockout axons form disorganized bundles at either side of the midline known as Probst bundles (Fothergill et al., 2014).

Netrin-1 stimulates axon growth by binding its receptor Deleted in Colorectal Cancer (DCC) (Buscaglia et al., 2021; Hill et al., 2012; Varadarajan and Butler, 2017; Dent, 2004; Shekarabi and Kennedy, 2002; Li et al., 2002). Like Netrin-1 deletion, DCC knockout mice fail to form commissures indicating that Netrin-1 signaling through DCC is essential for axon guidance across the midline (Yung et al., 2015; Fothergill et al., 2014). In addition, axonal response to Netrin-1 depends upon the microtubule cytoskeleton (Buscaglia et al., 2021; Gasperini et al., 2017; Piper et al., 2015). Microtubules are dynamic polymers of α-and β-tubulin heterodimers that undergo periods of growth and depolymerization. The dynamic instability of microtubules helps drive axon extension or retraction in response to a guidance cue. Agenesis or hyperplasia of the corpus callosum and other commissures is associated with mutations that disrupt neuronally expressed tubulin in humans and mice indicating that microtubules are important for axon guidance in response to Netrin-1 (Buscaglia et al., 2021; Gartz Hanson et al., 2016; Bahi-Buisson et al., 2014; Bahi-Buisson and Maillard, 1993; Aiken et al., 2017). Microtubule stabilization is also required for axon response to guidance cues (Piper et al., 2015; Buck and Zheng, 2002). However, the mechanisms underlying how Netrin-1 affects microtubule properties are not understood.

Post-translational modifications (PTMs) may be a mechanism to rapidly change microtuble properties in response to guidance cues. PTMs to tubulin can alter microtubule properties and affect the binding of certain microtubule-associated proteins (MAPs) (Verhey and Gaertig, 2007; Chakraborti et al., 2016; Janke and Magiera, 2020). PTMs and MAPs regulate the stability of the microtubule polymer and affect axon growth (Dema et al., 2024; Friocourt et al., 2003; Jean et al., 2012; Bonnet et al., 2001; Jentzsch et al., 2024; Xu et al., 2017; Portran et al., 2017). Tubulin PTMs provide temporal and spatial control along the microtubule by altering MAP binding, kinesin activity, and intrinsic tubulin interactions (Bonnet et al., 2001; Xu et al., 2017; Portran et al., 2017; Marcos et al., 2009; Utreras et al., 2008; Lessard et al., 2019). The addition of glutamate residues to the α-and β-tubulin carboxy-terminal tails (polyglutamylation) alters the localized charge of the microtubule lattice (Janke and Magiera, 2020; Janke, 2014; Janke et al., 2008; Audebert et al., 1993; Bodakuntla et al., 2021; Bodakuntla et al., 2021; Ruse et al., 2022). Therefore, microtubule polyglutamylation changes the binding activity of specific MAPs and alters the trafficking of motor proteins to affect axonal growth (Friocourt et al., 2003; Bonnet et al., 2001; Bigman and Levy, 2020; Bodakuntla et al., 2020). This raises the possibility that Netrin-1 may regulate microtubule dynamics via altering polyglutamylation. In neurons, microtubule polyglutamylation is important for neuronal survival and function (Bodakuntla et al., 2021; Bodakuntla et al., 2020; Bedoni et al., 2016; Magiera et al., 2018; Shashi et al., 2018; Wang and Morgan, 2007). Furthermore, microtubule polyglutamylation affects MAP binding and motor trafficking rates, which could affect axon growth (Bonnet et al., 2001; Lessard et al., 2019; Bigman and Levy, 2020; Bodakuntla et al., 2020)., This demonstrates that polyglutamylation levels can regulate microtubule networks. Additionally, microtubule polyglutamylation levels are rapidly tuned in cells (Torrino et al., 2021), on a similar timescale to changes in axon length in response to Netrin-1. Together, these data support the premise that polyglutamylation could regulate the microtubule cytoskeleton for axon response to axon guidance cues like Netrin-1.

Polyglutamylation is controlled by Tubulin Tyrosine Ligase Like (TTLL) proteins that add glutamate residues to tubulin heterodimers and cytosolic carboxypeptidase (CCP) proteins that remove glutamate residues (Janke et al., 2005). TTLL proteins can initiate the branch point glutamate residue or elongate a glutamate chain. TTLL1 extends glutamate chains on α-and β-tubulin (Ping et al., 2023; Wu et al., 2022). TTLL1 is highly expressed in the brain (Janke et al., 2005). TTLL1 is necessary and sufficient to increase microtubule polyglutamylation in neurons, suggesting that TTLL1 polyglutamylates microtubules in neurons (Bodakuntla et al., 2020). Together, these data form the premise for the hypothesis that polyglutamylation may regulate microtubule response to guidance cues.

In this study, we show Netrin-1 increases microtubule polyglutamylation in the axon. We show that TTLL1 is required for axonal response to Netrin-1. We should that two important MAPs, MAP1b and DCX, localize to the axon in response to Netrin-1. Finally, we show that the localization of MAP1B and DCX to the axon in response to Netrin-1 depends upon TTLL1. These data suggest that TTLL1 is important for axonal response to Netrin-1.

Materials and methods

Animal care

C57Bl6 (RRID:IMSR_JAX:000664) mice were housed in pathogen-free facilities approved by AALAC. Procedures were performed under protocol 139 approved by the IACUC at The University of Colorado, Anschutz Medical Campus. Mice were kept on a 14:10 h light:dark cycle with *ad libitum* access to food and water. Mice were set up in breeding pairs. Pups were taken between postnatal day (P) 0 and P4 for all experiments.

Primary cortical neuron dissections, nucleofection, and culture

For primary neuronal cultures, mice were taken between P0 and P4 for dissection. The head was sprayed down with 70% ethanol and a decapitation was performed. The brain was removed and placed on a plate containing Hanks Balanced Salt Solution (Gibco Cat# 14175095) with 200 mL kynurenic acid, referred to as Dissection Media (DM). The hindbrain was resected. The brain was split along the midline and the meninges was removed. Next, the cortex was isolated and split into small pieces. These pieces were placed into a conical containing 3 mL of DM. Cortical pieces were then moved to a conical containing DM supplemented with papain, L-cysteine, and kynurenic acid. The conical was placed into a 37°C incubator for 45 min. After 45 min the papain solution was aspirated and replaced with 4 mL of plating media containing DMEM with glucose and sodium pyruvate, Glutamax, and pen/strep. Cells were resuspended and then allowed to settle before the media was aspirated again. Fresh plating media was added to the cells. Using a narrow bore Pasteur pipette, the cells were triturated between 10 and 20 times. This process

breaks down all the pieces into a single-cell suspension. Neurons were then spun down at 400 RCF for 5 min. Media was aspirated and the cells were resuspended for downstream processes. **Nucleofection:** Primary cortical neurons were co-nucleofected with equal volumes of marker plasmids and a different plasmid of interest. For example, 4 µg of GFP-CSAP (Dr. Chad Pearson, CU Anschutz) and 4 µg of myrTdTomato (Dr. Santos Franco, CU Anschutz) were added together to *Nucleofector Solution for Mouse Neurons with Supplement 1* (Lonza Cat# VPG-1001) in the same tube to create lipid droplets with both plasmids. For experiments manipulating TTLL1, neurons were nucleofected with 4 µg of TTLL1 OE plasmid or 4 µg TTLL1 shRNA plasmid along with 4 µg of a plasmid expressing a fluorescence marker (either myrTdTomato or GFP-CSAP). The solution was mixed by pipetting. Primary neurons were centrofuged and media was removed. Cells were resuspended in 50 µL of *Nucleofector solution with Supplement 1* and 50 µL of the plasmid/ Nucleofector solution before transfer to the nucleofection cuvette. Neurons were nucleofected using the O-03 setting on the Lonza Nucleofector 2b (Lonza Cat# 13458999). Nucleofected cells recovered in 2 mL of culture media with additional L-glutamine supplementation for 30 min in a 37°C incubator. Neurons were then plated for growth overnight in a 37°C incubator. After 24 h in culture, neurons were imaged on a Zeiss 900 microscope. 24 h after plating, the media was replaced with Neurobasal A without phenol red supplemented with B-27, 1X Glutamax, and b-FGF for all experiments.

Netrin-1 production and purification

Using an established protocol for Netrin-1 purification, Cos-7 cells were transfected with a Netrin-1 plasmid (OriGene Cat#: MG223704) using Lipofectamine 3,000 (Thermo Fisher Scientific Cat#: L3000015) (McCormick et al., 2024; Mutalik et al., 2024; Boyer et al., 2020; Plooster et al., 2017). Cells were incubated at 37°C with 5% CO_2 overnight. The next day, DMEM was removed, and cells were washed twice with PBS. OptiMEM serum-free media was added, and the cells were incubated for 24 additional hours. Next, the OptiMEM was removed and placed into a conical. The conical was spun down at 1400xg for 3 min to remove debris and dead cells. The media was then moved to a calibrated Amicon 30 kDa molecular weight cutoff centrifuge tube (Cat#: UFC903008). The tube was spun down at 3000xg at 4°C for 5 min and the flow-through was discarded. The tube was spun down for another 5 min and the flow-through was again discarded. Additional 1 min spins were performed until the filter portion of the tube contained ~500 µL of media. This was then removed and used for downstream experiments. The protein was then run on an SDS gel and stained with Coomassie blue to ensure the appropriate-sized band (80 kDa) was detected (Supplementary Figure S1). A BCA assay (Pierce) was used run to determine the concentration of the protein.

Western blots

Cortical neurons were dissected from mice between P0 and P3. Cells were plated in a 6-well plate coated with poly-d-lysine. Cultured primary neurons were exposed to 500 ng/mL of Netrin-1. Netrin-1 was left on the cells for either 5, 10, or 20 min before the media was removed. Cells were washed once with 2 mL of PBS to remove excess media. PBS was removed, 2 mL of fresh PBS was added, and neurons were scraped off the bottom of the dish using a cell scraper. Cells were spun down and PBS was removed. Cells were resuspended in RIPA buffer containing protease and phosphatase inhibitors. Protein abundance was determined using the Pierce BCA assay. Afterward, Lameli buffer was added to the samples. Western blots were performed using BioRad 4–20% gels and run at 65 V for 2 to 3 h. Protein was transferred using the BioRad Trans Blot Turbo system. Blots were washed in 1X TBS and then blocked for 1 h in 5% milk in 1X TBST. Primary antibodies were added and were left to incubate overnight on a shaker at 4°C. Primary antibodies were removed, and the membrane was washed with 1X TBST 3 times for 5 min each time. Secondary antibodies were diluted in 5% milk in 1X TBST and added to the membrane. The membrane was placed on a shaker for 1 h at room temperature. Secondary antibodies were removed, and the blot was washed with 1X TBST for 5 min 3 times. BioRad ECL developer was added to the blot for 5 min and left on a shaker before imaging of the blot was performed. All blots were imaged using a BioRad imaging system. Densitometry was analyzed using FIJI. Polyglutamylation levels were normalized to the amount of GAPDH protein expression seen on the blot. A ratio of polyglutamylation to GAPDH was used to determine the change in expression before and after the addition of Netrin-1.

Neuron growth rate experiments

Primary cortical neurons were nucleofected with 4 mg of MyrTdTomato. The neurons were plated and cultured for 24 h. Images were taken on a Zeiss 900 confocal microscope with a 20X air objective. Images were captured every 10 min before and every 10 min after 500 ng/mL of Netrin-1 was added to the media. Images were analyzed in FIJI using the line segment tool. Lengths were measured from the beginning of the axon to the longest tip of the growth cone. The change in length between each time point was calculated and graphed as DLength.

GFP-CSAP imaging

Primary cortical neurons were nucleofected with 4 µg of GFP-CSAP (Dr. Chad Pearson, CU Anschutz) and 4 µg of MyrTdTomato (Dr. Santos Franco, CU Anschutz). After 24 h in culture, neurons were imaged on a Zeiss 900 microscope. For additional GFP-CSAP experiments neurons were nucleofected with 4 µg of TTLL1 OE plasmid, TTLL1 shRNA, or scramble control plasmid. Images were taken every 10 min for 30 min before adding Netrin-1 at 500 ng/mL. Images were taken immediately and every 10 min for 30 min after Netrin-1 was added. Images were analyzed in FIJI, where a threshold was set and maintained individually per neuron and kept across every time point. ROIs were taken at 5 µm away from the soma, 20 µm away from the soma, 5 µm away from the growth cone and the growth cone for normal GFP-CSAP loacalization changes. For GFP-CSAP data collected in the Supplementary Figures the entire axon was measured as we previously observed changes in GFP-CSAP along multiple points of the axon.

Neuron morphology analysis

Neurons were nucleofected with 4 µg of MyrTdTomato and 4 µg of TTLL1 overexpression plasmid or scramble shRNA control. The neurons were plated and cultured for 24 h. Images were taken on a Zeiss 900 confocal microscope with a 20X air objective. Neurons were analyzed using a Scholl analysis plugin with FIJI. Images were cropped to include only the axon within the image. Primary branch points from the axon were counted and compared between the TTLL1 OE neurons and MyrTdTomato expressing neurons.

Immunofluorescence

Each mouse cortex was dissociated into single neurons which were divided between 8 wells on a cover slip (ThermoFisher product #177402). Plates were removed from the incubator to room temperature and 500 ng/mL Netrin-1 or an equal volume of vehicle was added. After either 5, 10, 20, or 30 min of Netrin-1 or vehicle exposure, media was aspirated and the cells were washed with PBS. Cells were fixed with a solution of 4% PFA and 0.1% glutaraldehyde in PBS for 10 min at room temperature. The fixation solution was removed, and cells were washed with PBS. Cells were then washed using 3% BSA with 0.2% Triton-X in PBS for 5 min. Next, cells were washed with a solution of 0.1% $NaBH_4$ in PBS for 7 min at room temperature on a shaker. The reducing buffer was removed and cells were washed 3 times with PBS for 5 min. Cells were blocked in 3% BSA with 0.2% Triton-X in PBS for 20 min at room temperature on a shaker. The blocking buffer was removed and primary antibodies were added and left overnight at 4°C on a shaker. The primary antibody was removed, and cells were washed with 0.2% BSA and 0.05% Triton-X in PBS 3 times for 10 min. Secondary antibodies were added and placed in the dark at room temperature for 30 min on a shaker. Cells were then washed with 0.2% BSA and 0.05% Triton-X in PBS 3 times for 10 min. One additional wash was performed with PBS. A coverslip was then placed on the slide along with Fluromount-G with DAPI. Slides were stored at 4°C in the dark until imaging was performed. Images were taken on a Zeiss 900 confocal microscope. Images were analyzed in FIJI. A threshold was set to eliminate background fluorescence and the cells were measured with one ROI containing the soma, one containing the entire axon, and one containing the growth cone. Mean fluorescence intensity is reported. MAP1B and DCX fluorescence intensity were compared between Netrin-1-treated and vehicle-treated controls at 5 and 10 min after exposure to account for any change in MAP1B or DCX that occurs due to mechanical force of liquid addition or time at room temperature. Statistics were performed in GraphPad Prism 10. Student's *t*-tests were performed between groups.

Statistical analyses

Statistics were performed in GraphPad Prism 10. Statistical significance was reported as a *p*-value of <0.05. Specific statistical analyses are reported in each figure legend. For all graphs mean ± SEM is shown unless otherwise noted. In comparisons between two groups a Student's *t*-test was performed.

To account for photobleaching of GFP-CSAP that occurred when imaging TTLL1 shRNA and scramble control neurons, we assessed the rate of decay before and after the addition of Netrin-1 in both samples. We estimate the following equation:

$$\log(y_{it}) = \alpha_i + \beta_1 time_t + \beta_2 time_t \times post_t + \beta_3 post_t + \beta_4 time_t \times ttll1_i + \beta_5 post_t \times ttll1_i + \beta_6 time_t \times post_t \times ttll1_i + \varepsilon_{it},$$

which measures the log of fluorescence for neuron *i* at minute t and represents an idiosyncratic error term. We allow for neuron-specific fluorescence with individual fixed effects (αi). We model the fluorescence decay allowing it to differ by ttll1shRNA both before and after the addition of Netrin-1, such that β_1 captures the decay rate for the control group before Netrin-1 is added, β_2 measures the change in the decay rate for the control after Netrin-1 is added, and β_3 measures any level shift in log fluorescence with the addition of Netrin-1. We measure the difference of each these measures for the ttll1 shRNA sample coefficients β_4, β_5, and β_6 respectively, paying particular attention to β_6, the difference between the ttll1 shRNA sample and the control sample in the change in the decay rate after adding Netrin-1. We cluster our standard errors by neuron so that our inference is robust to autocorrelation within the same neuron over time. While the rate of decay was exponential before Netrin-1 was added to both samples, it flattened after the addition of Netrin-1 in the control group but continued to decay in the treatment group.

Dissection Media
HBSS (Ca_{2+} and Mg_{2+} free) Cat. # 14170112
1 M HEPES Cat. # 15630106
Kynurenate solution
Plating Media
DMEM w/ glucose and sodium pyruvate Cat. # 11995065
Glutamax (100X) Cat. # 35050061
Pen/Strep (100X) Cat. # 15070063
Maintenance Media
Neurobasal A Cat. # 12349015
B27 (50X) Cat. # 17504001
Glutamax (100X) Cat. # 35050061
B-FGF (0.1 mg/mL) Cat. # 450–33-100UG
Borate Buffer
Boric Acid Cat. # B6768-500G
Sodium tetraborate
MilliQ Water (pH 8.5)
Plate Coating
Borate Buffer
Poly-D Lysine Stock Cat. # A3890401
Kynurenate Solution
Kynurenic Acid
10N NaOH Cat. # SS255-1
MilliQ Water
Papain Solution
HBSS (Ca_{2+} and Mg_{2+} free) Cat. # 14170112

Kynurenate (100 mM)
Papain
Cysteine (1 M)
DNAse I Cat. # 11284932001
Plasmids
MACF43-GFP
MyrTdTomato (Dr. Santos Franco, CU Anschutz)
GFP-CSAP (Dr. Chad Pearson, CU Anschutz)
Netrin-1 OE OriGene Cat#: MG223704
TTLL1 OE OriGene Cat#: NM_178869
TTLL1 shRNA Santa Cruz Biotech Cat#: sc-154786-SH
Control shRNA plasmid Santa Cruz Biotech Cat#:sc-108060
Antibodies
Rabbit Polyglutamylated Tubulin AdipoGen Cat#: AG-25B-0030-C050
Rabbit GAPDH Cell Signaling Technologies Cat#:2118
Goat Doublecortin Invitrogen Cat#: PA5-142704
Mouse MAP1B Santa Cruz Biotech Cat#: sc-135978
Rabbit Total Beta Tubulin: Invitrogen Cat # PA1-16947
Mouse Alpha Tubulin DM1A Sigma Cat #T6199
anti-Polyglutamylation Modification, mAb (GT335) AdipoGen Cat# AG-20B-0020-C100
Goat Anti-Rabbit IgG (H + L) Alexa Fluor Plus 555 Invitrogen Cat#:A32732
Goat Anti-Mouse Invitrogen Cat#:A11001
Donkey Anti-Goat IgG (H + L) Alexa Fluor Plus 647 Invitrogen Cat#:A32849
VECTASHIELD Vibrance Antifade Mounting Medium with DAPI Vector Laboratories Cat#:H-1800
Donkey Anti-Rabbit HRP Santa Cruz Biotech Cat#:sc-2313
Goat Anti-Mouse HRP Santa Cruz Biotech Cat#:sc-2005
Precision Protein StrepTactin-HRP Conjugate Bio-Rad Cat#:1610380

Results

Netrin-1 alters microtubule polyglutamylation along the axon

Netrin-1 increases axonal growth rate rapidly (Figure 1A) (Buscaglia et al., 2021). Axons could respond to Netrin-1 by increasing total polymerized tubulin or modifying established microtubules. We tested whether total tubulin levels in the axon increase in response to Netrin-1 by measuring total tubulin immunofluorescence and area in mouse primary cultured cortical neurons before and after exposure Netrin-1. Tubulin immunofluorescence did not increase after Netrin-1 exposure (Figures 1B,C). Furthermore, the total area of axonal tubulin fluorescence did not increase between neurons exposed to Netrin-1 and unexposed cultured neurons (Figure 1D). We hypothesized that neurons increase PTM abundance along the microtubule in response to Netrin-1. We quantified levels of polyglutamylation before and after Netrin-1 stimulation in primary cultured cortical neurons with western blots. Polyglutamylated tubulin normalized to GAPDH levels increased within 20 min following Netrin-1 stimulation and trended towards an increase after 10 min (Figure 1E). Centriole and Spindle-Associated Protein (CSAP) localizes to polyglutamylated microtubules (Bompard et al., 2018; Backer et al., 2012). For spatial and temporal resolution to visualize where and when polyglutamylation levels change with Netrin-1 stimulation, we used GFP-CSAP as a live polyglutamylation reporter and measured fluorescence intensity at multiple locations along the axon (Figures 1F–J; Supplementary Video S1) (Backer et al., 2012). Netrin-1 stimulation significantly increased the fluorescence intensity of GFP-CSAP in the axon shaft immediately following its application and for up to 20 min afterward (Figures 1F–I). There was no significant change in GFP-CSAP intensity in the growth cone at any point following Netrin-1 stimulation (Figure 1J). To distinguish between whether neurons increase the *initiation* of glutamylation or *extension* of glutamylated chains on tubulin tails in the axon in response to Netrin-1, we measured immunofluorescence of the GT335 glutamylation antibody, which recognizes the first two glutamates on the tubulin carboxy terminal tail, with and without Netrin-1 exposure. Immunofluorescence of GT335 in the axon did not increase with Netrin-1 exposure (Figures 1K,L) indicating that Netrin-1 does not induce new initiation of glutamylation chains on the tubulin tail. Rather, Netrin-1 stimulation increases the abundance of long glutamate side chains with 4 or more glutamate residues in the axon (Figure 1C). These data indicate that MTs are dynamically altered through post-translational modifications in response to Netrin-1. We next probed whether microtubule polyglutamylation is required for the axon growth rate increase in response to Netrin-1.

TTLL1 is required for axon growth response to Netrin-1

We hypothesized that precise control of polyglutamylation regulates microtubule stability to promote Netrin-induced growth response. TTLL1 extends polyglutamylation chains on the tubulin carboxy-terminal tails in neurons, while other TTLLs are responsible for initiation (Trichet et al., 2000; Wang et al., 2022). To test whether TTLL1 was required for the increase in axonal polyglutamylation in response to Netrin-1, we performed live imaging of neurons co-nucleofected with GFP-CSAP and TTLL1 shRNA plasmid. CSAP fluorescence decayed rapidly due to photobleaching even after addition of Netrin-1 in TTLL1 shRNA expressing neurons, while CSAP fluorescence flattens with the addition of Netrin-1 in scramble controls (Supplementary Figure S2). These data suggest that TTLL1 is required to increase polyglutamylation in response to Netrin-1. To determine if TTLL1 is required for axon growth in response to Netrin-1, we reduced TTLL1 expression in primary cortical neurons with TTLL1 shRNA and measured axon growth rate using a membrane-bound TdTomato protein before and after Netrin-1 stimulation. We measured the change in length from the soma to the most distal tip of the growth cone over time in TTLL1 shRNA and the scramble control. We observed that similar to previously published data (Buscaglia et al., 2021), control neurites increased in growth rate following Netrin-1 exposure (Figure 2A). TTLL1 knockdown abolished changes in neurite growth rate following the addition of Netrin-1 (Figure 2B).

We reasoned that polyglutamylation could be sufficient for an increase in growth rate. We overexpressed TTLL1 and measured CSAP fluorescence before and after Netrin-1. CSAP

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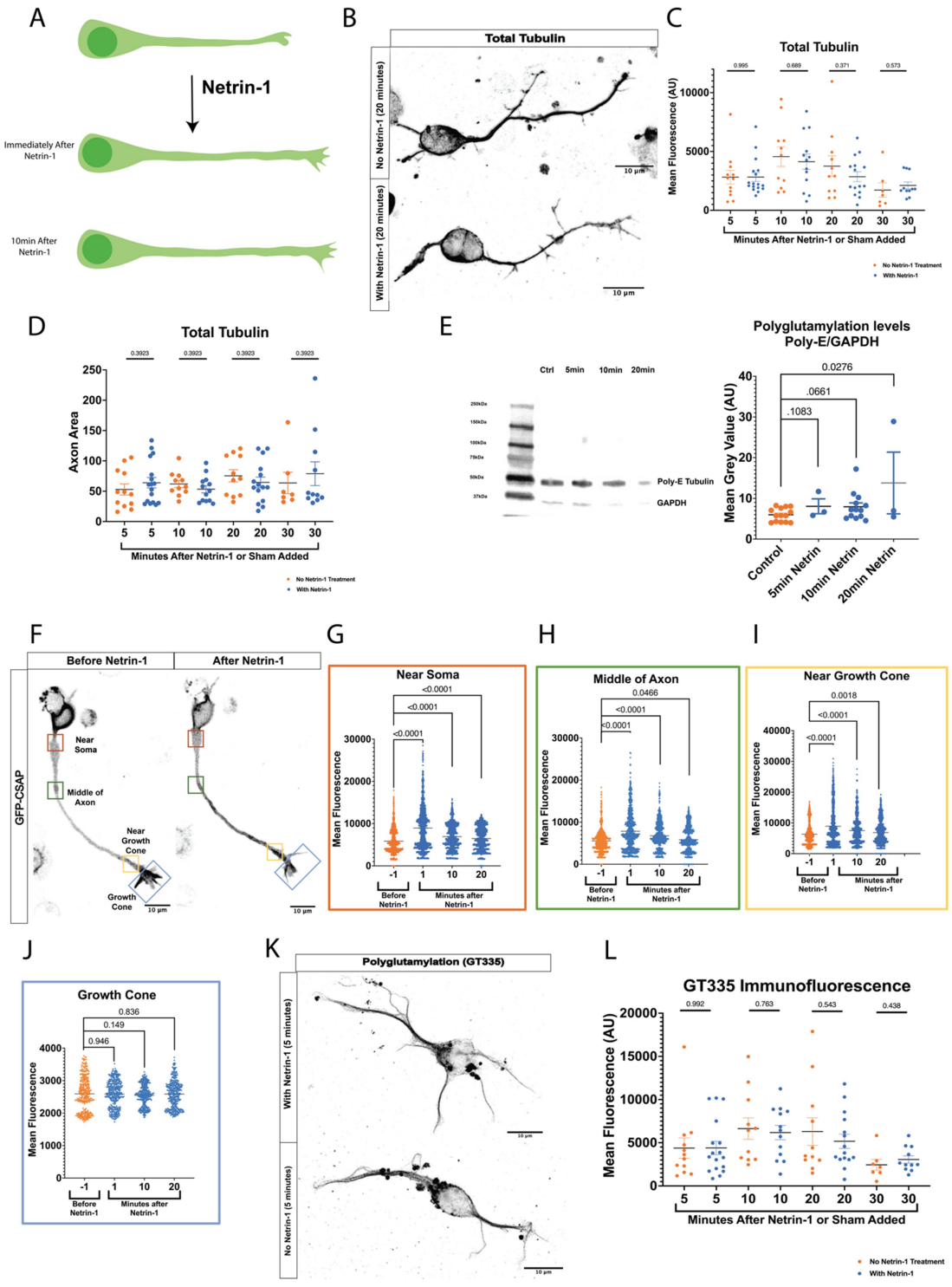

FIGURE 1
(A) Netrin-1 causes an increase in axon growth rate. However, it is unknown how the microtubule cytoskeleton is regulated to allow for this increase in growth rate. **(B)** Representative images of neurons stained for total tubulin. **(C)** Fluorescence data from total tubulin stained neurons shows no increase in total tubulin levels following Netrin-1 stimulation (*N* = cortical neurons from 6 mice). **(D)** Representative Western Blots and quantified densitometry show that polyglutamylation/GAPDH increases after Netrin-1 stimulation. *N* = cultured cortical neurons from 6 mice for no Netrin-1 and 10 min Netrin-1, *N* = cultured cortical neurons from 3 mice for 5 min Netrin-1, and 20 min Netrin-1. **(E)** Representative image showing example locations along the neuron where ROIs were selected. **(F)** Near Soma shows an increase in CSAP fluorescence intensity following Netrin-1 stimulation (*N* = 23 CSAP expressing cortical neurons from 8 mice). **(G)** The Middle of the Axon also experiences an increase in CSAP fluorescence intensity following Netrin-1 stimulation. **(H)** Near the Growth Cone also shows an increase in CSAP fluorescence intensity after Netrin-1 stimulation. **(I)** The Growth Cone showed no differences in GFP CSAP at any time following Netrin-1 addition to the media. **(J)** Representative images of neurons stained with the glutamylated tubulin antibody GT335 which mark glutamylated tubulin independent of glutamate chain length. **(K)** Quantified levels of GT335 immunofluorescence glutamylated tubulin show no increase in levels following Netrin-1 stimulation (*N* = cortical neurons from 6 mice).

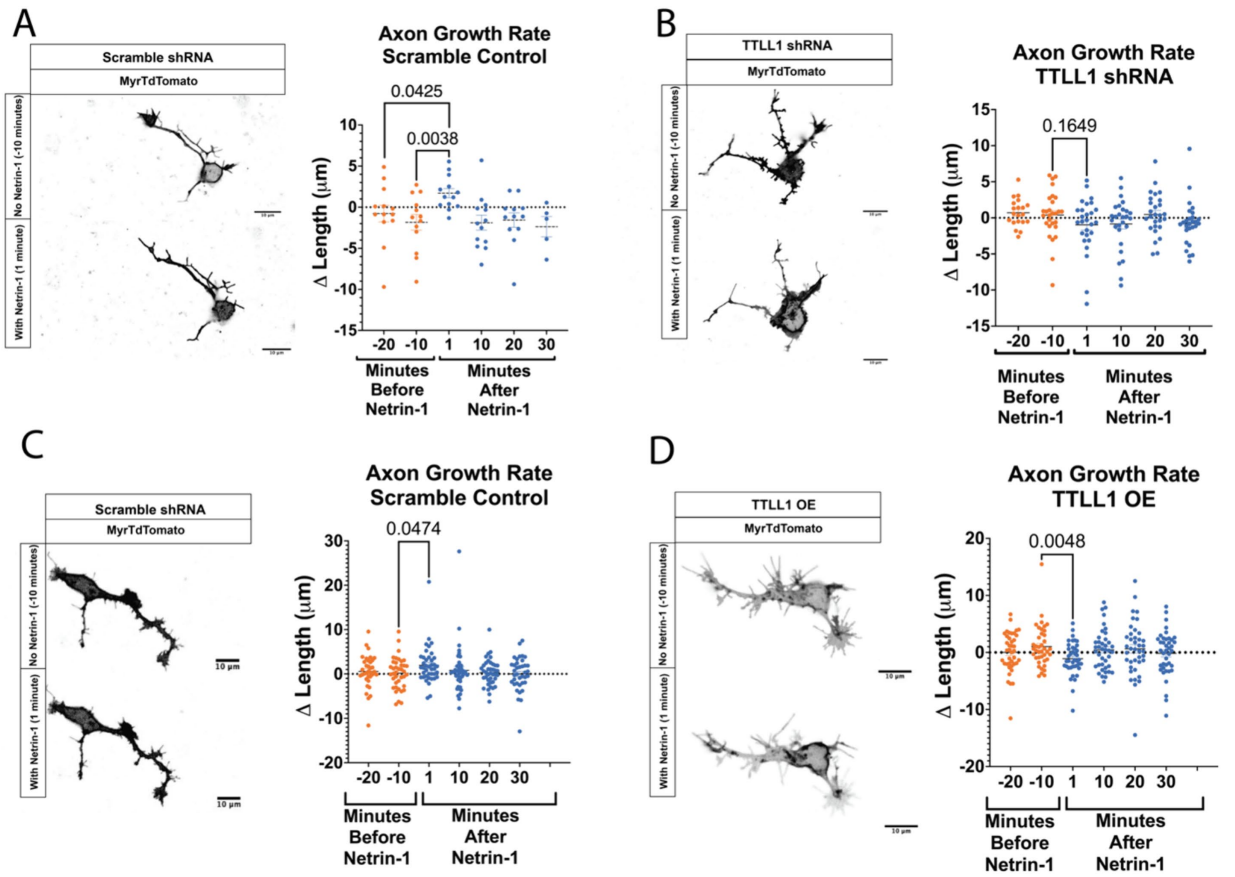

FIGURE 2
Growth rate following Netrin-1 stimulation is TTLL1 dependent. **(A)** Axons expressing a scramble shRNA that are time matched to those shown in B can respond to Netrin-1 exposure by increasing their growth rate ($N = 5$ or more neurons from 2 mice). **(B)** Axon growth no longer increases after Netrin-1 exposure in TTLL1 shRNA neurons (N = At least 20 neurons from 3 mice). **(C)** In neurons expressing a scramble shRNA performed at the same time as those in D experience an increase in growth rate following Netrin-1 exposure (N = at least 35 neurons from 3 mice). **(D)** In TTLL1 OE neurons, there is a significant decrease in the growth rate following Netrin-1 exposure ($N = 40$ neurons from 3 mice).

fluorescence does not increase in response to Netrin-1 in neurons that overexpress TTLL1 (Supplementary Figure S2). These data suggest that microtubule polyglutamylation does not increase in response to Netrin-1 in TTLL1 overexpressing neurons. To determine if a change in microtubule polyglutamylation is required for an increase in growth rate, we overexpressed TTLL1 in primary cortical neurons and measured neurite growth response to Netrin-1. Whereas control neurons increase in response to Netrin-1, TTLL1 overexpression significantly decreased neurite growth rate following the addition of Netrin-1 (Figures 2C,D). An abundance of TTLL1 inhibits neurite growth response to Netrin-1. These data support the hypothesis that TTLL1 is required to increase microtubule polyglutamylation for neurite growth response to Netrin-1. We observed that TTLL1 overexpression changes neuronal morphology. A Scholl analysis showed that TTLL1 overexpressing neurons significantly increases the number of branch points along the axon (Supplementary Figure S3). Altering the levels of TTLL1 in either direction is detrimental to axon response to Netrin-1 stimulation. We next wanted to determine whether MAPs that stabilize microtubules increase localization to the axon in response to Netrin-1.

Netrin-1 stimulation increases MAP abundance

Microtubule polyglutamylation alters the charge of the C-terminal tail thereby changing the binding affinity of certain MAPs for the microtubule surface. MAP1B is an essential MAP required for Netrin-1 signaling and commissure formation (del Río et al., 2004; Jayachandran et al., 2016; Meixner et al., 2000; Shi et al., 2019; Takei et al., 2000). To test if MAP1B localization changes with Netrin-1 stimulation, we fixed and stained neurons for MAP1B at multiple times after Netrin-1 or vehicle addition (Figures 3A–D). Netrin-1 addition significantly increases MAP1B fluorescence intensity in the soma, along the axon, and in the growth cone after 10 min of exposure (Figures 3B–D), while there is a trend towards a decrease in MAP1B fluorescence 10 min after vehicle addition.

Doublecortin (DCX) may be important for the axon response to guidance cues (Dema et al., 2024; Sébastien et al., 2023; Tint et al., 2009). To determine if DCX localization changes in response to Netrin-1, we stained primary neurons with Netrin-1 for DCX in a time course after stimulation with Netrin-1 (Figure 3E). Netrin-1 exposure significantly increases DCX in the axon (Figure 3G). DCX trends towards increasing in the growth cone after Netrin-1,

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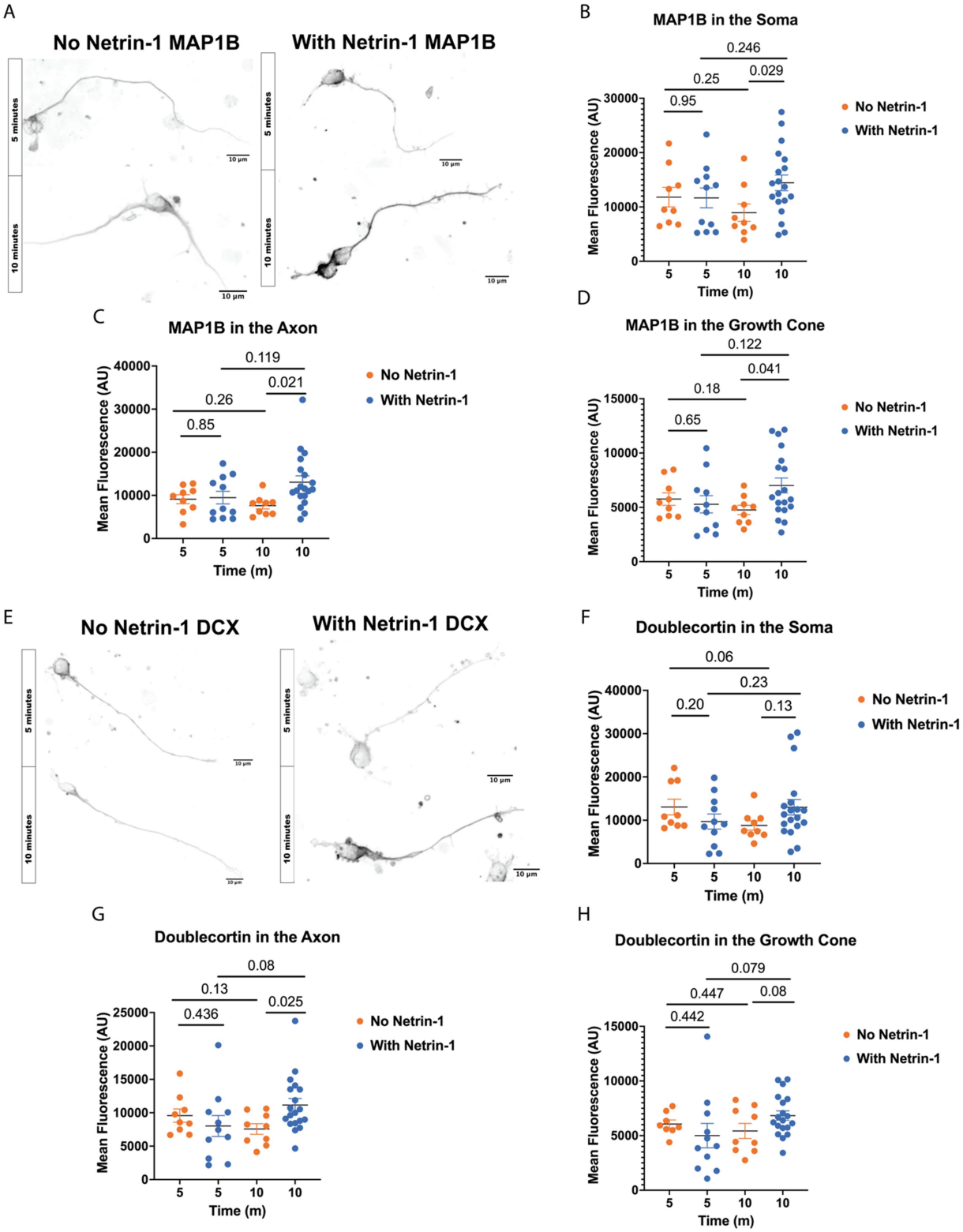

FIGURE 3
Netrin-1 stimulation changes MAP localization. **(A)** Representative images of MAP1B in neurons at DIV1 after fixation, Scale Bar 10 µm. **(B)** MAP1B in the soma changes with Netrin-1 after 10 min (*N* = 9 or more neurons from 2 animals). **(C)** MAP1B changes in the axon after 10 min of Netrin-1 stimulation (*N* = 9 or more neurons from 2 animals). **(D)** MAP1B increases in the growth cone following Netrin-1 stimulation. **(E)** Representative images of DCX in DIV1 neurons after fixation, Scale Bar 10 µm. **(F)** Doublecortin does not change in the soma following Netrin-1 stimulation (*N* = 9 or more neurons from 2 animals). **(G)** Doublecortin does increase in the axon following Netrin-1 stimulation for 10 min (*N* = 9 or more neurons from 2 animals). **(H)** Doublecortin trends towards an increase in the growth cone following 10 min of Netrin-1 stimulation (*N* = 9 or more neurons from 2 animals).

but does not alter DCX in the soma (Figures 3F–H). These results indicate that neurons increase the localization of DCX to the axon in response to Netrin-1. The increase in DCX and MAP1B fluorescence intensity occurs after the period when GFP-CSAP fluorescence increases following Netrin-1 stimulation (Figure 1), suggesting that increasing polyglutamylation levels may recruit or aid in trafficking MAP1B and DCX. Is Netrin-1-induced localization of MAP1B and DCX dependent on precise control of TTLL1 levels?

TTLL1 overexpression changes MAP localization in response to Netrin-1

To determine whether precise microtubule polyglutamylation regulation is necessary for MAP localization in response to Netrin-1, we overexpressed TTLL1 in cultured primary cortical neurons, stimulated them with Netrin-1, and stained neurons for MAP1B and DCX (Figures 4A,E). MAP1B staining does not increase in the axon after 10 min of Netrin-1 exposure in TTLL1 OE neurons as it does in control neurons (Figure 4C). TTLL1 overexpression prevents Netrin-1-induced MAP1B increases in the soma (Figure 4B). However, MAP1B still increases in the growth cone of TTLL1 OE neurons (Figure 4D). In TTLL1 OE neurons, DCX fluorescence in the axon, soma, and growth cone is reduced 5 min after Netrin-1 exposure compared to vehicle-exposed neurons at the same time point. There were no changes in DCX fluorescence 10 min following Netrin-1 exposure in any area measured in cortical neurons overexpressing TTLL1 compared to vehicle exposed neurons (Figures 4F–H). TTLL1 overexpression prevents Netrin-1-induced increases in polyglutamylation, MAP1B, and DCX localization to the axon, and neurite growth rate. Together, these data show that Netrin-1-induced MAP1B and DCX localization to the axon are dependent on TTLL1.

Discussion

Regulation of polyglutamylation is important for axon response to Netrin-1

The mechanism by which Netrin-1 communicates with the microtubule cytoskeleton has been a significant knowledge gap. Here, we show that microtubule polyglutamylation increases in response to Netrin-1 (Figure 1), and that increased polyglutamylation is required for the axon growth response to Netrin-1 (Figure 2). Both MAP1B and DCX increase in abundance along the axon in response to Netrin-1 (Figure 3). The increase in axon growth rate may be due to a stabilizing effect from MAP1B or DCX (Figures 3, 4). The localization changes of MAP1B and DCX in the axon require regulated TTLL1 activity (Figure 4). Our data supports the model that Netrin-1 stimulation rapidly increases TTLL1 activity to promote microtubule polyglutamylation. Polyglutamylation changes the microtubule charge to promote the binding of stabilizing MAPs such as MAP1B and DCX (Figure 5). We propose that the increase in microtubule polyglutamylation and MAP binding stabilizes the lattice, promoting increased axon growth (Figure 5).

Post-translational modifications

Post-translational modifications can regulate microtubule function in a myriad of ways, The increase in polyglutamylation along the axon after Netrin-1 exposure may regulate microtubule response to external stimuli. Previous work in the field has shown changes in microtubule polyglutamylation in response to external mechanical forces (Torrino et al., 2021). We see increases in GFP-CSAP fluorescence in response to Netrin-1 a minute after the chemotactic cue is added to the media and it stays elevated for at least 20 min (Figure 1). However, in western blot analysis, we do not see significant increases in polyglutamylation until 20 min after the addition of Netrin-1 (Figure 1). Additionally, the changes seen in polyglutamylation in the western blot are likely long glutamate chains, as Netrin-1 does not increase the immunofluorescence of GT335 glutamylation antibody, which marks the initial two glutamates added to the tubulin C-terminal tail (Figure 1). While we use GFP-CSAP as a proxy for polyglutamylation, it may also play a role in changing the polyglutamylation state of microtubules (Bompard et al., 2018). That confounding factor is a limitation of our study. Further studies could investigate how PTMs respond to guidance cues using nanobodies which would enable live-imaging with the necessary protein specificity (Barakat et al., 2022; Freise and Wu, 2015; Fu et al., 2018).

Netrin-1 stimulation increases microtubule polyglutamylation in the axon through the action of TTLL1. Netrin-1-stimulated DCC could directly or indirectly activate TTLL1. Netrin-1-induced increases in polyglutamylation are too rapid for transcription or translation of new TTLL1. One possibility is that Netrin-1 stimulation increases glutamate available to TTLL1 to add to microtubules or other substrates. Glutamine metabolism to generate glutamate is required for microtubule polyglutamylation (Torrino et al., 2021). Reducing available glutamate reduces microtubule stability suggesting that polyglutamylation increases microtubule stability directly or indirectly through changing affinity for MAPs (Torrino et al., 2021). Glutamate levels also regulate axon growth (Schmitz et al., 2009; Zheng et al., 1996; Kreibich et al., 2004). For example, glutamate stimulates axon growth in cultured dopaminergic neurons (Schmitz et al., 2009). Furthermore, cultured spinal cord neurites turn towards a glutamate source (Zheng et al., 1996). While we have thought of glutamate stimulating axonal growth through calcium, glutamate availability could be a limiting reagent for its addition to microtubules. How polyglutamylation-modifying enzyme activity is modulated in the context of Netrin-1 will be an exciting area of future research.

Extensive research has defined mechanisms by which Netrin-1 stimulates changes in the actin cytoskeleton for axon guidance (McCormick et al., 2024; Mutalik et al., 2024; Boyer et al., 2020; Plooster et al., 2017; Menon et al., 2021; Menon et al., 2015). Co-immunoprecipitations or BioID experiments could shed light on the mechanism by which Netrin-1 bound DCC stimulates TTLL1 activity. It is also a possibility that intermediate pathways facilitate signaling between the DCC receptor and the microtubule cytoskeleton. However, DCC interacts with β-tubulin and this may allow for nearby tubulin modifying

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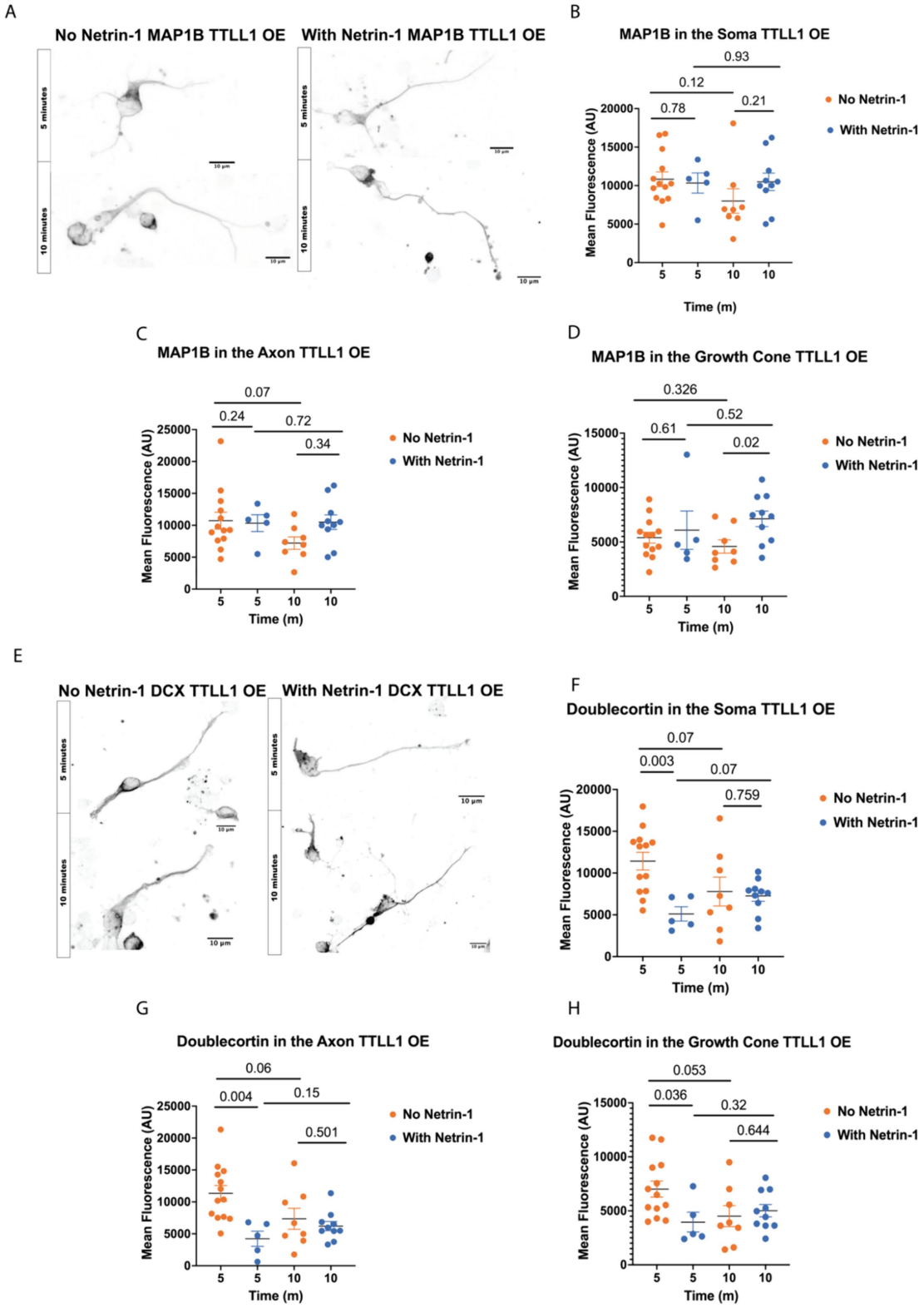

FIGURE 4
TTLL1 OE alters Netrin-1 induced changes in MAP localization. **(A)** Representative images of MAP1B in TTLL1 OE neurons at DIV1 after fixation, Scale Bar 10 μm. **(B)** MAP1B in the soma does not change with Netrin-1 after 10 min (*N* = Minimum of 5 neurons from 2 animals). **(C)** MAP1B does not change in the axon after 10 min of Netrin-1 stimulation (*N* = Minimum of 5 neurons from 2 animals). **(D)** MAP1B continues to increase in the growth cone following Netrin-1 stimulation in TTLL1 OE neurons (*N* = Minimum of 5 neurons from 2 animals). **(E)** Representative images of DCX in DIV1 TTLL1 OE neurons after fixation, Scale Bar 10 μm. **(F)** Doublecortin does not change in the soma following Netrin-1 stimulation (*N* = Minimum of 5 neurons from 2 animals). **(G)** Doublecortin does not increase in the axon following Netrin-1 stimulation for 10 min (*N* = Minimum of 5 neurons from 2 animals). **(H)** Doublecortin does not increase in the growth cone following 10 min of Netrin-1 stimulation (*N* = Minimum of 5 neurons from 2 animals).

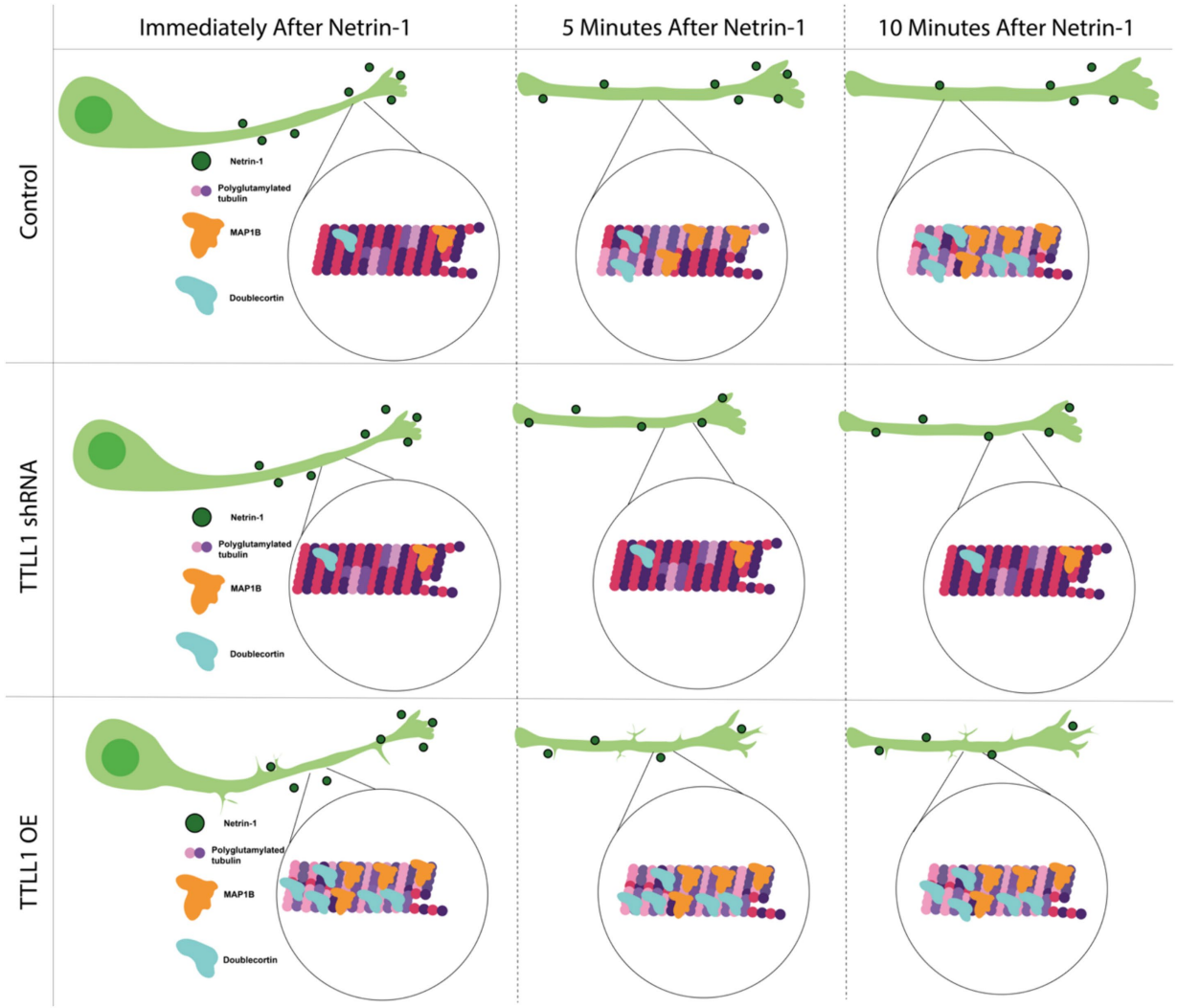

FIGURE 5
Model mechanism showing Netrin-1 stimulation increases polyglutamylation of microtubules over time. This increased polyglutamylation leads to increases in MAP1B and DCX in the axon, which stabilizes the microtubule cytoskeleton. This increased stability allows for improved axon growth following Netrin-1 stimulation.

enzymes to alter microtubule PTMs (Qu et al., 2013). This raises the possibility that there could be a complex including TTLLs and DCC to modify the microtubule cytoskeleton in response to receptor activation. This could be validated through future BioID or Co-IP experiments.

This study focused on polyglutamylation; however, numerous post-translational modifications can occur on the microtubule lattice. How additional microtubule modifications are altered in response to guidance cues is an intriguing area of future research that could deepen our understanding of cytoskeletal regulation during development. The tyrosination/detyrosination cycle is an interesting candidate for further study as it regulates pathfinding (Marcos et al., 2009). Additionally, MAP1B interacts with Tubulin Tyrosine Ligase protein which controls tubulin tyrosination (Utreras et al., 2008). Post-translational modifications of tubulin during axon guidance remain an exciting area of research. Microtubule PTMs can alter intrinsic lattice dynamics and how MAPs and motors bind.

Polyglutamylation recruits spastin, a microtubule severing enzyme that Is an important regulator of microtubule dynamics (Lacroix et al., 2010; Valenstein and Roll-Mecak, 2016). Interestingly, spastin breaks the microtubule lattice and increases local microtubule polymerization to regulate synapse formation (Aiken and Holzbaur, 2024). An increase in spastin activity causes branching in neurons (Yu et al., 2008). The increased axon branching phenotype observed in TTLL1 OE neurons may be due to increased spastin activity acting on hyper-glutamylated microtubules (Supplementary Figure S3). There is also the possibility that these branches are actin-mediated, as Netrin-1 has long been associated with changes in actin cytoskeleton regulation (Shekarabi and Kennedy, 2002; Li et al., 2002; Menon et al., 2021; Shekarabi et al., 2005; Boyer and Gupton, 2018). PTM control of microtubule properties continues to be an area of active research. Our study offers some insight into how microtubules are regulated in developing neurons. These results indicate important changes to polyglutamylation occur *in vitro* and in the specific cells that perform these migrations.

Microtubule-associated proteins in the developing brain

Microtubule-associated proteins offer another layer of regulation of the microtubule lattice. The variety of MAP functions can provide precise regional control over the stability and function of microtubules. We show that MAP1B fluorescence increases ten minutes after addition of Netrin-1, while it trends towards decreasing ten minutes after addition of vehicle. The trend towards a decrease in MAP1B in vehicle exposed neurons could be due to temperature changes or consequences of mechanical stimulation with the addition of media. Polyglutamylation increases microtubule stability and is localized to axons and growth cones (Bonnet et al., 2001; Lessard et al., 2019). MAP1B is required for axon response to Netrin-1 and stabilizes the microtubule cytoskeleton in neurites (del Río et al., 2004; Meixner et al., 2000; Li et al., 2006). MAP1B may preferentially bind to polyglutamylated microtubules (Bonnet et al., 2001) and this could be the mechanism through which Netrin-1 signaling promotes microtubule stability. Altering TTLL enzyme levels may change glutamate chain length, which could regulate the affinity of MAP1B for the microtubule lattice. The increase in MAP1B axonal localization in response to Netrin-1 could stabilize the microtubule lattice and allow for increased axon growth in response to Netrin-1 (Figure 2). The increase in GFP-CSAP and MAP1B in the axon following Netrin-1 are supports the model that Netrin-1 increases polyglutamylation which recruits MAP1B or aids in its localization to the axon. TTLL1 OE abolishes the increase in axon growth and MAP1B localization to the axon following Netrin-1 stimulation, supports the model that TTLL1 is required for axon growth and MAP1B localization to the axon. However, our results in the growth cone are not consistent with this model. While Netrin-1 does not measurably increase GFP-CSAP in the growth cone, Netrin-1 increases MAP1B in the growth cone (Figures 1J, 3D). Furthermore, Netrin-1 increases MAP1B in the growth cone of TTLL1 OE neurons (Figure 4D). These data indicate that MAP1B localization is either not dependent on polyglutamylation in the growth cone, or that GFP-CSAP does not localize to the growth cone adequately to assess changes in polyglutamylation. Another possibility is that overexpressing TTLL1 does not affect polyglutamylation in the growth cone.

We report an increase of DCX fluorescence in the axon ten minutes after Netrin-1 stimulation, while we observe a trend towards a decrease in DCX fluorescence ten minutes after addition of vehicle (Figure 3). We observe a trend towards an increase in DCX fluorescence in the growth cone after Netrin-1 stimulation. This raises the possibility that microtubule stability in the axon, behind the growth cone, is important for overall response to Netrin-1. DCX knockout mice have reduced polyglutamylation levels and fail to respond to guidance cues (Dema et al., 2024; Sébastien et al., 2023). DCX localizes to microtubules in the growth cone in a highly polarized fashion and stabilizes microtubule polymer (Dema et al., 2024; Friocourt et al., 2003). Neuronal DCX knockouts fail to respond to brain-derived neurotrophic factor gradients indicating an important role for DCX in axon guidance (Dema et al., 2024). Specific levels of microtubule polyglutamylation recruit spastin, a MAP that regulates axonal microtubule dynamics in specific localizations to facilitate appropriate axonal transport (Lacroix et al., 2010; Valenstein and Roll-Mecak, 2016). DCX is also important for actin response to Netrin-1 through its effect on actin-binding proteins, suggesting another mechanism by which polyglutamylation could control axon guidance (Fu et al., 2013). Thus, polyglutamylation could increase DCX and MAP1B binding to stabilize microtubules in the axon and reduce axon retraction during development. Additionally, the increase in polyglutamylation and MAP localization in response to Netrin-1 could be important for microtubule intrusion and polymerization into the growth cone for tuned response to Netrin-1. Our data indicates that DCX may help stabilize the microtubule cytoskeleton in response to Netrin-1. Because DCX strengthens the microtubule lattice, its increase in the growth cone could be an important response to Netrin-1 stimulation. Additionally, DCX localization increases in the actin-rich protrusions of the growth cone, which could be an important aspect of Netrin-1 response (Tint et al., 2009; Fu et al., 2013). Similar to MAP1B, overexpression of TTLL1 prevents the increase in DCX following Netrin-1 stimulation in the axon. This may cause axonal microtubules to be less stable and reduce the ability for the axon to grow in response to Netrin-1. TTLL1 regulation is important for proper Netrin-1 response. An overabundance of the protein may cause problems with tuning the levels of polyglutamylation and therefore there is a dampened response to Netrin-1 stimulation.

Our study shows that Netrin-1 increases microtubule polyglutamylation which is required for axons to grow more quickly. TTLL1 is required for the axon growth response to Netrin-1. However, increased levels of TTLL1 also inhibit the effects of Netrin-1 on growth. These data suggest that tight control of TTLL1 is important for axon response to Netrin-1 due to its role in extending glutamate chains on microtubules, which can lead to MAP binding and stabilization of the microtubule lattice.

Data availability statement

The original contributions presented in the study are included in the article/Supplementary material, further inquiries can be directed to the corresponding author.

Ethics statement

The animal study was approved by University of Colorado Anschutz Medical Campus IACUC. The study was conducted in accordance with the local legislation and institutional requirements.

Author contributions

KN: Investigation, Data curation, Formal analysis, Methodology, Validation, Visualization, Writing – original draft. JC: Writing – review & editing, Formal analysis. EB: Conceptualization, Funding acquisition, Investigation, Project administration, Supervision, Writing – review & editing.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

Supplementary material

The Supplementary material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fnins.2024.1436312/full#supplementary-material

SUPPLEMENTARY FIGURE S1
Netrin-1 was expressed in Cos-7 cells and purified for addition to cultured neurons.

SUPPLEMENTARY FIGURE S2
TTLL1 shRNA abolishes Netrin-1 induced increase in GFP-CSAP. GFP-CSAP fluorescence decays over time due to photobleaching. The decay of fluorescence signal significantly slows after addition of Netrin-1 in the control neurons **(A–C)** whereas decay does not change after addition of Netrin-1 in TTLL1 knockdown neurons **(C–F)**. GFP-CSAP does not significantly increase with Netrin-1 in TTLL1 overexpressing neurons **(G,H)**. GFP-CSAP fluorescence was not visible in scramble control neurons at the same laser power (data not shown).

SUPPLEMENTARY FIGURE S3
Neurons overexpressing TTLL1 have significantly more axonal branches than control neurons.

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GPR37 and its neuroprotective mechanisms: bridging osteocalcin signaling and brain function

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Osteocalcin (OCN) is a hormone secreted by osteoblasts and has attracted widespread attention for its role in regulating brain function. Clinical studies indicate a positive correlation between levels of circulating OCN and cognitive performance. Indeed, lower circulating OCN has been detected in various neurodegenerative diseases (NDs), while OCN supplementation under certain conditions may improve cognitive function. GPR37, a G protein-coupled receptor, has recently been identified as a receptor for OCN. It exhibits distinct expression patterns across various brain regions and cell types, potentially influencing its functional roles within the brain. Research indicates that GPR37 regulates neuronal migration, cell proliferation, differentiation, and myelination. Furthermore, GPR37 has been shown to mitigate inflammation and apoptosis through various mechanisms, exerting neuroprotective effects. However, its regulatory influence on brain function exhibits inconsistency, highlighting a duality in its actions. Therefore, this review thoroughly summarizes the roles and mechanisms of GPR37 in modulating cellular physiological activities and its involvement in immune responses, stress reactions, and neuroprotection. It aims to enhance the understanding of how GPR37 modulates brain function and facilitate the identification of novel therapeutic targets or strategies for related diseases.

KEYWORDS

osteocalcin, GPR37, brain function, inflammation, stress response, neuroprotection

1 Introduction

Osteocalcin (OCN), a protein composed of 44–56 amino acids, is secreted by osteoblasts (Komori, 2020; Nowicki and Jakubowska-Pietkiewicz, 2024) and was initially considered primarily involved in bone mineralization. Subsequent research has revealed that OCN can circulate through the bloodstream and enter various tissues and organs, such as skeletal muscle and liver. In these regions, OCN regulates insulin sensitivity, glucose and lipid metabolism, and skeletal muscle function (Komori, 2020; Nowicki and Jakubowska-Pietkiewicz, 2024). Furthermore, OCN crosses the blood-brain barrier (BBB) and exerts regulatory effects on the central nervous system (CNS), particularly regarding cognitive function and mood regulation (Shan et al., 2023). Several clinical studies have demonstrated a positive correlation between circulating levels of OCN and cognitive function. In various

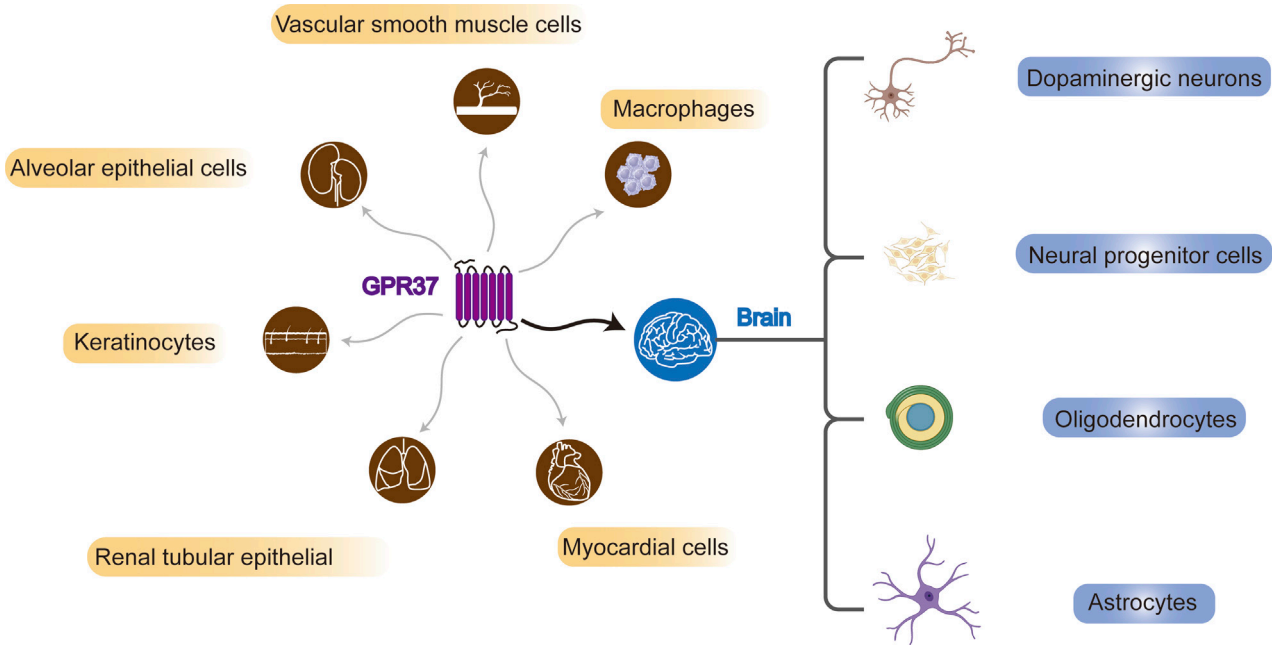

FIGURE 1
Systemic and brain distribution of GPR37. GPR37 is expressed in various cell types, including vascular smooth muscle cells, macrophages, alveolar and renal tubular epithelial cells, keratinocytes, and myocardial cells. In the brain, it is found in dopaminergic neurons, neural progenitors, oligodendrocytes, and astrocytes.

neurodegenerative diseases (NDs), such as Alzheimer's disease (AD) and Parkinson's disease (PD), lower circulating levels of OCN are observed (Hou et al., 2021; Liu et al., 2023). Mice deficient in OCN exhibit deficits in spatial learning and hippocampus-dependent memory (Oury et al., 2013). The supplementation of OCN can potentially improve spatial learning and memory by reducing amyloid-beta (Aβ) deposition and gliosis, elevating levels of monoamine neurotransmitters, and promoting neuroplasticity within the hippocampus and cortex (Shan et al., 2023).

The functions of OCN are contingent upon its receptors. To date, three OCN receptors have been identified in mammals: GPR37 (G protein-coupled receptor 37), GPR158, and GPRC6A, all classified as G protein-coupled receptors (Karsenty, 2023). These receptors exhibit distinct regional distributions and fulfill various functions within the body. This review focuses on GPR37, the most recently identified central receptor for OCN. Notably, GPR37 exhibits high expression in the brain and is significantly associated with the development and prognosis of various CNS diseases. The deficiency of GPR37 can result in dopaminergic neuronal damage and disrupt long-term potentiation (LTP) (Hertz et al., 2019; Zhang et al., 2020a). GPR37 may also exhibit bidirectional effects in certain physiological phenomena. In a stroke model, GPR37 negatively correlates with serum inflammatory factor levels (McCrary et al., 2019; Zhang et al., 2022). Conversely, in lipopolysaccharide (LPS)-induced inflammation models, the expression of GPR37 is significantly elevated, further activating glial cells and exacerbating the inflammatory response (Qian et al., 2022).

Given the complex and uncertain roles of GPR37 in various functions, along with the incomplete understanding of its regulations in the CNS, this review aims to summarize the roles and mechanisms of GPR37 to enrich the "bone-brain axis" theory further and offer new targets for the treatment of NDs.

2 Identification and distribution of GPR37

In 1997, GPR37 was identified by analyzing cDNA expression sequence tags from the human frontal cortex, utilizing RACE-PCR technology to study neuropeptide-specific receptor genes (Marazziti et al., 1997). Subsequent research has revealed that GPR37 is expressed in multiple brain regions of the CNS (Yang et al., 2016; Mouhi et al., 2022) and different types of cells, including substantia nigra dopaminergic neurons (Imai et al., 2001; Morato et al., 2021), neural progenitor cells (NPCs) (Berger et al., 2017; Owino et al., 2021), oligodendrocytes (OLs), and astrocytes (Bang et al., 2018). However, in microglia, GPR37 is unidentified (Bang et al., 2018). The expression of GPR37 may vary even in the same type of cells, which may depend on the stage of cell development. For example, GPR37 is highly expressed in mature OLs but not in oligodendrocyte precursor cells (OPCs) (Yang et al., 2016) (Figure 1).

Previous studies have demonstrated that OCN binds specifically to GPR37 but not to its homolog GPR37L1, as confirmed by affinity assays and immunoprecipitation techniques (Qian et al., 2021). Further investigations reveal that OCN is involved in myelination via GPR37. Exogenous injection of OCN in wild-type (WT) mice significantly decreases the levels of myelin-associated proteins—proteolipid protein 1 (PLP1) and myelin basic protein (MBP) in the corpus callosum and spinal cord. Notably, this effect is absent in GPR37−/− mice, indicating that OCN's actions are

mediated through GPR37 (Qian et al., 2021). In primary cultured OLs, inhibition of GPR37 using shRNA or antibodies significantly attenuates the OCN-induced reduction of PLP1 and MBP, whereas silencing GPR37L1 does not affect this downregulation (Qian et al., 2021). These *in vivo* and *in vitro* findings demonstrate that OCN exerts specific effects through GPR37, establishing a distinct ligand-receptor relationship between them.

3 Central regulatory functions of GPR37

The role of GPR37 can be traced back to studies on its homolog, SCGPR1, in chicken embryos. SCGPR1 demonstrates significant developmental expression in the neural tube, forebrain, midbrain, and spinal cord. This experiment suggests that GPR37 may be expressed in varying temporal and spatial patterns depending on the developmental stage and needs of the organism as it progresses from an embryo to an adult (Odani et al., 2007). The involvement of OCN in embryonic development offers insights into the developmental regulation of GPR37 expression. During pregnancy, maternal OCN crosses the placental barrier and enters the embryonic bloodstream, which plays a neuroprotective role by preventing apoptosis of hippocampal neurons (Oury et al., 2013). OCN levels synchronize with cognitive changes from growth and development to aging. Maternal and embryonic OCN contribute to establishing and maintaining body homeostasis in newborns and adult offspring, influencing brain development (Oury et al., 2013; Correa Pinto Junior et al., 2024). With aging, the decline in bone mass and OCN levels, along with a progressive decrease in the activity of critical molecules essential for cellular functions, such as nicotinamide adenine dinucleotide (NAD) and NRF2, collectively contribute to cognitive decline (Nishimoto et al., 1985; Silva-Palacios et al., 2018; Fania et al., 2019).

A deficiency in OCN contributes to a range of peripheral metabolic disorders and markedly reduces the expression of genes related to glucose metabolism in the brain. With advancing age, OCN−/− mice develop insulin resistance and glucose intolerance, while supplementation with OCN mitigates these metabolic disturbances (Ferron et al., 2008; Ferron et al., 2012; Zhang et al., 2020b; Paracha et al., 2024). Dysregulation of peripheral glucose metabolism is closely associated with central insulin resistance (Guo et al., 2020). Impaired insulin signaling in the brain—particularly involving the IRS/PI3K/Akt pathway—often exacerbates the pathogenesis of NDs (Dewanjee et al., 2022). These disruptions are associated with profound impairments in learning and memory during adulthood (Oury et al., 2013; Correa Pinto Junior et al., 2024). These findings highlight that maintaining optimal maternal skeletal health and adequate OCN levels during pregnancy may be critical strategies for ensuring physiological homeostasis in offspring and reducing the risk of neurodevelopmental disorders.

Furthermore, there appears to be a reciprocal interaction between brain development and bone formation during embryogenesis. Fetal chondrocytes produce OCN and differentiate into osteoblasts only when co-cultured with brain tissue, indicating a tissue-specific response (Groot et al., 1994). Additionally, the Wnt/β-catenin signaling pathway, pivotal in bone formation, shares overlapping mechanisms with GPR37-mediated signaling pathways involved in neuronal physiology (Jiang et al., 2014; Berger et al., 2017).

3.1 Cellular physiological activities

Research on olfactory ensheathing cells (OECs), a specialized type of glial cell primarily located in the olfactory bulb, has confirmed the pivotal role of GPR37 in facilitating neuronal migration and supporting the regeneration and repair of olfactory neurons. Treatment of primary OECs and embryonic cultures containing olfactory regions with the GPR37 inhibitor Macitentan significantly reduces the migration of gonadotropin-releasing hormone (GnRH) neurons and OECs. Conversely, the GPR37 agonist TX14A directly promotes the migration of GnRH neurons (Saadi et al., 2019). These functions of GPR37 were also validated in GPR37−/− mice, where GPR37 knockout resulted in reduced migration capacity of OECs and GnRH cells (Saadi et al., 2019). The impact of GPR37 on cell migration may be linked to reduced Akt phosphorylation or decreased RhoA-GTPase activity in OECs, which disrupts cytoskeletal reorganization and impairs GnRH cell migration (Saadi et al., 2019).

In megalencephalic leukoencephalopathy with subcortical cysts (MLC), GPR37 preserves the stability of intercellular connections by negatively regulating the expression and function of glial MLC1 and glial cell adhesion molecule (GlialCAM), thereby ensuring normal cell adhesion and signal transmission (Pla-Casillanis et al., 2022). In NPCs, knocking down GPR37 reduces the expression of doublecortin (Dcx), a neuronal marker, and the number of terminally differentiated Microtubule-associated protein 2 (MAP2)-positive cells, a marker of mature neurons. At the same time, increasing the expression of chondroitin sulfate proteoglycan 4 (Cspg4), a microglial marker (Massey et al., 2008). These findings demonstrate the crucial role of GPR37 in neuronal and glial differentiation and neurogenesis. OCN/GPR37 is involved in the differentiation of NPCs, primarily through alterations in Wnt signaling (Berger et al., 2017). Wnt signaling is more active in younger individuals and declines significantly with age, showing an age-dependent reduction (Inestrosa et al., 2020). Activating the Wnt/β-catenin pathway can prevent Aβ-induced damage to brain endothelial cells, promote BBB repair (Wang et al., 2022), and enhance hippocampal synaptic plasticity (Hu et al., 2019). Inhibition of Wnt signaling disrupts the expression of genes closely associated with the differentiation, such as vimentin (VIM), leading to excessive activation of glial cells, which interferes with neurite extension and synaptic plasticity (Pebworth et al., 2021). The changes in VIM are analogous to the perspective that molecular drivers of AD vary with age: compared to normal aging, VIM is significantly enriched in elderly patients with AD. Furthermore, the increase in VIM is more pronounced in younger AD patients than in their older counterparts (Panizza and Cerione, 2024). Excessive activation of GPR37 has been implicated in aberrant cell proliferation, particularly in tumor cells. Research indicates that GPR37 is highly expressed in gliomas, where it plays a crucial role in promoting tumor cell proliferation and migration. Its overexpression is correlated with poor clinical outcomes and is linked to the activation of critical oncogenic signaling pathways,

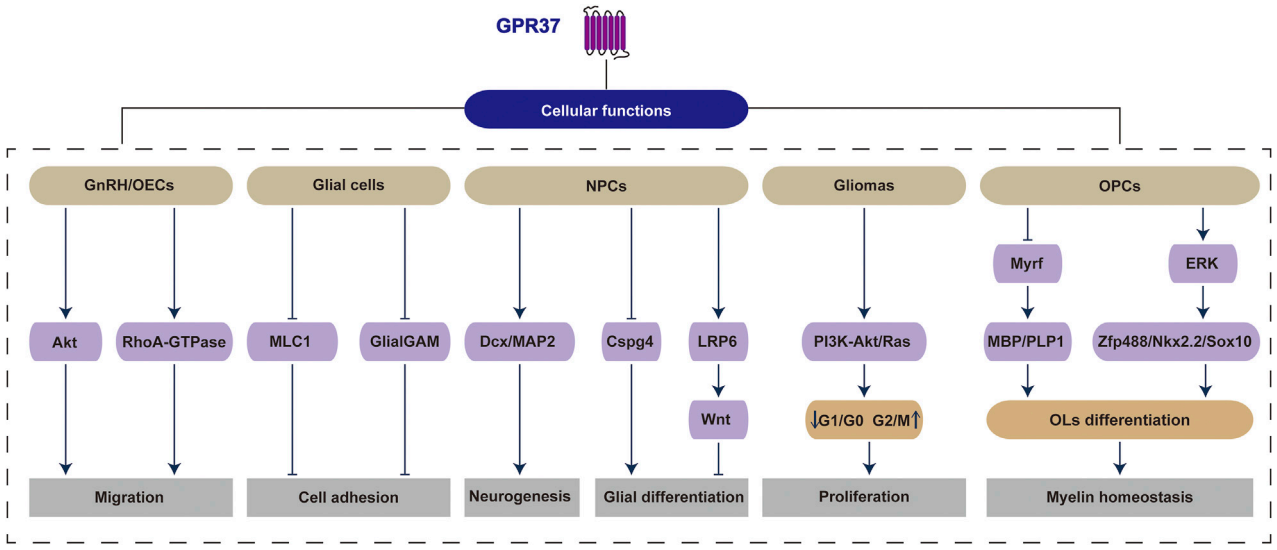

FIGURE 2
GPR37 regulates a range of physiological and pathological activities within cells. Specifically, GPR37 influences critical cellular functions across various cell types, including migration in GnRH/OECs, cell adhesion in glial cells, neurogenesis and glial differentiation in NPCs, proliferation in gliomas, as well as OLs differentiation and myelin homeostasis in OPCs.

including the PI3K-Akt and Ras pathways. Conversely, silencing GPR37 has been shown to suppress these malignant behaviors (Liang et al., 2023). In cultured human glioma U251 cells, GPR37 expression is significantly upregulated after 2 days. This phenomenon correlates with a decreased proportion of cells in the G1/G0 phase and an increased proportion in the S and G2/M phases, thus driving accelerated cell proliferation. This proliferation is further supported by a marked increase in phosphorylated Akt (Ser473) levels (Zhang and Wang, 2018).

The OCN/GPR37 signaling pathway also maintains myelin homeostasis (Smith et al., 2017; Qian et al., 2021). The absence of OCN could lead to excessive myelination in the CNS, characterized by the abundant expression of MBP and PLP1, along with an increased number of OLs. The underlying mechanism may involve the regulation of OCN on the expression of myelin-associated gene Myrf, which is a crucial transcription factor for OLs myelination and myelin maintenance. This regulation may inhibit OPCs differentiation into mature OLs (Qian et al., 2021). This process may be closely related to the effects of GPR37 on maintaining low-density lipoprotein receptor-related protein 6 (LRP6) levels and Wnt signaling in NPCs. Research indicates that the knockdown of GPR37 in NPCs leads to decreased levels of LRP6 and a reduction in the expression of Sp5, a target gene of Wnt. Furthermore, in LRP6-deficient HEK293 cells, neither GPR37 nor GPR37-1TM (the N-terminal domain of GPR37) can activate Wnt signaling unless LRP6 is reintroduced, which subsequently reactivates Wnt signaling (Berger et al., 2017). Additionally, GPR37 can promote OLs differentiation and myelination through ERK signaling (Yang et al., 2016). The influence of GPR37 on OLs differentiation is also modulated by the zinc finger transcription factor Zfp488, Nk homology domain protein Nkx2.2, and Sox10 (Schmidt et al., 2024).

GPR37 exerts a significant and broad regulatory influence on various cellular activities within the CNS. It involves cell proliferation, migration, differentiation, and myelination processes through diverse signaling pathways and molecular mechanisms. However, its dual role as a therapeutic target under different physiological and pathological conditions warrants further investigation (Figure 2).

3.2 Inflammation and immune responses

GPR37 is a crucial factor closely associated with inflammation and immune responses. Activation of GPR37 through neuroprotectin D1 (NPD1) and artesunate (ARU) has been shown to decrease serum interleukin-6 (IL-6) levels in WT mice infected with LPS, *Listeria*, and malaria parasites, thereby mitigating inflammation and reducing mortality (Bang et al., 2021). However, it failed to resolve inflammation in GPR37−/− mice (Bang et al., 2021). Research indicates that inflammatory pain, encompassing thermal hyperalgesia and mechanical allodynia, is notably delayed in GPR37−/− mice. These mice demonstrate significantly elevated levels of the pro-inflammatory cytokine IL-1β alongside reduced levels of the anti-inflammatory cytokines IL-10 and transforming growth factor-β (TGF-β) in the skin of their hind paws (Bang et al., 2018). Additionally, GPR37 activation could reduce the degree of cardiac ischemia-reperfusion injury by upregulating the activity of the JNK/PPAR-γ pathway, promoting phagocytic function of cardiac macrophages, M2-type polarization, and expression of anti-inflammatory factors (Zeng et al., 2019).

Acute inflammation and edema frequently occur following injury or infection, initially involving polymorphonuclear neutrophils (PMNs) infiltration. During this process, GPR37 can bind to specialized pro-resolving mediators (SPMs) to exert anti-

inflammatory effects (Park et al., 2020), which may be related to macrophage activation. In macrophages, OCN treatment significantly reduces IL-6 and tumor necrosis factor-alpha (TNF-α) induced by LPS while upregulating the expression of anti-inflammatory factors such as IL-10, TGF-β, and Arginase 1 (Arg1). However, in GPR37−/− macrophages, OCN fails to exert the anti-inflammatory effects (Qian et al., 2022). Furthermore, GPR37 has the potential to activate the calcium signaling pathway, leading to an increase in intracellular calcium levels and an enhancement of the phagocytic activity of WT macrophages (Bang et al., 2018; Bang et al., 2021). This process is mainly dependent on Gi protein-coupled signaling. Notably, pretreatment of macrophages with pertussis toxin (PTX), a Gi/o protein inhibitor, abolishes the rapid alterations in intracellular Ca^{2+}, cAMP, and pERK levels that OCN triggers in WT macrophages (Qian et al., 2022).

Further studies have linked OCN/GPR37 to neuroinflammation caused by brain dysfunction. In PD models, OCN treatment has been shown to mitigate dopaminergic neuron loss, significantly decreasing the numbers of astrocytes and microglia in the substantia nigra and striatum, along with reductions in TNF-α and IL-1β (Guo et al., 2018). Lower serum GPR37 levels and higher levels of inflammatory markers such as S100β, neuron-specific enolase (NSE), IL-1β, and TNF-α are observed in stroke patients compared to healthy controls. In addition, GPR37 levels are significantly negatively correlated with the NIH Stroke Scale (NIHSS) scores (Li et al., 2024). Animal studies further substantiate the link between GPR37 and neuroinflammation. In a model of fetal alcohol spectrum disorders (FASD) induced by alcohol exposure, significant increases in the expression of pro-inflammatory cytokines, including IL-1β, TNF-α, and chemokine CCL2, were observed in the cerebellum, accompanied by a notable decrease in *GPR37* (Kane et al., 2021). These findings suggest that GPR37 may regulate brain dysfunction by modulating central inflammation.

GPR37−/− mice exhibit significant changes in glial and progenitor cell dynamics in the middle cerebral artery occlusion (MCAO) lesion area. These alterations include a reduction in astrocyte response (McCrary et al., 2019) and increased NPCs and OPCs (Owino et al., 2021). Notably, at earlier time points within 24 h post-stroke, microglial M1 polarization is significantly enhanced, accompanied by elevated levels of pro-inflammatory cytokines like TNF-α, IL-1β, IL-6, and chemokines C-C motif chemokine ligand 2/3 (CCL2/3) (McCrary et al., 2019). CCL2/3 may contribute to the recruitment and infiltration of macrophages into the lesion of brain injury (Ciechanowska et al., 2020; Popiolek-Barczyk et al., 2020). Though these infiltrating macrophages exhibit functional similarities to microglia, they originate from distinct sources (Davies and Miron, 2018). In certain inflammatory conditions, such as multiple sclerosis (MS), macrophages collaborate with microglia, contributing to the pathological processes (Dong and Yong, 2019). Extensive studies in macrophages have established the role of the OCN-GPR37 axis in counteracting peripheral inflammation (Qian et al., 2022). Moreover, findings from GPR37−/− models suggest that GPR37 exerts significant anti-inflammatory effects on the CNS (McCrary et al., 2019). Nevertheless, direct evidence demonstrating the anti-inflammatory function of OCN through GPR37 in the brain remains limited despite the strong plausibility of this mechanism.

While GPR37 is primarily recognized for its substantial anti-inflammatory effects, some individual studies present opposing views. For instance, in glioma, elevated GPR37 is positively correlated with increased infiltration of M2 macrophages, which is associated with a poor prognosis (Liang et al., 2023). In an LPS-induced inflammation model, the enhanced reactivity of enteric glial cells is accompanied by increased GPR37 expression, whereas this response is diminished in GPR37−/− mice (Robertson et al., 2024a) (Table 1)

3.3 Stress responses

Emerging evidence indicates that GPR37 activation is crucial in protecting primary astrocytes from H_2O_2-induced cell death. Notably, this protective function is substantially compromised when endogenous GPR37 expression is downregulated (Meyer et al., 2013). In ischemic stroke models of MCAO, the absence of GPR37 results in elevated apoptosis and autophagy, accompanied by a pronounced increase in infarct size within the damaged region (McCrary et al., 2019). Furthermore, in these regions, GPR37 has been shown to mitigate neuronal apoptosis, promote cell survival, and shrink infarct size through the PI3K/Akt/ASK1 signaling pathway (Yu et al., 2024).

The involvement of GPR37 in cell survival appears to be intricately linked to oxidative stress and endoplasmic reticulum (ER) stress (ERS). A CHIP-Seq experiment in human neuroblastoma cells identified GPR37 as a downstream target gene of NRF1. As a transcription factor, NRF1 is intricately associated with mitochondrial function and oxidative stress, suggesting that GPR37 plays a significant role in the cellular responses to oxidative stress (Satoh et al., 2013). Clinically, elevated levels of GPR37 have been detected in the cerebrospinal fluid (CSF) of patients with medulloblastoma. Moreover, metabolomic profiling reveals that under hypoxic conditions, cyclooxygenase metabolites are almost absent in the CSF, while epoxygenase products and the lipid hormone 12,13-DIHOME, which promotes β-oxidation, are significantly upregulated (Reichl et al., 2020). This increase may reflect a tumor self-regulatory mechanism aimed at reducing inflammation by increasing GPR37 expression, facilitating adaptation to hypoxia, and enhancing invasiveness. While GPR37 overexpression might contribute to tumor progression, it also underscores its protective role in stress-related cellular processes.

A multitude of proteins undergo folding and modification within the ER. When incorrectly folded or improperly assembled, proteins accumulate in the ER lumen, triggering ERS. To mitigate ERS, cells initiate the unfolded protein response (UPR) and activate ER-associated degradation (ERAD), facilitating the retrotranslocation of misfolded proteins to the cytosol for degradation. Consequently, the accumulation of proteins in the cytosol directly results from ER protein aggregation and ERS (Hwang and Qi, 2018). The overexpression of GPR37 further exacerbates protein accumulation in the cytosol, intensifying ERS and promoting neuronal apoptosis (Imai et al., 2001; Marazziti et al., 2009). In PD models, this overexpression activates ERS, enhances

TABLE 1 Effects of GPR37 on inflammation or immune response.

Species	Model 1 (GPR37)	Model 2	Tissue/ cell	Phenotype		Treatment (GPR37)	Phenotype after treatment	Reference
				GPR37	Inflammation			
Mice	WT	Peripheral inflammation	Serum	-	↑	↑	Macrophage ablation↓ Inflammation↓ Survival rate↑	Bang et al. (2021)
			Macrophage					
Mice	KO	Inflammatory pain	Hind paw skin	-	↑	-	Delayed pain↑	Bang et al. (2018)
Human	WT	Stroke	Serum	↓	↑	-	NIHSS↑	Li et al. (2024)
Mice	WT	FASD	Cerebellum	↓	↑	-	Inflammation↑	Kane et al. (2021)
Mice	KO	MCAO	Brain	-	↑	-	Inflammation↑	McCrary et al. (2019), Owino et al. (2021)
	WT			↓			-	
Mice	WT	LPS	Serum	↓	↑	↑	Survival rate↑ Inflammation↓	Park et al. (2020), Qian et al. (2022)
			Macrophage					
	KO		Serum	-			Survival rate↓ Inflammation↑	
			Macrophage					
-	-	-	Cardiac macrophage	-	-	↑	M2-type polarization↑	(Zeng et al., 2019)
Mice	KO	LPS	Enteric glial cells	-	↓	-	Reactivity of enteric glial cells↓	Robertson et al. (2024a)

autophagy, and selectively degenerates GPR37-expressing neurons by converting LC3-I to LC3-II (Marazziti et al., 2009). Conversely, reducing GPR37 expression can inhibit ERS (Kubota et al., 2006). Dexmedetomidine, an alpha-2 adrenergic receptor (A2AR) agonist, significantly reduces ERS by preventing the accumulation of GPR37 and decreasing the activity of the procaspase-3/CHOP apoptotic pathway in the hippocampus of neonatal mice exposed to buprenorphine (Lin et al., 2021). Two fundamental mechanisms are involved in the role of GPR37 in alleviating ERS. First, the degradation of cytosolic GPR37 represents a pivotal mechanism in mitigating ERS. Research has elucidated that the ubiquitin ligase HRD1 facilitates the ubiquitination and proteasomal degradation of GPR37, thereby attenuating GPR37-mediated ERS and preventing apoptosis (Kaneko, 2016). Second, by promoting the translocation of GPR37 from the cytosol to the plasma membrane (Hertz et al., 2019), the ERS inhibitor 4-phenylbutyric acid effectively reduces the accumulation of misfolded proteins, including GPR37. As a result, it alleviates ERS and mitigates cytosolic protein aggregation and related stress responses (Kubota et al., 2006). In contrast to the potential adverse effects of GPR37 accumulation in the cytosol, the transmission of GPR37 signaling may positively influence ER function. GPR37 facilitates the maturation of LRP6, a glycoprotein essential for maintaining ER homeostasis, thereby ensuring effective Wnt/β-catenin signaling. Additionally, GPR37 protects LRP6 from ER-associated degradation. Consequently, GPR37 mitigates cellular damage induced by ERS (Berger et al., 2017).

In summary, GPR37 can potentially alleviate cellular damage induced by oxidative stress or ERS in challenging environments. However, excessive GPR37 expression may exacerbate stress responses and hasten disease progression in specific scenarios, underscoring its dual functionality. This paradox indicates that the functional regulation of GPR37 is highly dependent on the cellular environment and the nature of the stressors. Further investigation is essential to elucidate its therapeutic potential across various pathological conditions (Figure 3).

3.4 Neuronal functions

GPR37 was initially identified as related to PD in NDs and was termed the parkin-associated endothelin receptor-like receptor (Pael-R) (Marazziti et al., 2004). Subsequent research has revealed that the function of GPR37 extends beyond PD, playing roles in various physiological processes, including neuroprotection, neurodevelopment, and, notably, synaptic plasticity. In GPR37−/− mice, lower levels of dopamine and dopamine transporter (DAT) have been observed, along with significantly reduced phosphorylation of the AMPA receptor subunit GluA1 and the NMDA receptor subunit GluN2B (Zhang et al., 2020a). These mice also exhibit impaired LTP in striatal neurons, reduced synaptic plasticity, and pronounced motor function deficits (Zhang et al., 2020a). Moreover, the activation of GPR37 by various factors, including OCN, has been shown to exert neuroprotective effects (Meyer et al., 2013; Qian et al., 2021).

The cytoplasmic accumulation of proteins can trigger cytotoxic effects through autophagic overload, stress, and inflammatory responses, collectively leading to cellular dysfunction and

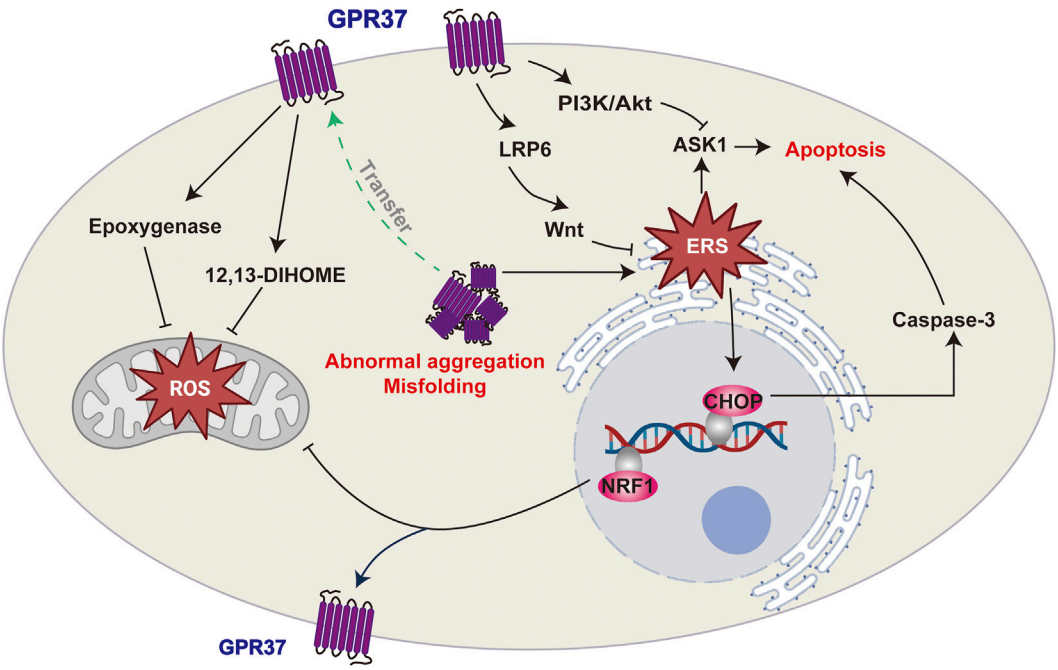

FIGURE 3
GPR37 and its roles in cellular stress responses. GPR37 regulates oxidative stress and endoplasmic reticulum stress (ERS), which can lead to apoptosis. It influences ROS production in mitochondria and modulates ERS, with misfolding or abnormal aggregation potentially triggering apoptotic pathways.

potentially accelerating disease progression. Adequately folded and membrane-localized GPR37 exerts neuroprotective effects, whereas misfolded and aggregated GPR37 has been associated with neurodegenerative changes in PD (Zhang et al., 2020a). In a neurotoxicity rat model induced by subcutaneous kainic acid injection, GPR37 was initially strongly expressed in the cytoplasm of Purkinje cells. Still, its levels significantly decreased a few days post-injection (Li et al., 2017). This reduction may be attributed to either increased degradation of cytoplasmic GPR37 or enhanced translocation to the plasma membrane. Inhibition of GPR37 aggregation within the ER or facilitation of its translocation to the plasma membrane may enhance cell viability (Dunham et al., 2009; Lundius et al., 2014). Furthermore, treatment with GM1, a brain-expressed ganglioside, significantly improved the survival of cells stably expressing GPR37 compared to WT cells lacking GPR37 in an MPP + -induced N2a PD cell model (Hertz et al., 2021). These findings indicate that GPR37 is crucial for cell survival. However, PCR analysis showed no significant alterations in GPR37 RNA expression following GM1 treatment, suggesting that the levels of GPR37 expression may not be the determining factor. Instead, forming plasma membrane complexes involving GPR37 may be instrumental in this process (Hertz et al., 2021).

Like other GPCRs, GPR37, located on the plasma membrane, is crucial for signaling recognition and response to external signals, regulating cellular functions, and as a drug target. When it binds to its ligand, such as OCN, GPR37 exerts neuroprotective effects through GPCR-mediated signaling pathways. GPR37 regulates the activity of proteins, including PI3K, Akt, and CaMKII, and promotes Ca^{2+} influx via transient receptor potential (TRP) family Ca^{2+} channels, facilitating cell mitosis (Rezgaoui et al., 2006). Additionally, GPR37 engages the ERK signaling pathway to promote neuroprotective functions such as OLs differentiation and myelination (Yang et al., 2016). The interaction between GPR37 and membrane proteins is crucial during signal transduction, particularly in modulating synaptic plasticity. While no significant changes in long-term depression (LTD) are observed in striatal and hippocampal neurons in the absence of GPR37, chronic blockade of the A2AR under GPR37−/− conditions enhances LTD and motor sensitization in the striatum (Hertz et al., 2019; Morato et al., 2019). GPR37 also regulates the DAT and dopamine D2 receptors (D2R), influencing dopamine neurotransmission (Leinartaite and Svenningsson, 2017). Loss of GPR37 results in increased DAT expression on the plasma membrane and enhanced DAT-mediated dopamine uptake, which may exacerbate symptoms in patients with PD (Marazziti et al., 2007).

Some studies suggest that downregulation of GPR37 may reduce apoptosis and improve cell survival in PD models, with apoptosis rates decreasing from 39.1% to 29% and cell survival increasing from 56% to 63% when GPR37 is downregulated (Zou et al., 2012). Additionally, though GPR37 inhibits DAT in PD and is beneficial for restoring dopamine signaling, the loss of GPR37 might have positive implications from an addiction treatment perspective. In GPR37−/− mice, the conditioned place preference response to amphetamine and cocaine is significantly reduced. These findings suggest that the absence of the GPR37 affects the reward response to stimulants, which may be beneficial for addiction treatment (Marazziti et al., 2011).

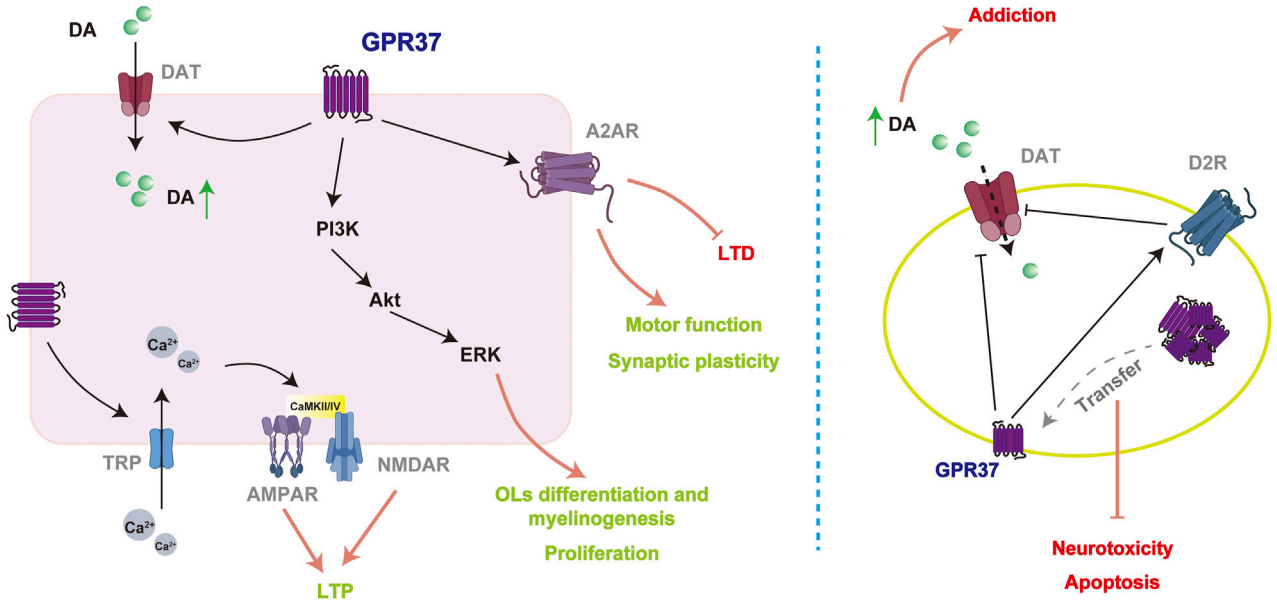

FIGURE 4
Potential mechanisms of GPR37 in mediating neuroprotective effects. GPR37 enhances synaptic plasticity by regulating multiple ion channels and receptors (left). However, in disease models (right), its mechanisms of action may be reversed entirely compared to physiological conditions, potentially leading to an increase in addictive behaviors.

The role of GPR37 in the nervous system is complex. The expression and localization of GPR37 significantly influence the regulation of the dopamine system, the maintenance of synaptic plasticity, and the response to neuroprotective factors such as OCN. When GPR37 is translocated to the plasma membrane and interacts with its ligands, it can exert neuroprotective effects through GPCR signaling pathways, including regulating the PI3K/Akt and ERK signaling pathways. However, dysfunction of GPR37 or its abnormal accumulation within cells can weaken its neuroprotective functions and is associated with developing various NDs (Figure 4).

4 OCN/GPR37 and NDs

Lower OCN levels are associated with alterations in brain microstructure (Puig et al., 2016). Mutations in the runt-related transcription factor 2 (RUNX2), which acts as an upstream regulator of OCN, result in cleidocranial dysplasia, frequently presenting as cognitive impairment (Takenouchi et al., 2014). In NDs, research on OCN has primarily focused on PD and various forms of dementia. In PD rat models, CSF OCN levels were significantly reduced, while OCN treatment mitigated the loss of tyrosine hydroxylase, a key enzyme involved in DA synthesis within the nigrostriatal pathway (Guo et al., 2018). Additionally, OCN was shown to reduce apoptosis of dopaminergic neurons in PD mouse models, alleviate neurotoxicity, and improve motor function impairments by modulating the Akt/glycogen synthase kinase 3beta (GSK3β) signaling pathway (Hou et al., 2021). A Mendelian randomization study explored the causal relationship between OCN and various forms of dementia, including AD, PD, Lewy body dementia (LBD), and vascular dementia (VD). The findings indicated that OCN exerts a significant impact on dementia, with its potential protective effect being more pronounced in AD compared to other types (Liu et al., 2023). Furthermore, animal studies demonstrated that intraperitoneal injection of OCN reduced Aβ levels in the hippocampus and cortex of AD mouse models, enhanced the power of high gamma band in medial prefrontal cortex, and improved anxiety-like behavior and cognitive dysfunction (Shan et al., 2023).

Remarkably, OCN supplementation has been demonstrated to ameliorate diabetes-associated cognitive deficits in a dose-dependent manner, an effect abrogated by the administration of Akt inhibitors (Zhao et al., 2024). In AD, OCN enhances cognitive function by reducing Aβ accumulation and upregulating glycolysis in glial cells (Shan et al., 2023). Moreover, alterations in glucose metabolism across multiple brain regions indicate the abnormal distribution of α-synuclein aggregates, contributing to the progression of PD (Scholefield et al., 2023). In Huntington's disease (HD) models, neuropathological alterations and motor deficits are accompanied by the progression of glucose intolerance and tissue wasting (Duan et al., 2003; Patassini et al., 2016). These findings indicate that OCN may play a crucial role in modulating cognitive function associated with aging and NDs, potentially through its influence on glucose metabolism.

The GPR37 is integral to the pathological processes underlying various brain disorders, with its deletion shown to impair oligodendrocyte function and elevate susceptibility to demyelinating diseases, notably MS (Smith et al., 2017). Additionally, proteomic analyses of brain tissue have identified that the s100 calcium-binding protein A5 (S100A5), implicated in mood disorders, exhibits marked alterations in the absence of GPR37, underscoring GPR37's potential role as a biomarker for

neurological damage (Nguyen et al., 2020). Interestingly, a GPR37-Del321F mutation was detected in the unaffected father of an individual with autism spectrum disorder (ASD), while the GPR37-R558Q mutation was present in the affected brother and the unaffected mother (Fujita-Jimbo et al., 2012). The pathophysiological impact of the R558Q mutation is likely due to its interference with GPR37's synaptic localization, as it prevents co-localization with synaptic scaffolding proteins multi-PDZ domain protein 1 (MUPP1) and contactin-associated protein-like 2 (CASPR2), leading to GPR37 retention within the endoplasmic reticulum and a consequent increased ASD risk (Tanabe et al., 2015).

Although the extent to which GPR37 mediates the functions of OCN remains uncertain, several studies have shed light on the complex role of GPR37. Similar to OCN, GPR37 is involved in the regulation of DA levels. In GPR37−/− mice, striatal DA levels were reduced to 60% of those in control groups. Conversely, in GPR37-overexpressing mice, striatal levels of 3,4-dihydroxyphenylacetic acid and vesicular DA were elevated (Imai et al., 2007). Additionally, GPR37−/− mice displayed dopaminergic neuron loss, LTP deficits, and increased susceptibility to neurotoxicity induced by 6-hydroxydopamine (Zhang et al., 2020a) along with pronounced anxiety- and depression-like behaviors (Mandillo et al., 2013). Notably, under pathological conditions, particularly in NDs, GPR37 activation may aggravate disease progression. Overexpression of GPR37 has been found to increase the vulnerability of dopaminergic neurons to chronic DA toxicity and promote apoptosis (Imai et al., 2007; Kitao et al., 2007). In contrast, the downregulation of GPR37 enhanced cell survival in PD models (Zou et al., 2012).

GPR37 shows potential as a biomarker for NDs. Both the correlations and distinctions in the unique processing mechanisms of GPR37 across various types of NDs (Argerich et al., 2024). In the striatum of AD patients, GPR37 levels were significantly elevated, though no corresponding increase was observed in CSF. In contrast, PD patients exhibited significantly higher levels of GPR37 in the CSF, suggesting that GPR37 might serve as a biomarker for PD progression rather than AD. Notably, this elevation was restricted to patients with slow progressive PD (Morato et al., 2021; Argerich et al., 2024). Beyond NDs, GPR37 expression also varies across psychiatric conditions. It was markedly downregulated in major depressive disorder but significantly upregulated in bipolar disorder (Tomita et al., 2013). Additionally, GPR37 plays a pivotal role in myelination, making it relevant to MS, a disorder characterized by progressive axonal demyelination in the central nervous system. These findings offer valuable insights into the roles of OCN and GPR37 in disease pathogenesis and progression, underscoring the importance of further investigation into their mechanisms.

5 Conclusion and prospective

Current evidence underscores the predominantly beneficial role of OCN in regulating brain function. This effect is linked to several signaling pathways, including RhoA/GTPase, PI3K/Akt/ASK1, ERK, Wnt/β-catenin, IP3/CaMKII, and cAMP/PKA. Under most physiological conditions, GPR37 serves a complementary or mediating role in enhancing the effects driven by OCN. The absence of either OCN or GPR37 results in excessive myelination, with GPR37 mediating the effects of OCN (Qian et al., 2021). In inflammatory responses, both OCN and GPR37 have predominantly demonstrated anti-inflammatory effects (McCrary et al., 2019; Qian et al., 2021), though the anti-inflammatory role of OCN in the central nervous system has yet to be fully validated. Additionally, both OCN and GPR37 display neuroprotective properties in NDs. Nevertheless, GPR37 may also display roles that diverge from OCN. For instance, the intracellular accumulation of GPR37 has been linked to aggravated stress responses (Marazziti et al., 2009). Furthermore, GPR37 is highly expressed in peripheral inflammatory models (Robertson et al., 2024b) and certain NDs, where it has been identified as a potential prognostic biomarker (Morato et al., 2021; Argerich et al., 2024).

While evidence has supported a connection between OCN and GPR37, their multi-receptor and multi-ligand interactions warrant further investigation to clarify whether their effects are synergistic or divergent. In addition to OCN, GPR37 binds a range of ligands including head activator (Rezgaoui et al., 2006), prosaposin (Bhattacharya et al., 2023; Yu et al., 2024), regenerating islet-derived family member 4 (Wang et al., 2016), NPD1(Bang et al., 2018), and the agonist ARU (Bang et al., 2021). The diversity of ligands increases the complexity of GPR37 in brain cognitive function and may explain the dual roles of GPR37 under different physiological and pathological conditions. Understanding the effects of these ligands will provide a theoretical foundation for developing novel therapeutic strategies based on the OCN/GPR37 axis, potentially achieving significant breakthroughs in treating cognitive dysfunctions and NDs.

Future research should prioritize exploring the specific signaling pathways and molecular mechanisms through which OCN affects GPR37, particularly its dual roles in different brain regions and pathological states. Understanding how to regulate OCN levels and GPR37 activity is crucial for future studies. Exercise is currently recognized as the most effective non-invasive strategy for enhancing circulating and brain OCN levels, with evidence suggesting that this elevation is independent of exercise modality, duration, gender, or age (Chahla et al., 2015; Armamento-Villareal et al., 2020; Hiam et al., 2021; Mohammad Rahimi et al., 2021; Koltun et al., 2024). However, further investigation is required to identify the specific exercise type that optimally promotes OCN secretion and GPR37 activation. Moreover, the recent discovery of GPR158 as an additional central receptor for OCN raises the possibility of functional overlap with GPR37 (Khrimian et al., 2017). Elucidating the relationship and functional differentiation between these two receptors is a critical area of ongoing research.

Author contributions

XB: Resources, Writing–original draft, Writing–review and editing. YW: Writing–original draft. WZ: Writing–original draft. CY: Supervision, Writing–original draft, Writing–review and editing. JL: Funding acquisition, Supervision, Writing–original draft, Writing–review and editing, Resources.

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Conflict of interest

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Glossary

A2AR	alpha-2 adrenergic receptor
AD	Alzheimer's disease
Arg1	Arginase 1
ARU	artesunate
ASD	autism spectrum disorder
Aβ	amyloid-beta
BBB	blood-brain barrier
CCL2/3	chemokines C-C motif chemokine ligand 2/3
CNS	central nervous system
CSF	cerebrospinal fluid
Cspg4	chondroitin sulfate proteoglycan 4
D2R	D2 receptors
DAT	dopamine transporter
Dcx	doublecortin
ERS	endoplasmic reticulum stress
FASD	fetal alcohol spectrum disorders
GlialCAM	glial cell adhesion molecule
GnRH	gonadotropin-releasing hormone
GPR37	G protein-coupled receptor 37
LPS	lipopolysaccharide
LRP6	lipoprotein receptor-related protein 6
LTD	long-term depression
MAP2	Microtubule-associated protein 2
MBP	myelin basic protein
MCAO	middle cerebral artery occlusion
MLC	megalencephalic leukoencephalopathy with subcortical cysts
NDs	neurodegenerative diseases
NPCs	neural progenitor cells
NPD1	neuroprotectin D1
NSE	neuron-specific enolase
OCN	Osteocalcin
OECs	olfactory ensheathing cells
OLs	oligodendrocytes
OPCs	oligodendrocyte precursor cells
Pael-R	parkin-associated endothelin receptor-like receptor
PD	Parkinson's disease
PLP1	proteolipid protein 1
PMNs	polymorphonuclear neutrophils
PTX	pertussis toxin
SPMs	specialized pro-resolving mediators
TGF-β	transforming growth factor-β
TNF-α	tumor necrosis factor-alpha
TRP	transient receptor potential
VIM	vimentin

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Implications of draxin in neurological disorders

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Axon guidance proteins not only play a role in the formation of proper neural circuits but also have other important functions, such as cell survival, migration, and proliferation in the brain. Therefore, mutations in the genes encoding these proteins frequently cause various types of neurological disorders, including psychiatric disorders and neurodegenerative diseases. We previously identified an axon guidance protein, draxin, that is essential for the development of several neural circuits and cell survival in the brain. Recently, the deletion of the *draxin* gene was identified in an inbred BTBR T^{+} $Itpr3^{tf}$/J (BTBR/J) mouse, which is a widely used model of Autism Spectrum Disorder (ASD), suggesting that *draxin* deletion is a genetic factor for ASD-like characteristics in BTBR/J mice. In this review, I summarize the neuroanatomical abnormalities in *draxin* knockout mice by comparing them to BTBR/J mice and discuss the possible contributions of draxin to anatomical and behavioral phenotypes in BTBR/J mice.

KEYWORDS

axon guidance, draxin, BTBR mouse, ASD, corpus callosum

Introduction

Draxin was first identified as an axon guidance protein that regulates commissural axons in the spinal cord and the forebrain. It is a secreted protein that shares no homology with other known proteins (Islam et al., 2009; Miyake et al., 2009). Draxin has been shown to bind to netrin-1 and its receptors, including Deleted in colorectal cancer (Dcc) and Neogenin (Neo1) (Ahmed et al., 2011; Shinmyo et al., 2015). Previous studies have suggested that draxin regulates the outgrowth of axons originating from various types of neurons *in vitro* (Islam et al., 2009; Naser et al., 2009; Ahmed et al., 2010; Ahmed et al., 2011; Chen et al., 2013; Meli et al., 2015; Shinmyo et al., 2015). *Draxin* knockout (KO) mice show developmental abnormalities in various neural circuits, including the corpus callosum, the hippocampal commissure, the anterior commissure, the fornix, and the thalamocortical axons (Islam et al., 2009; Zhang et al., 2010; Shinmyo et al., 2015). Thus, draxin may control the development of neural circuits in the brain through the netrin-1 receptors or by modulating netrin-1-mediated axon guidance.

Previous human and animal studies have shown that axon guidance proteins are associated with structural changes in neuronal connections during neurological disorders (Nugent et al., 2012; Van Battum et al., 2015). In addition, because axon guidance cues have other important functions in the brain, such as cell survival, migration, and proliferation (Mehlen et al., 2011), mutations in the genes encoding axon guidance proteins can cause many neurological disorders. Indeed, draxin and/or netrin signaling has been shown to be associated with several neurological disorders, including psychiatric disorders, gliomas, and neurodegenerative diseases (Infante et al., 2015; Vosberg et al., 2020; Ahn et al., 2021; Jasmin et al., 2021; Cai et al., 2024). Recently, an 8-bp frameshift deletion of the *draxin* gene

was identified in an inbred BTBR T^+ $Itpr3^{tf}$/J (BTBR/J) mouse, a widely used model of Autism Spectrum Disorder (ASD) (Morcom et al., 2021; Arslan et al., 2023). Furthermore, *draxin* deletion in BTBR/J mice was shown to contribute to the dysgenesis of the corpus callosum, which is a neuroanatomical abnormality characteristic of human ASD (Arslan et al., 2023). In this review, I summarized the neuroanatomical abnormalities in *draxin* KO mice by comparing them to BRBR/J mice.

Dysgenesis of the corpus callosum in human ASD

ASD is a neurodevelopmental disorder defined by impairments in social interactions, communication deficits, and repetitive behaviors with restricted interests (Lai et al., 2014). Identifying abnormalities in brain structures in ASD is critical for developing more precise and objective diagnoses and for creating effective new treatments. One prominent mechanism that has been suggested to contribute to the underlying pathology of ASD is abnormal long-range neuronal connectivity. This is because numerous MRI studies have demonstrated reduced fractional anisotropy in major white matter tracts in individuals with ASD, including the cingulum, uncinate fasciculi, occipitotemporal tracts, and, most consistently, the corpus callosum (Barnea-Goraly et al., 2004; Alexander et al., 2007; Keller et al., 2007; Frazier and Hardan, 2009; Kumar et al., 2010; Weinstein et al., 2011).

The corpus callosum is a large bundle of nerve fibers that connects the left and right hemispheres of the brain. Variable corpus callosum abnormalities have been reported in the anterior, midbody, and posterior regions of the forebrain in ASD (Egaas et al., 1995; Saitoh et al., 1995; Haas et al., 1996; Piven et al., 1997; Manes et al., 1999; Hardan et al., 2000). These observations suggest that the abnormal development of the corpus callosum is associated with ASD. This is consistent with recent results from mega-analyses comparing white matter microstructural differences between healthy participants and those with psychiatric disorders, showing that patients with schizophrenia, bipolar disorder, or ASD disorder have common alterations in the corpus callosum (Koshiyama et al., 2020).

The corpus callosum plays a critical role in the transmission and integration of information between the left and the right hemispheres. The anterior corpus callosum connects regions of the prefrontal cortex and is associated with higher-order cognitive, emotional, and social functions. The midbody of the corpus callosum connects multiple regions, including the primary motor and sensory cortices, and is involved in sensory and motor processing. The posterior corpus callosum links the occipital lobes and is crucial for the processing and integration of visual information. Abnormal development in specific regions of the corpus callosum may be associated with the specific cognitive and behavioral characteristics of ASD. However, abnormalities in brain structures in patients with ASD have been observed not only in the corpus callosum but also in other regions. Therefore, to understand the causes of behavioral abnormalities in ASD accurately, it is important to analyze animal models of specific anatomical and functional abnormalities.

TABLE 1 Anatomical abnormalities in brains of *draxin* KO and BTBR mice.

	Draxin KO	BTBR/J
Aberrant neural circuits		
Corpus callosum	+	+
Hippocampal commissure	+	+
Anterior commissure	+	+
Thalamocortical axons	+	+
Corticofugal axons	+	?
Fornix	+	?
Other abnormalities in the brain		
Shrinkage of the hippocampus	+	+
Reduced size of the amygdala	?	+

+ Abnormal development; ?, not investigated.

BTBR mouse, an idiopathic animal model of ASD

Characteristic behavioral phenotypes of ASD have been modeled in mice. One such model is the inbred BTBR/J mouse, which is the most extensively researched and the most commonly reproduced inbred strain (Nadler et al., 2006; Bolivar et al., 2007; Moy et al., 2007). BTBR/J mice exhibit impaired in social interactions and high levels of repetitive behaviors (Moy et al., 2007; McFarlane et al., 2008; Dodero et al., 2013). Furthermore, this strain is characterized by the absence of the corpus callosum and a smaller-to-absent hippocampal commissure (Wahlsten et al., 2003). A previous study identified several genomic regions in BTBR/J mice that distinctly influenced their ASD-like characteristics (Jones-Davis et al., 2013). Recently, an 8-bp frameshift deletion of the *draxin* gene, leading to the loss of draxin function, was identified in BTBR/J mice (Morcom et al., 2021; Arslan et al., 2023). The *draxin* gene is located in a genomic region that was previously identified as contributing to commissural abnormalities in BTBR/J mice (Jones-Davis et al., 2013). Since *draxin* KO mice display malformations of the corpus callosum and the hippocampal commissure, draxin is a promising candidate for explaining the defects in these commissures in BTBR/J mice. Consistently, abnormal development of the corpus callosum was partially restored in BTBR/J mice with a heterozygous knock-in that reverted the 8 bp *draxin* deletion to the wild-type, suggesting that the draxin deletion contributes to agenesis of the corpus callosum in BTBR/J mice (Arslan et al., 2023).

Similarities in neuroanatomical phenotypes between *draxin* KO and BTBR mice

Since previous studies have suggested that BTBR/J mice are characterized by multiple genetic aberrations, it is important to clarify the contribution of draxin to the anatomical and behavioral phenotypes of BTBR/J mice. *Draxin* KO mice show various developmental abnormalities in the brain similar to those observed in BTBR/J mice. BTBR/J mice exhibit an absence of the

corpus callosum, and reductions in the hippocampal and the anterior commissures (Table 1) (Wahlsten et al., 2003; Ellegood et al., 2015). Similar to BTBR/J mice, *draxin* KO mice show severe defects in all forebrain commissures, the corpus callosum, the hippocampal commissure, and the anterior commissure (Islam et al., 2009). Given that the abnormal development of the corpus callosum was partially rescued in BTBR/J mice with a heterozygous knock-in that reverted the 8 bp *draxin* deletion to the wild-type, the *draxin* deletion contributes to the absence of the corpus callosum in BTBR/J mice (Arslan et al., 2023). However, this observation suggests that additional genetic factors contribute to the absence of the corpus callosum in BTBR/J mice. Both *draxin* KO mice and BTBR mice with a C57Bl/6J genetic background display variable penetrance of the corpus callosum defect, suggesting that other genetic factors modify the corpus callosum phenotype driven by the *draxin* mutation (Morcom et al., 2021).

Draxin KO mice also show severe defects in the thalamocortical and corticofugal projections (Shinmyo et al., 2015). During normal brain development, corticofugal and thalamocortical axons meet in the internal capsule and depend on each other for their guidance to the thalamus and neocortex, respectively (Lopez-Bendito and Molnar, 2003). Corticofugal axons grow from the cortex into the internal capsule in wild-type mice. In contrast, some corticofugal axons of *draxin* KO mice do not enter the internal capsule but instead grow toward the external capsule. Thalamocortical axons in *draxin* KO mice grow normally toward the internal capsule. However, some of them do not enter the cortex and instead either stall or turn laterally toward the external capsule, whereas others enter the cortex with an abnormal topographic organization. Visualization of the cortical sensory regions revealed disruptions in the spatial positions of thalamocortical axon terminals in *draxin* KO mice (Shinmyo et al., 2015). Thus, draxin is essential for guiding thalamocortical axons from the internal capsule to the cortex, as well as for their region-specific connections between the thalamus and cortex. Importantly, the topography of thalamocortical projections changes in BTBR/J mice, in which the primary somatosensory and visual cortical areas are medially shifted (Fenlon et al., 2015). Therefore, abnormalities in the topographic organization of thalamocortical projections are a common feature of *draxin* KO and BTBR/J mice, although this phenotype in *draxin* KO mice requires further investigation. Another similarity in the anatomical phenotype between *draxin* KO mice (Zhang et al., 2010) and BTBR/J mice (Mercier et al., 2012) is the shrinkage of the hippocampus. In addition to the hippocampus, the size of the amygdala nuclei is reduced in BTBR/J mice (Mercier et al., 2012). However, it remains unclear whether the anatomy of the amygdala is altered in *draxin* KO mice or not. Collectively, *draxin* deletion is likely to be the primary genetic factor underlying the neuroanatomical phenotypes in BTBR/J mice.

Discussion

In this review, I have summarized the similarities in neuroanatomical phenotypes between *draxin* KO and BTBR/J mice. In addition to their phenotypical similarities, recent studies have suggested that draxin contributes to neuroanatomical phenotypes in BTBR/J mice (Morcom et al., 2021; Arslan et al., 2023). However, the contribution of draxin to the behavioral phenotypes of BTBR/J mice remains unclear. To address this issue, it is necessary to perform behavioral analyses in *draxin* KO mice and *draxin* knock-in BTBR mice.

It is important to determine the neuroanatomical abnormalities responsible for the behavioral phenotypes of ASD. Previous studies on humans with ASD and BTBR/J mice have suggested that dysgenesis of the corpus callosum is strongly associated with behavioral abnormalities in ASD. However, there is no direct evidence supporting this idea because dysgenesis of the corpus callosum is generally accompanied by other anomalies in brain structures in both humans and mice. For example, patients with corpus callosum anomalies frequently display dysgenesis of the hippocampal commissure (Hetts et al., 2006). Therefore, to examine whether the behavioral phenotypes characteristic of ASD are caused by anomalies in the corpus callosum, a mouse model with a specific defect in the corpus callosum is required. Surgical lesions of the corpus callosum at an early postnatal stage do not affect the juvenile play or adult social behaviors, nor do they increase repetitive self-grooming (Yang et al., 2009). This evidence does not support the hypothesis that disconnection of the corpus callosum is a causal factor for ASD-like behaviors in mice. However, experimental lesions at the postnatal stage may not replicate congenital corpus callosum anomalies. Both BTBR/J and *draxin* KO mice show corpus callosum agenesis with similar misprojections of the callosal axons. In these mice, callosal axons fail to cross the midline; instead, they form ipsilateral "Probst" bundles that run parallel to the midline (Islam et al., 2009; Fenlon et al., 2015). Since this aberrant neuronal circuitry is retained throughout adulthood, it may contribute to ASD-like behaviors in mice.

Furthermore, both *draxin* KO and BTBR/J mice have abnormalities in the topographic organization of connections between the thalamus and the cortex (Fenlon et al., 2015; Shinmyo et al., 2015). This suggests that the alteration in cortical area patterning caused by the deletion of the *draxin* gene contributes to the previously observed sensory and behavioral deficits in BTBR/J mice (Moy et al., 2007; McFarlane et al., 2008). It is critical to generate conditional *draxin* KO mice with specific neural structural abnormalities and perform behavioral analyses to investigate these possibilities. Recently, it was reported that BTBR TF/ArtRbrc (BTBR/R) mice, a sister strain of BTBR/J, show core symptoms of ASD despite having an intact *draxin* gene and preserved forebrain commissures (Lin et al., 2023). BTBR/R mice will be useful for understanding the draxin-independent mechanisms that cause ASD-like behaviors.

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Conflict of interest

The author declares that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Arx revisited: involved in the development of GABAergic interneurons

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The aristaless-related homeobox (Arx) transcription factor, located on the X chromosome, has been implicated in a wide range of neurological disorders, including intellectual disability and epilepsy, as well as diabetes and pancreatic developmental disorders. In the mouse brain, Arx is expressed not only in the olfactory bulb (OB) and cerebral cortex progenitor cells but also in these gamma-aminobutyric acid (GABA)-releasing interneurons. In the initial study, constitutive Arx knockout (KO) mice showed aberrant migration and a reduction in GABAergic interneurons in the neonatal OB. However, constitutive Arx KO mice with perinatal lethality preclude further analysis in adolescent or adult mice. To overcome this, Arx-floxed mice have been crossed with Cre driver mice to generate conditional KO mice with selective Arx deletion in distinct interneuron progenitors. These studies have identified Arx as a key transcriptional regulator involved in the generation, fate determination, and migration of cortical interneurons. This review focuses on the critical role of Arx in the development of progenitor cells and the migration of interneurons in the mouse OB and cerebral cortex, and discusses differences in Arx mutant-based abnormality between mouse mutants and human patients.

KEYWORDS

Arx, transcription factor, olfactory bulb, cerebral cortex, interneuron

1 GABAergic interneurons in the olfactory bulb

In the olfactory system, odorants are detected by olfactory sensory neurons (OSNs) that express specific odorant receptors in the olfactory epithelium (OE) (Mori and Sakano, 2011; Mori and Sakano, 2021). The axons of OSNs project to distinct glomeruli in the olfactory bulb (OB), where they interact with excitatory projection neurons, promoting the development of dendrites in specific subsets of inhibitory interneurons (Mori and Sakano, 2011; Lepousez et al., 2013; Figueres-Oñate et al., 2014; Mori and Sakano, 2021). OB interneuron progenitors are generated in the ventricular-subventricular zone (V-SVZ) on the lateral ventricle wall, not only during early development but also throughout adulthood (Tong and Alvarez-Buylla, 2014; Figure 1A). These progenitors migrate via the rostral migratory stream (RMS) to the OB, where they differentiate into gamma-aminobutyric acid (GABA)-releasing inhibitory interneurons, including granule cells (GCs) and periglomerular cells (PGCs) (Alvarez-Buylla et al., 2008; Lledo et al., 2008; Whitman and Greer, 2009; Adam and Mizrahi, 2010; Kaneko et al., 2010; Sakamoto et al., 2011; Sequerra, 2014; Figure 1B). In the OB, GCs and PGCs form reciprocal synapses with mitral and tufted cells (M/TCs), receiving

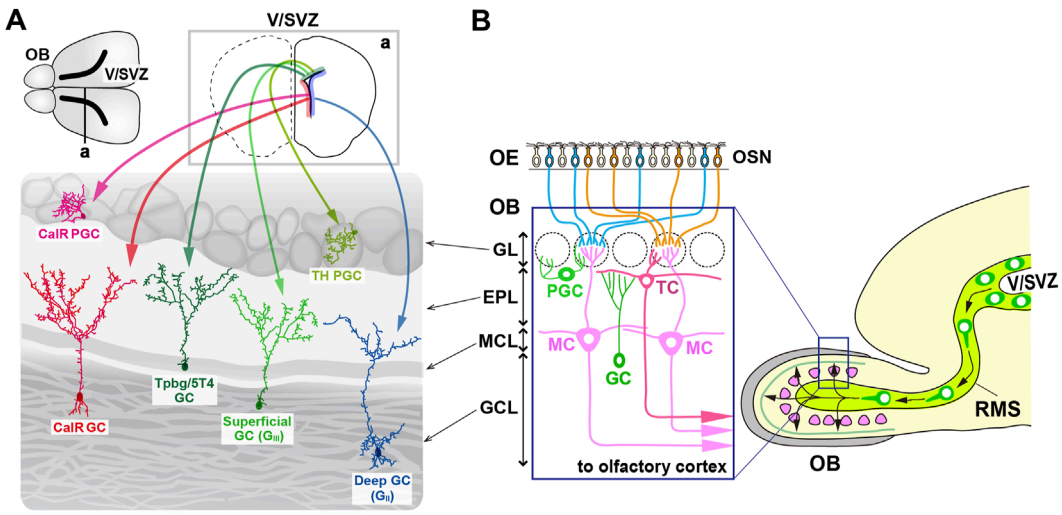

FIGURE 1
Multiple subtypes of olfactory bulb (OB) interneurons. **(A)** The mammalian OB is structured into distinct layers: the glomerular layer (GL), external plexiform layer (EPL), mitral cell layer (MCL), and granule cell layer (GCL). Olfactory sensory signals from olfactory sensory neurons (OSN) in the olfactory epithelium (OE) are transmitted by excitatory projection neurons such as mitral cells (MCs) and tufted cells (TCs) to inhibitory interneurons like granule cells (GCs) and periglomerular cells (PGCs). **(B)** Distribution of neural stem cells in the ventricular-subventricular zone (V/SVZ) in specific areas. Adult OB interneurons are generated in different subregions of the V/SVZ (upper row; a), migrate through the rostral migratory stream (RMS), and subsequently differentiate into distinct subtypes of mature interneurons in the OB, including PGCs (TH and CalR) and GCs (G_{II}, G_{III}, Tpbg/5T4, and CalR).

glutamatergic inputs from their dendrites and returning GABAergic outputs to their dendrites (Burton, 2017).

GCs are the most abundant non-axonal interneurons in the OB and release GABA from their spiny apical dendrites, which extend into the external plexiform layer (EPL) to interact with the lateral dendrites of M/TCs (Burton, 2017). In contrast, PGCs, which are also non-axonal, have small soma and spatially restricted dendritic branches, and release GABA (and sometimes dopamine) to modulate local glomerular activity (Kosaka and Kosaka, 2011; Galliano et al., 2018). Based on the location of dendritic arborization in the EPL, GCs are further classified into "superficial," "intermediate," and "deep" (Mori et al., 1983; Greer, 1987; Takahashi et al., 2018; Figure 1B). Additionally, different subsets of GCs are distinguished by biochemical markers such as calretinin (CalR), $Ca2^{+}$ calmodulin-dependent protein kinase II α (CaMKIIα), oncofetal trophoblast glycoprotein (Tpbg, also known as 5T4), metabotropic glutamate receptor 2 (mGluR2), and neurogranin (Imamura et al., 2006; Batista-Brito et al., 2008; Gribaudo et al., 2009; Merkle et al., 2014; Nagayama et al., 2014; Malvaut et al., 2017). PGCs are further divided into two types: Type 1 expressing tyrosine hydroxylase (TH), the rate-limiting enzyme in dopamine synthesis, and Type 2 expressing calbindin (CalB), CalR, or Tpbg/5T4 (Kosaka et al., 1995; Parrish-Aungst et al., 2007; Toida, 2008; Yoshihara et al., 2012; Nagayama et al., 2014; Figure 1B). CalR and CalB, calcium-binding proteins with EF-hand motifs, maintain calcium homeostasis within neurons and are involved in synaptic plasticity and neurotransmission regulation. Based on the functional properties of CalR and CalB, it might be possible to distinguish the subtypes of GCs and PGCs within the OB.

Embryonic neurogenesis begins around embryonic day (E) 10, when neural epithelial cells in the ventricular zone (VZ) of the lateral ventricle differentiate into radial glial cells (RGCs) (Götz and Huttner, 2005; Turrero García and Harwell, 2017). From E13 to E14, the SVZ is formed via the multiplication of RGCs, and becomes the primary proliferative region. The earliest OB interneurons are generated mainly from the lateral ganglionic eminence (LGE) between E12.5 and E14.5 (Wichterle et al., 1999; Wichterle et al., 2001; Tucker et al., 2006; Kohwi et al., 2007; Batista-Brito et al., 2008). Progenitor cells from the dorsal LGE, expressing transcription factors such as Dlx2, Gsh2 (Gsx2), and Er81 (Etv1), give rise to all major OB interneuron subtypes (Wichterle et al., 2001; Stenman et al., 2003; Qin et al., 2017). Mutations in these and other transcription factors, such as Arx or Sp8, lead to a significant reduction in the number of GABAergic interneurons in both the GC layer (GCL) and glomerular layer (GL) (Stenman et al., 2003; Yun et al., 2003; Yoshihara et al., 2005; Waclaw et al., 2006; Li et al., 2018; Guo et al., 2019).

OB interneuron neurogenesis continues after birth, peaking within the first few weeks of life (Batista-Brito et al., 2008; Figure 1A). Although the rate of neurogenesis declines with age, the ability to generate new neurons persists throughout adulthood in the SVZ, which remains a proliferative region (Alvarez-Buylla and Garcia-Verdugo, 2002; Tramontin et al., 2003; Obernier and Alvarez-Buylla, 2019). Fate mapping studies have shown that the postnatal SVZ contains heterogeneous pools of neural stem cells

Abbreviations: Arx, aristaless-related homeobox; ASD, autism spectrum disorder; cKO, conditional knockout; FCM, fibrocellular mass; GABA, gamma-aminobutyric acid; GC, granule cell; HD, homeodomain; ID, intellectual disability; M/TC, mitral and tufted cell; OB, olfactory bulb; OE, olfactory epithelium; OSN, olfactory sensory neuron; PAE, poly-Ala expansion; PGC, periglomerular cell; RGC, radial glial cell; RMS, rostral migratory stream; TH, tyrosine hydroxylase; V-SVZ, ventricular-subventricular zone.

originating from the medial ganglionic eminence (MGE), LGE, and embryonic cortical regions, which remain quiescent until activated in adulthood (Young et al., 2007; Fuentealba et al., 2015; Furutachi et al., 2015). LGE- and cortical-derived progenitors give rise to distinct populations of OB interneurons, with cortical progenitors predominantly producing CalR-positive interneurons, but with LGE progenitors producing CalB-positive interneurons. Both progenitor pools contribute to the generation of TH-expressing interneurons (Young et al., 2007).

2 Aristaless-related homeobox (Arx) transcription factor

Aristaless-related homeobox (Arx) is a transcription factor containing a paired homeodomain (HD) that is located on the X chromosome. It functions as both an activator and a repressor (Miura et al., 1997; Friocourt and Parnavelas, 2010; Olivetti and Noebels, 2012). In addition to the HD, Arx includes a conserved aristaless domain, an octapeptide domain, and four poly-alanine (Ala) tracts (Friocourt and Parnavelas, 2010; Figure 2A). Mutations in Arx are associated with a broad spectrum of phenotypes, which can be categorized into three primary groups: (1) mutations resulting in truncated proteins, which cause severe intellectual disabilities (ID), autism spectrum disorders (ASD), epilepsy, and brain malformations, particularly the deletion of the corpus callosum (Scheffer et al., 2002; Strømme et al., 2002a; Uyanik et al., 2003), (2) mutations that cause ID, ASD, and epilepsy without structural brain malformations, and (3) missense mutations and in-frame expansions of the first two poly-Ala tracts (Strømme et al., 2002a; Strømme et al., 2002b; Kato et al., 2003). Poly-Ala tract expansion (PAE) mutations have been identified in nine genes, eight of which, including Arx, encode transcription factors (Albrecht and Mundlos, 2005; Messaed and Rouleau, 2009). Unlike polyglutamine repeats, which are more commonly studied, PAEs are typically short (less than 20 Ala residues) and cause developmental defects similar to those seen in Arx, suggesting a shared underlying molecular or genetic mechanism for PAE-related disorders (Albrecht and Mundlos, 2005; Messaed and Rouleau, 2009).

Arx is expressed during development in the nervous system, pancreas, and testes, with its expression continuing in the brain, muscles, heart, and liver in adult mice (Kitamura et al., 2002; Colombo et al., 2004). In the brain, Arx is not only expressed in progenitor cells of the cerebral cortex but also in postnatal GABA-containing interneurons, indicating a potential role in interneuron migration and the development of the cerebral cortex (Colombo et al., 2004; Friocourt et al., 2008; Colasante et al., 2015). Knockout (KO) mouse models, as well as Arx HD mutations, have recapitulated severe epilepsy phenotypes observed in Arx-related disorders.

3 Abnormalities in the olfactory system due to Arx deficiency

In the initial study, constitutive Arx KO mice at postnatal day (P) 0 show aberrant migration and a reduction in GABAergic interneurons in the OB (Yoshihara et al., 2005; Figure 2D). Several abnormalities in cell organization, differentiation, and axonal projection were observed in the developing olfactory system of Arx KO mice. OB interneurons, including GCs and PGCs, arise from progenitors in the LGE and migrate rostrally through the RMS to the OB (Luskin, 1998; Wichterle et al., 2001). Arx is strongly expressed in these interneurons and their progenitors, including radial glial cells (RGCs), in the OB and RMS (Figures 2B, C). In Arx KO mice, the proliferation and migration of interneurons to the OB are severely impaired, leading to accumulation of OSN axons at the entrance to the OB. This is similar to the phenotype in the neocortex, to which migration of their interneurons from the MGE is disordered (Kitamura et al., 2002). While the birthplaces, migration routes, and final destinations of interneurons in the cerebral cortex and OB differ, a common mechanism underlying directional neuronal migration likely involves Arx, which may regulate the expression of downstream genes in a cell-autonomous manner. However, the expression patterns of candidate downstream molecules (PSA-NCAM, Robo/Slit, Eph/ephrin, integrin, and Dcc), which may control the migration of OB interneurons, do not differ between Arx KO and wild-type mice (Yoshihara et al., 2005). Additionally, both wild-type and Arx KO mice exhibit a rudimentary RMS glial tube composed of RGCs and astrocytes extending from the SVZ to the OB (Hartfuss et al., 2001; Yoshihara et al., 2005).

In Arx KO mice, the subpopulations of GABAergic interneurons and TH-positive cells were completely absent from the OB (Yoshihara et al., 2005). Furthermore, the expression of Nurr1, a transcription factor crucial for the differentiation of TH-positive OB interneurons, was absent in the mutant mice (Backman et al., 1999; Liu and Baker, 1999). These findings suggest that Arx deficiency disrupts the differentiation of specific interneuron subtypes in the OB. One plausible explanation is that Arx acts upstream of Nurr1 and TH in the differentiation cascade, though it is also possible that progenitors of TH-positive interneurons fail to receive appropriate differentiation signals from the OB due to impaired migration.

Although Arx is not expressed in mitral cells (MCs), abnormalities in the MC layer (MCL) were observed in Arx KO mice, including a thicker and irregular outline of the layer (Yoshihara et al., 2005). Given that an increased number of interneurons from the RMS contributes to OB expansion during late embryonic stages, the disruption of the MCL in Arx KO mice may stem from a reduction in the GCL caused by the failure of interneurons to migrate into the OB. It is also possible that Arx plays a role in the progenitor cells of OB projection neurons, as RGCs serve as progenitors for many brain neurons (Anthony et al., 2004). Abnormal layer formation in OB projection neurons could thus result from cell-autonomous defects in RGCs due to Arx deficiency. Alternatively, the defect could involve a failure of signaling from OB interneurons, RGCs, or OSNs that normally guide the projection pattern of OSNs. In Arx KO mice, most OSN axons fail to reach the OB, terminating instead in a disorganized structure called the fibrocellular mass (FCM), located in front of the OB (Figure 2D).

Several members of the Dlx transcription factor family (Dlx1, Dlx2, Dlx5) play critical roles in the development of the olfactory system. These factors are expressed sequentially, differentially, and in overlapping patterns in OB interneurons and their progenitors

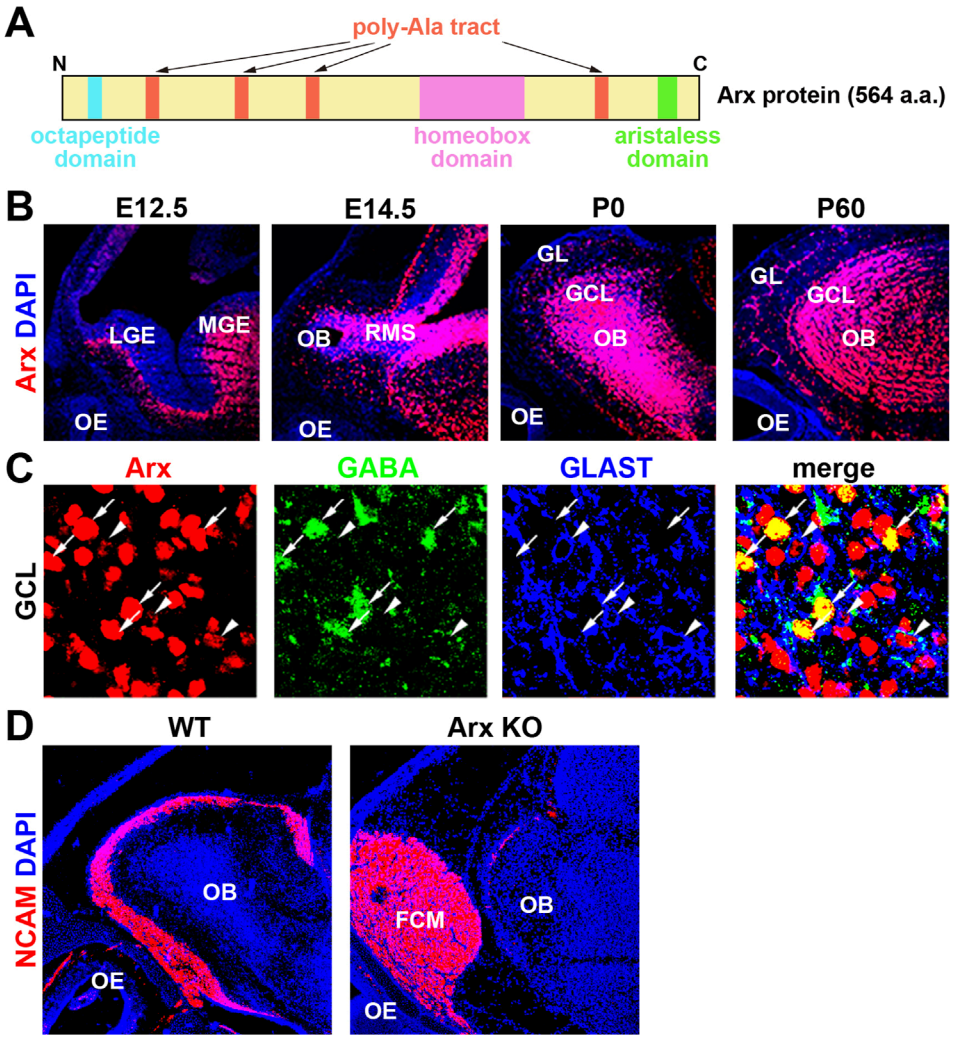

FIGURE 2
Axons of olfactory sensory neurons (OSNs) fail to enter the OB in Arx KO mice. **(A)** Schematic representation of the homeobox transcription factor Arx. **(B)** Arx is expressed throughout development in the OB but not the OE. Sagittal sections of E (embryonic day) 12.5, E14.5, P (postnatal day) 0, and P60 wild-type mice were labeled with anti-Arx antibody (red) and counterstained with DAPI (4′,6-diamidino-2-phenylindole, blue: nuclei). LGE: lateral ganglionic eminence, MGE: medial ganglionic eminence, RMS: rostral migratory stream. GL: glomerular layer. These figures were taken from Figure 1 of Yoshihara et al. (2005), with permission from the journal. **(C)** Arx is expressed in interneurons (GABA+) and radial glial cells (RGCs, GLAST+) of the OB. Enlarged view of the granule cell layer (GCL), triple-labeled sections of Arx (red), GABA (green: GCs), and GLAST (glutamate transporter, blue: RGCs). Arrows: Arx+ and GABA + GCs. Arrowheads: Arx+ and GLAST + RGCs. These figures were taken from Figure 1 of Yoshihara et al. (2005), with permission from the journal. **(D)** Immunofluorescence labeling of NCAM (neural cell adhesion molecule, red: olfactory axons) and DAPI staining (blue: nucleus) on parasagittal sections of wild-type and Arx-deficient mice at P0. In Arx mutant mice, OSN axons fail to reach the OB and terminate in an axon-tangled structure, termed the fibrocellular mass (FCM). These results suggest that Arx regulates the axonal projection of OSNs through the proper development of either RGCs or interneurons in the OB.

(Bulfone et al., 1998; Levi et al., 2003; Long et al., 2003). In Dlx1/Dlx2 double KO mice, severe defects in the proliferation and migration of OB interneuron are observed, with these interneurons being completely absent (Bulfone et al., 1998). In contrast, Dlx5 KO mice exhibit milder phenotypes, which resemble those observed in Arx KO mice, including reduced OB size, impaired migration of OB interneurons, disrupted the MCL, and abnormal axonal projection of OSNs that form the FCM (Levi et al., 2003; Long et al., 2003). It has been reported that Dlx1/2 have key roles in guiding the fate specification and migration of OB interneurons by promoting Arx, Etv1, Pbx3, Prokr2, Sp8, Sp9, and Tshz (Yoshihara et al., 2005; Waclaw et al., 2006; Long et al., 2007; Guo et al., 2019).

In Arx KO mice, the projection pattern of OSNs shows defects in a non-cell autonomous manner: most of the OSN axons fail to reach the OB and terminate in the FCM. The possibility of reciprocal influences between the OE and OB during induction and development has been proposed and widely studied (López-Mascaraque and de Castro, 2002). In rats, the arrival of pioneer OSN axons in the OB regulates cell cycle dynamics and the rate of differentiation of neural progenitor cells, inducing the formation of the OB (Gong and Shipley, 1995). These studies suggest that the OE somehow affects the development of the OB. Is FCM formation due to a deficit originating from the OB rather than the OE? This has been primarily investigated using extratoes (Xt/Xt)

mice (St John et al., 2003), which carry a Gli3 mutation. In these mice, the OB is entirely absent, and the sparse OB projection neurons on the rostral surface of the forebrain undergo apoptosis (Hui and Joyner, 1993; St John et al., 2003). In contrast, the OE develops normally in terms of its gross morphology and the expression of signaling molecules, including odorant receptors (Sullivan et al., 1995). However, OSN axons fail to reach the telencephalon and instead terminate in an abnormal structure known as the FCM (St John et al., 2003). These findings suggest that while the OB does not influence cell proliferation or differentiation in the lateral OB, it may play a crucial role in directing OSN axon guidance.

In Arx KO mice, only a small proportion of OSN axons contact the OB, while most fail to reach the OB and terminate in the FCM (Figure 2D). Since Arx is not expressed in OSNs, it has been hypothesized that Arx regulates the expression of one or more guidance signals produced by interneurons and RGCs in the OB to ensure proper OSN axon innervation. To further investigate the molecular mechanisms underlying these observations, microarray was performed to compare gene expression levels between the OBs of wild-type and Arx KO mice. Differential expression analysis revealed alterations (decrease) in genes implicated in neuronal proliferation and migration, such as the cell adhesion molecule Plexin C1 and the cell proliferation regulator Prc1 (polycomb repressive complex 1), including Ring1B that may regulate the differentiation potential of neural stem cells to neurons and glia (Román-Trufero et al., 2009). To determine whether these candidate genes directly regulate interneuron proliferation and migration in the OB, future studies should employ loss- and gain-of-function experiments.

4 Abnormalities in the cerebral cortex due to Arx deficiency

Cortical interneurons constitute a diverse population with widely varying morphology, connectivity, and activity patterns (Kepecs and Fishell, 2014). These neurons originate from progenitor cells located in the embryonic proliferative zones known as the MGE, caudal ganglionic eminence (CGE), and LGE (Kepecs and Fishell, 2014). Each ganglionic eminence gives rise to a distinct subset of interneurons; however, the genetic programs governing interneuron fate specification and maintenance remain incompletely understood. The first signs of interneuron diversity appear in the region-specific expression of a limited set of transcription factors within the basal ganglia primordium (Yun et al., 2003; Flames et al., 2007). For instance, the homeobox transcription factor Nkx2.1 is expressed throughout the MGE but is absent in the CGE and LGE (Shimamura et al., 1995). In contrast, the LIM-homeodomain transcription factor Lhx8 is expressed only in specific subdomains of the MGE (Flames et al., 2007). Nevertheless, how these initial heterogeneities contribute to the extensive diversity of adult interneurons remains unclear, further complicated by the fact that many subcortical projection neurons, such as those in the basal ganglia, are also generated from these regions (Zhao et al., 2003; Nóbrega-Pereira et al., 2010).

Arx is a crucial transcription factor in cortical interneuron development, and its mutations are associated with neurodevelopmental disorders such as developmental epilepsies, ID, and ASD in humans (Lim, 2023). For instance, induction of Arx can rescue loss of MGE-derived somatostatin (Sst) and parvalbumin (Pvalb) cortical interneurons in Lhx6 KO mice (Vogt et al., 2014). Nkx2.1, which is critical for the regional specification of the MGE, in turn induces Lhx6 expression to promote Sst and Pvalb interneuron fate in the cortex (Sandberg et al., 2018). Understanding the role of Arx and its associated transcriptional networks is essential for elucidating the underlying mechanisms of these pathologies. Perinatal lethality of constitutive Arx KO mice precludes further analysis in adolescent or adult mice (Kitamura et al., 2002). Several driver mice in which Cre had been inserted so that its expression would mimic that of genes known to shape the emerging identity, function, and positioning of GABAergic cortical interneurons were created (Taniguchi et al., 2011). Then, Arx-floxed mice have been crossed with the Dlx5/6-Cre driver to generate conditional KO (cKO) mice with selective Arx deletion in interneuron progenitors (Marsh et al., 2016). Dlx5/6-Cre cKO male mice (Arx−/Y) show its deficiency in cortical interneuron progenitors, leading to perinatal lethality. However, Dlx5/6-Cre cKO female mice (Arx−/X) show a reduction in the number of interneurons in the cerebral cortex at perinatal and early postnatal stages.

More recently, based on Arx cKO mice with several Cre drivers, Lim et al. (2024) have identified Arx as a key transcriptional regulator involved in the generation, fate determination, and migration of cortical interneurons by modulating gene transcription networks during brain development. For instance, Arx directly or indirectly regulates genes involved in proliferation and the cell cycle (e.g., Bub3, Cspr3), fate specification (e.g., Nkx2.1, Maf, Mef2c), and migration (e.g., Nkx2.1, Lmo1, Cxcr4, Nrg1, ErbB4). First, the loss of Arx in the SVZ of the ganglionic eminences delays cell cycle exit, presumably disrupting the transition from proliferation to differentiation (Lim et al., 2024). This delay is consistent with the aberrant upregulation of Csrp2 (Zhang et al., 2023), a gene known to promote stem cell-like properties, and Bub3 (Silva and Bousbaa, 2022), a cell cycle checkpoint protein frequently overexpressed in tumor cells. As direct transcriptional targets of Arx, the upregulation of these genes in Arx-deficient interneuron progenitors likely sustains a proliferative state and impairs differentiation. Second, a dramatic reduction in Arx-deficient cortical interneurons is observed, particularly within the marginal zone (MZ) stream (Lim et al., 2024). Nkx2.1, a direct target of Arx, is among the most upregulated genes in the MGE cluster. Given that the downregulation of Nkx2.1 is necessary for post-mitotic cortical interneurons to migrate along the cortical migratory stream (Nóbrega-Pereira et al., 2008), defects in interneuron migration in Arx cKO mice may stem, at least in part, from the failure to downregulate Nkx2.1. Third, another direct target of Arx involved in cortical interneuron migration is Lmo1. The expression of Lmo1 is consistently elevated in Arx cKO, constitutive KO, and Arx $(GCG)^7$ mutant mice (Lee et al., 2014). Interestingly, ChIP-seq analysis and slice culture electroporation studies indicate that Lmo1 directly represses Cxcr4 expression (Lim et al., 2024). The loss of Cxcr4 in Arx-deficient interneurons, along with the ectopic upregulation of the inductive signal Nrg1/ErbB4 (a direct target of Arx), contributes to the failure of interneurons to enter the cortical

MZ. These findings offer novel insights into the role of Arx in cortical interneuron development and its disruption in disease.

5 Abnormalities in mice vs. humans due to Arx deficiency

Mutations in Arx, an X-linked gene, are implicated in various neurological disorders, including ID, ASD, and epilepsy in humans (Lim, 2023). While mouse models have demonstrated the critical role of Arx in cortical development and interneuron migration, they do not fully recapitulate the phenotypes observed in human patients. For instance, mice with Arx deletion in cortical projection neuron progenitors exhibit hyperactivity and abnormal behavior but do not develop seizures (Simonet et al., 2015). In contrast, mice with a knock-in Arx poly-Ala expansion (PAE) mutation show a reduction in GABAergic interneurons within the cerebral cortex (Kitamura et al., 2009; Lee et al., 2017) and develop seizures (Price et al., 2009; Mattiske et al., 2016; Loring et al., 2021). Furthermore, epilepsy in many patients with Arx PAE mutations is drug-resistant, underscoring the necessity of developing novel therapeutic strategies. Despite the valuable insights gained from these mouse models, they fail to fully capture the role of Arx in human brain development.

Nieto-Estevez et al. (2024) utilized human neural organoid models derived from male patients with Arx PAE, which harbors eight additional Ala residues in the second poly-Ala tract of Arx. In human cortical organoids that have been generated from induced pluripotent stem cells derived from the patients, Arx PAE causes premature differentiation of RGCs and a depletion of these progenitor cells at the initial stage, followed by a subsequent reduction in GABAergic cortical interneurons at the later stage (Nieto-Estevez et al., 2024). As interneurons originate in the ganglionic eminence and migrate tangentially, the reduction of interneurons in the cortex suggests that Arx affects neuronal migration. Arx PAE promotes the expression of Cxcr4 and accelerates interneuron migration (Beguin et al., 2013); yet, accelerated migration does not lead to increased interneurons in the cortex. It is possible that interneurons with Arx PAE keep moving because they fail to encounter their final target. Defects in GABAergic cortical interneurons contribute to hyperactivity, mirroring the phenotypes observed in Arx mutant mouse models and human patients. Such *in vitro* studies provide valuable insights into the pathological mechanisms underlying Arx PAE mutations and offer a promising human-based platform for developing potential therapeutic interventions.

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AT: Writing–original draft, Writing–review and editing. SY: Writing–original draft.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Generative AI statement

The author(s) declare that no Generative AI was used in the creation of this manuscript.

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Slit-Robo signaling supports motor neuron avoidance of the spinal cord midline through DCC antagonism and other mechanisms

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Axon pathfinding and neuronal migration are orchestrated by attractive and repulsive guidance cues. In the mouse spinal cord, repulsion from Slit proteins through Robo family receptors and attraction to Netrin-1, mediated by the receptor DCC, control many aspects of neural circuit formation. This includes motor neuron wiring, where Robos help prevent both motor neuron cell bodies and axons from aberrantly crossing the spinal cord midline. These functions had been ascribed to Robo signaling being required to counter DCC-mediated attraction to Netrin-1 at the midline, either by mediating repulsion from midline-derived Slits or by silencing DCC signaling. However, the role of DCC in promoting motor neuron and axon midline crossing had not been directly tested. Here, we used *in vivo* mouse genetics and *in vitro* axon turning assays to further explore the interplay between Slit and Netrin signaling in motor neuron migration and axon guidance relative to the midline. We find that DCC is a major driver of midline crossing by motor axons, but not motor neuron cell bodies, when *Robo1* and *Robo2* are knocked out. Further, *in vitro* results indicate that Netrin-1 attracts motor axons and that Slits can modulate the chemotropic response to Netrin-1, converting it from attraction to repulsion. Our findings indicate that Robo signaling allows both motor neuron cell bodies and axons to avoid the midline, but that only motor axons require this pathway to antagonize DCC-dependent midline attraction, which likely involves a combination of mediating Slit repulsion and directly influencing Netrin-DCC signaling output.

KEYWORDS

axon guidance, neuronal migration, spinal cord, motor neuron, robo signaling, crosstalk, floor plate

Introduction

Assembly of neural circuits during embryonic development requires the guidance of nascent axons to their correct targets. This process of axon pathfinding is instructed by molecular cues that signal through receptors on the leading process of the axon, the growth cone (Kolodkin and Tessier-Lavigne, 2011). While axon guidance cues are often categorized as either attractants or repellants, some of them can exert both attractive or repulsive effects,

depending on context. A classic example is the secreted protein Netrin-1, which can signal attraction through the receptor Deleted in Colorectal Cancer (DCC) and repulsion via Unc5 family members (Keino-Masu et al., 1996; Leonardo et al., 1997). While the complement of available Netrin receptors is a key determinant of a neuron's response to this cue, the level of cAMP in the growth cone also influences the valence of Netrin-1's effects on axon extension (Song et al., 1998), and extracellular signals, such as laminin, can modulate the intracellular cAMP concentration to switch Netrin-mediated attraction to repulsion (Höpker et al., 1999). Hence, multiple intrinsic and extrinsic factors dictate how an axon will respond to a given cue. This concept extends to cross-regulatory interactions between guidance cues. Growing axons *in vivo* usually integrate information from several cues that either collaborate to steer a growth cone in the same direction or exert opposite effects on axon extension. While simple summation of the attractive and repulsive effects of multiple ligands through parallel signaling pathways is observed in some cases, signal crosstalk can drive synergistic, permissive, or hierarchical integration of guidance information (Morales and Kania, 2017). Netrin-1 repulsion, for instance, synergizes with ephrin-B2 repulsion in motor axon pathway choice in the developing vertebrate limb (Poliak et al., 2015), and motor axon attraction to Netrin-1 in the spinal cord has been proposed to be silenced by axon repellants of the Slit family through hierarchical receptor interactions (Bai et al., 2011). The contexts in which different mechanisms of guidance cue integration drive axon pathfinding *in vivo* have not been fully delineated.

Netrin-mediated attraction and Slit-dependent repulsion control axonal crossing of the nervous system midline in bilaterians, including nematode worms, flies, mice, and humans (Dickson and Zou, 2010). In the mouse spinal cord and hindbrain, floor plate cells at the ventral midline secrete both Netrin-1 and all three Slit paralogs – Slit1, Slit2, and Slit3 (Kennedy et al., 1994; Brose et al., 1999). Netrin-1 is also produced by radial glia and deposited at the pial surface, and the combined attractive and growth-promoting effects of floor plate- and radial glia-derived Netrin-1 guide commissural axons towards and across the ventral midline (Serafini et al., 1994; Serafini et al., 1996; Dominici et al., 2017; Varadarajan et al., 2017; Moreno-Bravo et al., 2019; Wu et al., 2019). Slit proteins signal axon repulsion through receptors of the Robo family (Blockus and Chedotal, 2016), and floor plate-derived Slits help expel commissural axons from the midline after crossing and prohibit their re-crossing (Zou et al., 2000; Long et al., 2004) while also preventing ipsilaterally projecting neurons from sending axons across the midline in the first place (Farmer et al., 2008). How spinal cord neurons integrate signaling from Netrin-1 and Slits remains incompletely understood.

Motor neurons in the spinal cord and hindbrain project axons towards their muscle targets in the body periphery, and they express both Netrin and Slit receptors during development (Bonanomi and Pfaff, 2010). Limb-innervating motor neurons belonging to the lateral motor column (LMC) use Netrin-1 expressed in the limb mesenchyme to select the correct dorso-ventral axon trajectory; this involves DCC-mediated attraction of motor axons originating from the lateral subdivision of the LMC and Unc5c-dependent repulsion of medial LMC axons (Poliak et al., 2015). Netrin-1 and DCC also regulate earlier aspects of motor neuron development, such as the dorso-ventral positioning of motor neuron cell bodies and of the motor exit points (MEPs) where motor axons leave the central nervous system; here, Netrin-mediated attraction to the midline and Slit-mediated repulsion appear to balance each other, as genetic disruption of either of these signaling pathways has opposing effects on motor neuron and MEP positioning (Kim et al., 2015; Kim et al., 2017). In mice lacking the Slit receptors Robo1 and Robo2, motor neuron cell bodies and axons can even be observed entering the ventral midline, which they usually avoid (Bai et al., 2011; Kim et al., 2015; Kim et al., 2017; Gruner et al., 2019). In one study, ectopic motor axon midline crossing in *Robo1*$^{-/-}$; *Robo2*$^{-/-}$ (*Robo1/2*$^{-/-}$) double knockout mice was attributed to a gain of Netrin-mediated midline attraction rather than a loss of Slit-mediated midline repulsion, invoking a hierarchical crosstalk model where Slit signaling through Robos suppresses attraction via Netrin-DCC (Bai et al., 2011). However, this model has not been validated by phenotypic rescue of motor neuron cell body and axon crossing of the midline via inactivation of DCC; other, Slit/Robo-independent DCC silencing mechanisms have been identified (Bonanomi et al., 2019); and different studies also report conflicting findings regarding the baseline ability of motor neurons to respond to Netrin-1 *in vitro* (Varela-Echavarría et al., 1997; Bai et al., 2011; Poliak et al., 2015; Kim et al., 2017). The precise roles, and interplay, of the Netrin-DCC and Slit-Robo pathways in motor neuron migration and axon guidance relative to the spinal cord midline have therefore remained somewhat enigmatic.

Here, we combined mouse genetics and *in vitro* axon guidance assays to revisit the functions of Netrin-DCC and Slit-Robo signaling in spinal motor neurons. Our results indicate that aberrant midline crossing by motor neuron cell bodies and axons in mice lacking Robo1 and Robo2 are not interdependent and that DCC contributes to axon, but not cell body entry into the ventral commissure. Further, we find that motor axons are attracted by Netrin-1 and that Slits can convert this attractive effect to repulsion. These results support a hierarchical relationship between Slit-Robo and Netrin-DCC signaling in motor axon guidance that goes beyond a silencing interaction and involves a Slit-induced change in the valence of axonal responses to Netrin-1.

Materials and methods

Animals

All experimental procedures had institutional approval through Brown University's Institutional Animal Care and Use Committee (current protocol number 24-11-0002) and followed the guidelines provided by the National Institutes of Health. Null alleles for *Robo1* (Long et al., 2004), *Robo2* (Grieshammer et al., 2004), and *DCC* (Fazeli et al., 1997) have been described before, and mice carrying these mutations were genotyped by PCR as originally reported. Mice were maintained on a CD-1 background. *Robo1*$^{+/-}$; *Robo2*$^{+/-}$; *DCC*$^{+/-}$ triple heterozygous animals were generated by crossing mice carrying the closely linked *Robo1* and *Robo2* knockout alleles (Chen et al., 2008) to *DCC*$^{+/-}$ mice, and experimental litters for phenotype analysis were generated by intercrossing of triple heterozygotes. For timed pregnancies, the day of vaginal plug was defined as embryonic day (E) 0.5, and littermate embryos of either sex were used for all experiments.

Immunohistochemistry

All spinal cord transverse cryosections were collected from brachial level (i.e., cervical and upper thoracic spinal cord segments with visible limb buds in the same sections). Immunohistochemistry (IHC) on 20-µm-thick cryosections was performed as previously described (Jaworski et al., 2010). Antibody labeling of neuronal cultures following live imaging in Dunn chambers was performed essentially as reported before (Pak et al., 2020). Primary antibodies used for IHC were rabbit polyclonal antibodies against class III β-tubulin (TuJ1) (Biolegend, 1:500) (Hu et al., 2006), Peripherin (Prph) (Millipore, 1:200) (Xiao et al., 2008), and FoxP1 (Abcam, 1:500) (Sheng et al., 2019), and mouse monoclonal antibodies against neurofilament (NF) (DSHB, 1:200) (Dodd et al., 1988) and Islet (Isl) 1/2 (DSHB, 1:200) (Tsuchida et al., 1994). Secondary antibodies (all from Invitrogen; 1:200) were Alexa488-conjugated donkey anti-rabbit, Alexa594-conjugated donkey anti-rabbit, Alexa488-conjugated donkey anti-mouse, and Alexa594-conjugated donkey anti-mouse. Hoechst 33342 (Molecular Probes, 1:1,000) was added with the secondary antibodies. Images were acquired on a Nikon Ti-E microscope.

Dunn chamber axon turning assay

Dunn chamber axon turning assays were adapted for motor neurons but essentially performed as previously described for spinal commissural neurons (Pak et al., 2020), with few modifications. E10.5 ventral spinal cord was dissected and dissociated as previously described (Suter et al., 2020), pooling tissue from multiple embryos. Cells were plated on nitric acid-washed and baked 18-mm coverslips coated with 100 µg/mL PDL and 5 µg/mL laminin, cultured in motor neuron media [1x penicillin/streptomycin/glutamine, 2% B-27 (both Gibco), 0.5% glucose, 10 ng/mL BDNF (Cell Sciences), 10 ng/mL NT-3 (Sigma) in Neurobasal-A medium (Gibco)], and used for experiments 16–26 hours (h) after plating. The age of neurons at the time of the experiment was therefore E10.5 + 1 day *in vitro* (DIV). Media from pre-culturing of neurons was reused in Dunn chambers, recombinant mNetrin-1 or mSlit2-N (both Biotechne/R&D Systems) was added at indicated concentrations to media in the Dunn chamber outer well, and ≈30–40 visual fields [containing 8–20 analyzable motor neurons per experimental replicate (n)] covering the bridge region of each chamber were imaged repeatedly over 2 h. For studying effects of mSlit2-N bath application on axon turning in response to mNetrin-1, mSlit2-N was added at 1 µg/mL to media used for Dunn chambers (inner and outer well), while 250 ng/mL mNetrin-1 was added to the outer well only. Images were acquired on a Nikon Ti-E microscope.

Quantification and statistical analysis

Quantification of motor neurons entering the midline

To count mispositioned motor neuron cell bodies at the spinal cord midline, brachial spinal cord sections were immunolabeled for Isl1/2 and Tuj1. The number of Isl1/2-positive (Isl1/2$^+$) cells in the ventral midline, defined by the area enclosed by the Tuj1$^+$ commissural axon bundle and the ventral edge of the central canal, from 6 to 15 sections per animal was quantified and normalized to the total number of sections per animal. Means across multiple animals of the same age and genotype (n = 3–5 animals) were calculated and used for statistical comparison after confirming normal distribution of the data. Statistical significance across multiple groups was assessed using a one-way ANOVA with post-hoc Holm's test for multiple comparisons (α = 0.05). Pairwise comparisons between groups were analyzed using two-tailed unpaired t-test (p = 0.05). To determine the molecular identity of motor neurons in the midline, E10.5 *Robo1/2*$^{-/-}$ brachial spinal cord sections were immunolabeled for Isl1/2 and the LMC-specific transcription factor FoxP1. The number of medial motor column (MMC; Isl1/2$^+$/FoxP1$^-$) and LMC (Isl1/2$^+$/FoxP1$^+$) cells in the ventral midline from 3–8 sections per animal (n = 3 animals) was quantified, normalized to the total number of sections per animal, and expressed as mean per animal. All motor neuron counts were performed blinded to animal identities.

Quantification of total motor neurons

To count total motor neurons, brachial spinal cord sections were immunolabeled for Isl1/2 and Tuj1. Isl1/2$^+$ cells in the ventral horn were counted and expressed as neurons per hemisection, averaging 6–16 hemisections per animal. Means across multiple animals of the same age and genotype (n = 5–9 animals) were calculated and used for statistical comparison after confirming normal distribution of the data. Pairwise comparison between groups was performed using a two-tailed unpaired t-test (p = 0.05).

Quantification of motor axons crossing the ventral midline

To count motor axons in the ventral midline, brachial spinal cord sections were immunolabeled for Prph and NF. The number of Prph$^+$ axons entering the midline from 5 to 15 sections per animal was quantified and normalized to the total number of sections per animal. Means across multiple animals of the same age and genotype (n = 4–9 animals) were calculated and used for statistical comparison after confirming normal distribution of the data. Statistical significance across multiple groups was assessed using a one-way ANOVA with post-hoc Holm's test for multiple comparisons (α = 0.05). Pairwise comparisons between groups were analyzed using two-tailed unpaired t-test (p = 0.05). Analyses were performed blinded to animal identities.

Quantification of axon turning in dunn chambers

Quantitative analysis of axon turning in Dunn chambers was performed as described previously (Pak et al., 2020). All analyses were performed blinded to experimental conditions. Motor neurons were identified by post-hoc immunostaining of the imaged coverslip for Isl1/2. For each experimental replicate, axon turning angles from all analyzable motor neurons were averaged, and means across multiple replicates per condition were analyzed for statistical significance using a one-way ANOVA with post-hoc Holm's test for multiple comparisons (α = 0.05) (n and p are indicated in figure legends) after confirming normal distribution of the data.

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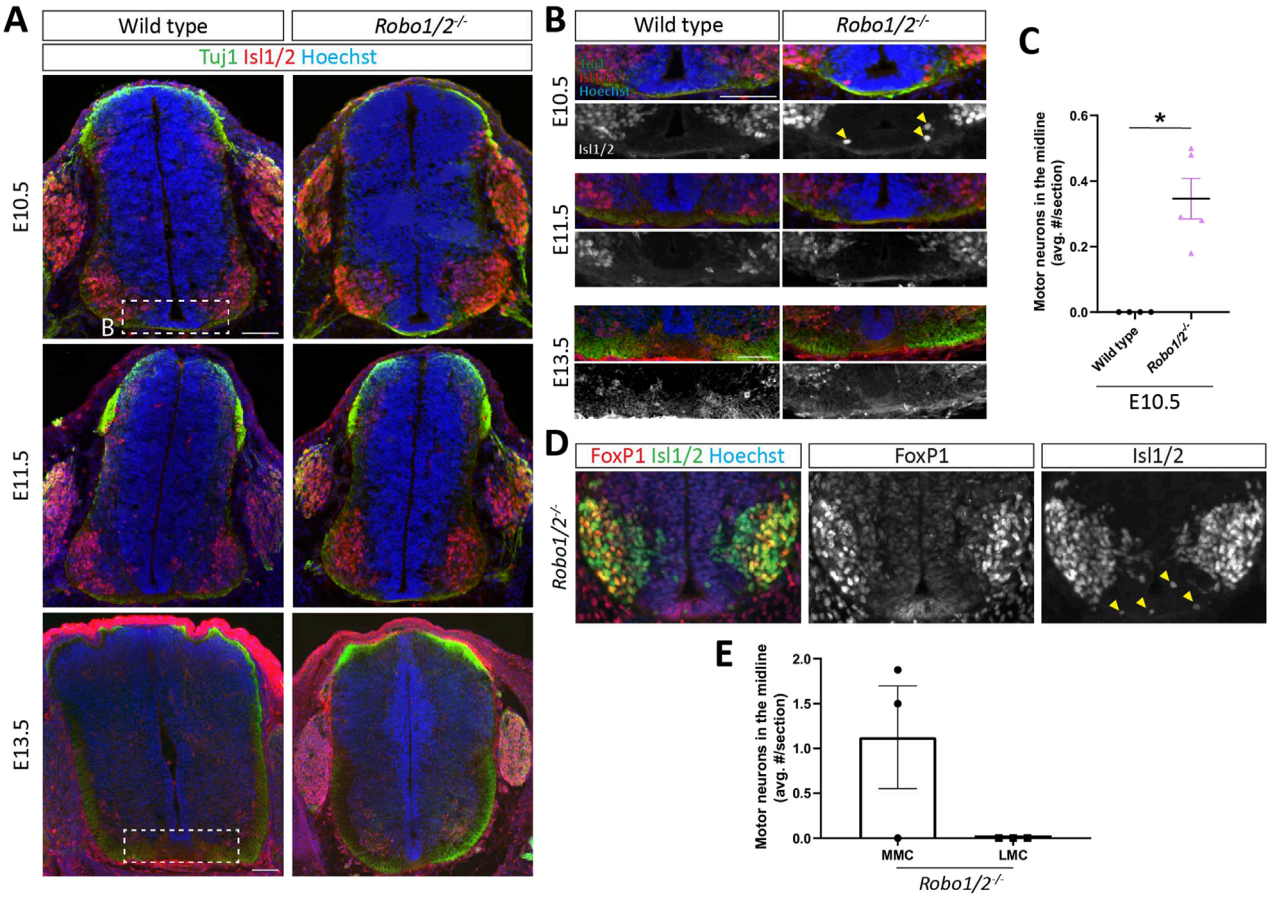

FIGURE 1
Motor neuron entry into the midline of *Robo1/2*$^{-/-}$ mice. **(A)** Transverse spinal cord sections of E10.5, E11.5, and E13.5 wild-type and *Robo1/2*$^{-/-}$ mice, stained for Isl1/2 and Tuj1. White boxes indicate regions shown in **(B)**. **(B)** Higher magnification views of E10.5, E11.5, and E13.5 wild-type and *Robo1/2*$^{-/-}$ mice. Isolated greyscale channel shows Isl1/2 staining. Yellow arrowheads indicate motor neurons infiltrating the midline. **(C)** The average number of Isl1/2^{+} cells in the midline was quantified in E10.5 wild-type and *Robo1/2*$^{-/-}$ mice. Motor neurons enter the midline in E10.5 *Robo1/2*$^{-/-}$ mice, which is not observed in wild-type sections (n = 4-5 animals/group, $p = 0.0159$). **(D)** Spinal cord sections from E10.5 Robo1/2$^{-/-}$ mice, stained for Foxp1 and Isl1/2. Isolated Foxp1 and Isl1/2 channels are also shown in greyscale. Motor neurons infiltrating the midline stained exclusively for Isl1/2. **(E)** The number of motor neurons in the midline belonging to the MMC (Isl1/2^{+}/FoxP1^{-}) and LMC (Isl1/2^{+}/FoxP1^{+}) was quantified (n = 3 animals). Data are represented as means ± SEM. Scale bar for E10.5 and E11.5 sections = 100 μm. Scale bar for E13.5 sections = 100 μm.

Results

Robo1 and Robo2 prevent MMC motor neurons from entering the spinal cord midline

Previous studies have reported that hindbrain and spinal cord motor neurons aberrantly migrate into the nervous system midline in mice lacking Robo1 and Robo2 (Kim et al., 2015; Gruner et al., 2019). In the spinal cord, this phenotype had been observed at E9.5 and E10.5, but not E12.5, and the columnar origin of the mispositioned motor neurons had not been determined (Kim et al., 2015). We sought to recapitulate this defect and examine its dependence on DCC-mediated midline attraction. First, we performed IHC using an antibody against the transcription factors Isl1 and Isl2, which mark motor neurons in the ventral spinal cord, and the panaxonal marker TuJ1 on transverse sections of brachial spinal cord from E10.5, E11.5, and E13.5 *Robo1/2*$^{-/-}$ mice and their wild-type littermates. At E10.5 and E11.5, motor neurons still migrate into the ventral horn, and their axons extend into the periphery, while motor neuron generation has ceased by E13.5, and motor axons start innervating their targets (Shirasaki et al., 2006; Wang et al., 2011). Across all ages in both wild-type and *Robo1/2* knockout embryos, most Isl1/2^{+} motor neurons occupy the spinal cord ventral horn, but in E10.5 *Robo1/2* double mutants, a small number of motor neurons is mispositioned within the floor plate area at the ventral midline, which is never observed in wild type (Figures 1A–C). We examined this phenotype more closely by co-labeling with antibodies against the LMC-specific marker FoxP1. We found that, in the spinal cord ventral horn of *Robo1/2* mutant mice, MMC (Isl1/2^{+}/FoxP1^{-}) and LMC (Isl1/2^{+}/FoxP1^{+}) motor neurons are spatially segregated as they are in wild type, and that motor neurons in the midline of *Robo1/2*$^{-/-}$ mice are exclusively of MMC, not LMC, identity (Figures 1D, E). The total number of motor neurons in E10.5 spinal cord is comparable between the two genotypes

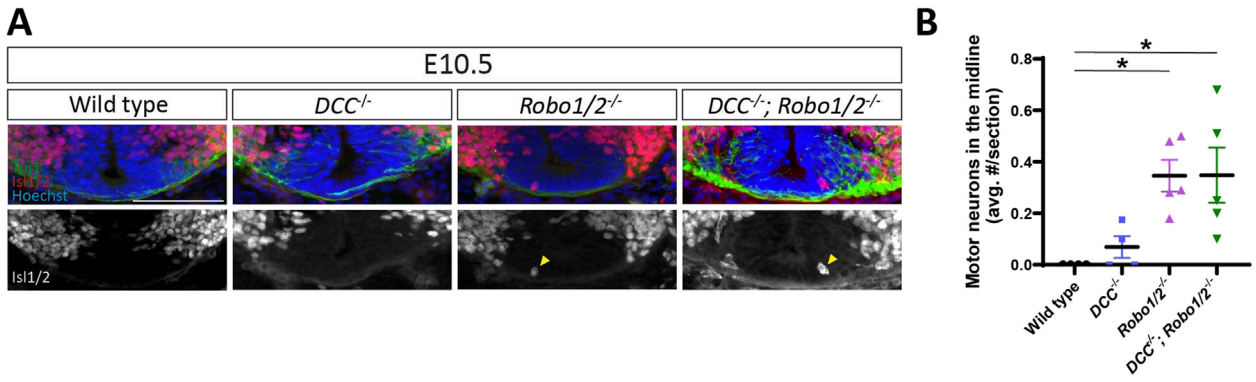

FIGURE 2
Mispositioning of motor neurons in *Robo1/2*$^{-/-}$ mice is not DCC-dependent. **(A)** Transverse sections of E10.5 wild-type, *DCC*$^{-/-}$, *Robo1/2*$^{-/-}$, and *DCC*$^{-/-}$; *Robo1/2*$^{-/-}$ mice, stained for Isl1/2 and Tuj1. Isolated Isl1/2 channel is shown in greyscale. Yellow arrowheads indicate Isl1/2^{+} motor neurons in the midline. **(B)** Quantification of motor neurons in the midline of E10.5 wild-type, *DCC*$^{-/-}$, *Robo1/2*$^{-/-}$, and *DCC*$^{-/-}$; *Robo1/2*$^{-/-}$ mice shows no significant differences in the number of mispositioned motor neurons between wild-type and *DCC*$^{-/-}$ mice (n = 4 animals/group, $p = 0.7842$). However, both *Robo1/2*$^{-/-}$ and *DCC*$^{-/-}$; *Robo1/2*$^{-/-}$ mice have significantly more mispositioned motor neurons in the midline compared to wild type (n = 4-5 animals/group, $p = 0.0261$ and $p = 0.0261$, respectively). No difference is observed in the average number of motor neurons in the midline between *Robo1/2*$^{-/-}$ and *DCC*$^{-/-}$; *Robo1/2*$^{-/-}$ groups (n = 5, $p = 0.9838$). Data are represented as means ± SEM. Scale bar = 100 μm.

(Supplementary Figure S1), and Isl1/2^{+} neurons are excluded from the midline in wild-type and *Robo1/2* double knockout mice at E11.5 and E13.5 (Figure 1A; Supplementary Figure S2). Thus, Robo1 and Robo2 prevent a subset of MMC motor neurons from migrating into the spinal cord ventral midline, without controlling overall motor neuron number or columnar organization, and aberrantly positioned motor neurons in the midline of *Robo1/2* double knockout mice do not persist past E10.5.

DCC is not required for motor neuron entry into the midline of *Robo1/2* mutant mice

Netrin-DCC signaling contributes to the ventral positioning of motor neuron cell bodies in the spinal cord (Kim et al., 2015), and the balance between DCC-mediated attraction to floor plate-derived Netrin and Robo1/2-mediated repulsion from midline Slits has been implicated in specifying the dorso-ventral position of MEPs where motor axons emerge from the spinal cord (Kim et al., 2017). To determine whether unbalanced DCC-mediated floor plate attraction causes motor neuron migration into the midline of *Robo1/2* knockout mice, we analyzed triple mutant mice lacking DCC, Robo1, and Robo2, as well as their wild-type, *DCC*$^{-/-}$, and *Robo1/2*$^{-/-}$ littermates. We found that motor neurons aberrantly enter the floor plate region in E10.5 *DCC*$^{-/-}$; *Robo1/2*$^{-/-}$ embryos, just as they do in *Robo1/2*$^{-/-}$ mice, and they are largely excluded from the midline in *DCC*$^{-/-}$ and wild-type littermates (Figure 2A). Motor neurons are never observed in the midline at E11.5 and E13.5, irrespective of genotype (Supplementary Figure S2). Quantification revealed that loss of DCC does not significantly change the number of mispositioned motor neurons in the *Robo1/2* mutant background (Figure 2B). These results indicate that midline entry of motor neurons in the absence of Robo1 and Robo2 is not driven by DCC signaling.

Robo1 and Robo2 inhibit motor axon crossing of the ventral commissure

Previous reports indicate that motor axons aberrantly project across the ventral midline of the hindbrain and spinal cord in mice lacking Robo1 and Robo2 (Bai et al., 2011; Kim et al., 2017; Gruner et al., 2019), and loss of Robo-dependent silencing of DCC-mediated floor plate attraction had been invoked as a driver of this phenotype (Bai et al., 2011). Because the timecourse of motor axon growth through the ventral commissure in *Robo1/2*$^{-/-}$ mice and its relationship to motor neuron cell body migration into the midline had not been characterized, we first examined motor neuron projections in E10.5, E11.5, and E13.5 *Robo1/2* double knockout embryos and their wild-type littermates. To this end, we stained transverse sections of brachial spinal cord with antibodies against the type III intermediate filament protein Prph, which labels axons in the peripheral nervous system, including motor axons. We found that, at E10.5, motor axons rarely enter the ventral commissure in wild-type embryos, but they are frequently observed in the midline of mice lacking Robo1 and Robo2 (Figures 3A–C); at E11.5, the incidence of motor axons in the commissure has increased in both genotypes, but it is still significantly elevated by about 2-fold in *Robo1/2*$^{-/-}$ embryos when compared to wild type (Figures 3A–C). Hence, ectopic motor axon midline crossing in *Robo1/2* knockout embryos persists longer than motor neuron cell body invasion of the commissure. By E13.5, the number of midline-crossing motor axons has returned to low, comparable levels in both wild-type and *Robo1/2* mutant animals (Figure 3A; Supplementary Figure S3). These results indicate that Robo1 and Robo2 temporarily suppress the tendency of motor axons to cross the midline, although a small number of motor neurons transiently project their axons into the ventral commissure even during normal development in wild-type embryos.

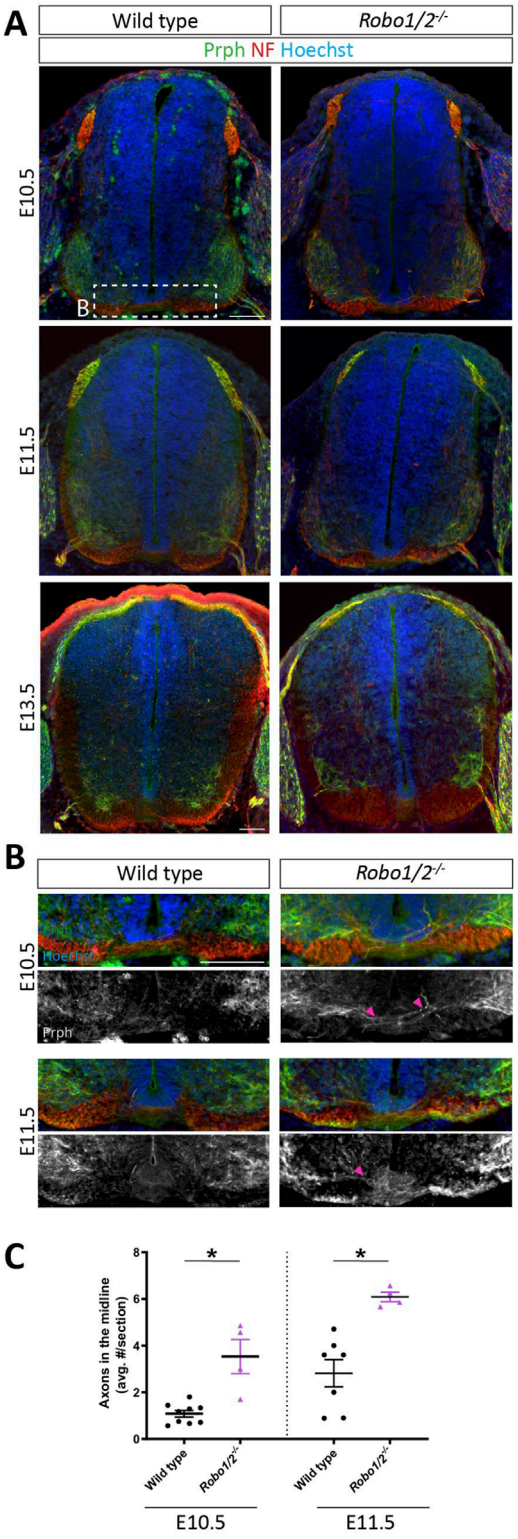

FIGURE 3
Motor axons aberrantly cross the midline in *Robo1/2*$^{-/-}$ mice. **(A)** Transverse E10.5, E11.5, and E13.5 wild-type and *Robo1/2*$^{-/-}$ mouse spinal cord sections, stained for NF and Prph. **(B)** Higher magnification views of E10.5 and E11.5 wild-type and *Robo1/2*$^{-/-}$ mouse spinal cord sections. Isolated greyscale channel shows Prph staining. Magenta arrowheads indicate Prph^{+} axons entering the midline. **(C)** Quantification of Prph^{+} axons crossing the midline of E10.5 and E11.5 wild-type and *Robo1/2*$^{-/-}$ mice shows significantly increased axon midline crossing in mutants compared to wild type at both ages (E10.5, p= 0.0056; E11.5, p= 0.0030). Data are represented as means ± SEM (n = 4–9 animals/group). Scale bar for E10.5 and E11.5 sections = 100 μm. Scale bar for E13.5 sections = 100 μm.

DCC accelerates motor axon midline crossing in *Robo1/2* mutant mice

Entry of motor axons into the ventral commissure of *Robo1/2* double knockout mice could be caused by DCC-mediated attraction to floor plate-derived Netrin-1, which is normally either balanced by Robo-mediated repulsion from midline Slits or directly silenced by Robo-DCC inhibitory crosstalk (Bai et al., 2011; Kim et al., 2017). Alternatively, loss of Robo-dependent midline repulsion alone might explain the *Robo1/2* knockout motor axon phenotype without a contribution of DCC signaling, as is the case for motor neuron cell body migration into the midline. To distinguish between these possibilities, we analyzed motor axon crossing of the ventral commissure in *DCC*$^{-/-}$*; Robo1/2*$^{-/-}$ embryos and their wild-type, *DCC*$^{-/-}$, and *Robo1/2*$^{-/-}$ littermates. We found that, at both E10.5 and E11.5, the number of midline-crossing motor axons in *DCC*$^{-/-}$ mice is similar to wild type (Figures 4A–C), indicating that DCC is not required for the low level of axon midline entry observed in wild-type embryos. The amount of motor axon midline crossing in *DCC*$^{-/-}$*; Robo1/2*$^{-/-}$ mice at E10.5 is significantly reduced when compared to *Robo1/2* double knockouts, and it is indistinguishable from wild-type and *DCC*$^{-/-}$ mice (Figures 4A, C), indicating a full phenotypic rescue. Thus, DCC is required for aberrant motor axon midline entry in *Robo1/2* mutant mice at E10.5. At E11.5, however, the severity of the phenotype in mice lacking DCC, Robo1, and Robo2 is similar to *Robo1/2* mutant mice (Figures 4B, C), and, at E13.5, the number of motor axons projecting through the ventral commissure is comparable across all genotypes (Figure 4C; Supplementary Figure S3). Hence, DCC is a major driver of early, but not late, motor axon midline crossing in *Robo1/2* knockout mice. Together, these results indicate that DCC signaling accelerates motor axon midline entry in the absence of Robo1 and Robo2, but it is not strictly required for motor axons to cross the commissure.

Slit2 converts Netrin-1-mediated motor axon attraction to repulsion

The phenotypic rescue of aberrant motor axon midline crossing by loss of DCC in *Robo1/2* knockout mice, albeit transient, reveals an antagonistic relationship between Netrin-DCC and Slit-Robo signaling in motor axon guidance relative to the midline. This could mean that Netrin-mediated attraction and Slit-dependent repulsion by the floor plate act in parallel, with motor axons passively integrating these opposing signals, or it could indicate that Slits actively suppress motor axon attraction to Netrin via hierarchical crosstalk between the signaling pathways, as previously proposed (Bai et al., 2011). To directly study the effects of these guidance cues and their possible crosstalk on motor axons, we examined

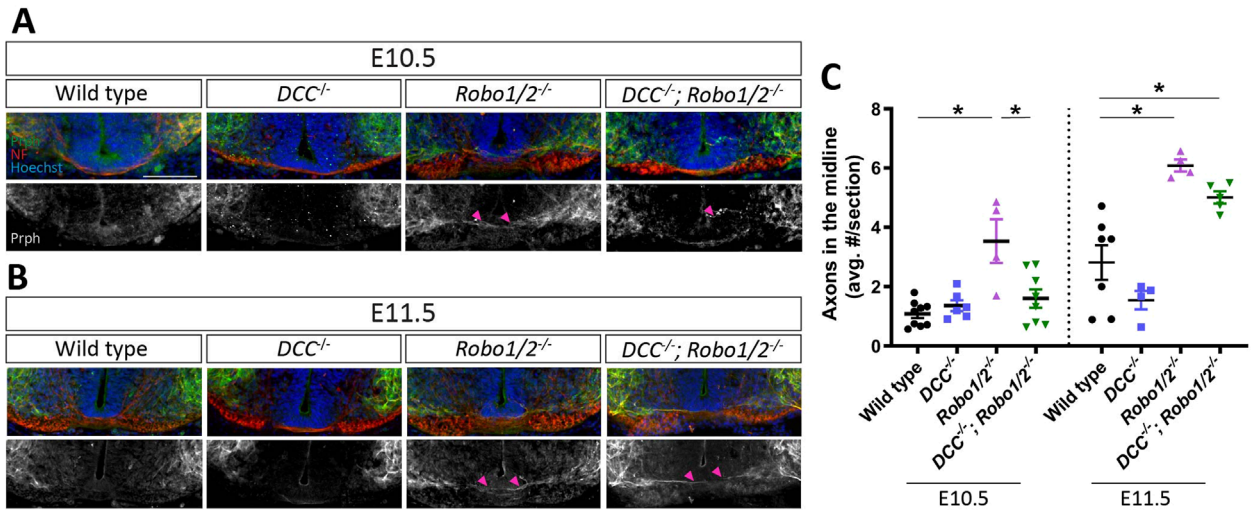

FIGURE 4
DCC mediates motor axon midline crossing in *Robo1/2*$^{-/-}$ mice at E10.5, but not E11.5. **(A, B)** Transverse E10.5 **(A)** and E11.5 **(B)** wild-type, *DCC*$^{-/-}$, *Robo1/2*$^{-/-}$, and *DCC*$^{-/-}$; *Robo1/2*$^{-/-}$ embryo sections, stained for NF and Prph. Isolated Prph channel is shown in greyscale. Magenta arrowheads indicate motor axons crossing into the midline. **(C)** Quantification of Prph^{+} axons crossing the midline of E10.5 *Robo1/2*$^{-/-}$ mice shows that aberrant midline crossing is significantly higher compared to wild-type (n = 4–8 animals/group, $p = 0.0002$) and *DCC*$^{-/-}$; *Robo1/2*$^{-/-}$ mice (n = 4–8 animals/group, $p = 0.0022$). E11.5 *Robo1/2*$^{-/-}$ mice also have higher numbers of midline-crossing motor axons compared to wild-type (n = 4–7 animals/group, $p = 0.0005$), but not to *DCC*$^{-/-}$; *Robo1/2*$^{-/-}$ mice (n = 4-5 animals/group, $p = 0.1366$). Additionally, E11.5 *DCC*$^{-/-}$; *Robo1/2*$^{-/-}$ mice have significantly more midline-crossing motor axons compared to wild type (n = 5–7 animals/group, $p = 0.0063$). Data are represented as means ± SEM. Scale bar = 100 μm.

the responses of E10.5 motor neurons to gradients of Netrin-1 and Slit2 by live imaging in Dunn chamber axon turning assays (Yam et al., 2009). We used the N-terminal, Robo-binding fragment of Slit2 (Nguyen Ba-Charvet et al., 2001) for these experiments, as Slit2 is prominently expressed by both floor plate and motor neurons at the time when Robo1 and Robo2 prevent ectopic motor axon midline crossing (Brose et al., 1999; Jaworski and Tessier-Lavigne, 2012). First, we established dose-response relationships for each cue. We found that Netrin-1 elicits motor axon attraction, as indicated by axon turning towards the high end of the protein gradient [producing positive turning angles], at peak concentrations of 250 ng/mL and above (Figures 5A, B). Slit2, on the other hand, appeared to repel motor axons at peak concentrations of 100 ng/mL and above, although this effect did not quite reach statistical significance (Figures 5C, D). These results support the idea that floor plate Netrin-1 and Slits can elicit midline attraction and repulsion, respectively, in motor axons.

To determine whether Slits can directly silence, rather than just balance, the attractive effect of Netrin-1 on motor axons, we exposed motor neurons to a gradient of Netrin-1 (peak concentration of 250 ng/mL), either in the presence or absence of Slit2; here, Slit2 was not presented as a gradient but instead uniformly added to the media at 1 μg/mL, a concentration that likely far exceeds the threshold needed for Slit-mediated repulsion and should provide an excess of Slit even under full saturation of available Robo receptors. As expected, we again observed motor axon attraction to Netrin-1 (Figures 5E, F; Supplementary Figure S4) and found that bath-applied Slit2 on its own does not induce axon turning (Figures 5E, F; Supplementary Figure S4). Surprisingly, however, simultaneous exposure to a Netrin-1 gradient and evenly distributed Slit2 not only abolishes motor axon attraction to Netrin-1 but causes strong turning away from the source of Netrin-1 (Figures 5E, F; Supplementary Figure S4). Thus, Slit2 can convert Netrin-1's attractive effect on motor axons to repulsion. This result is consistent with the idea that loss of Robo1 and Robo2 *in vivo*, while reducing or eliminating Slit-mediated midline repulsion, causes motor axons to gain attraction to floor plate-derived Netrin-1. It also suggests that wild-type motor axons might be repelled by Netrin-1 *in vivo*, as long as they are exposed to sufficient amounts of Slit proteins.

Discussion

During development, spinal motor neurons extend axons to targets in the body periphery while their cell bodies remain anchored in the ventral horn of the spinal cord. A multiplicity of factors allows motor axons to leave the central nervous system, including mechanisms that prevent these axons from being attracted to inappropriate targets within the spinal cord (Suter and Jaworski, 2019). Previous work had demonstrated that genetic inactivation of the Slit receptors Robo1 and Robo2 in mice causes a subset of motor axons to remain within the central nervous system and extend across the floor plate at the ventral midline; similarly, motor neuron cell bodies leave the ventral horn and enter the commissure in *Robo1/2* double knockout mice (Bai et al., 2011; Kim et al., 2015; Kim et al., 2017; Gruner et al., 2019). To what extent these phenotypes are driven by loss of Slit-mediated repulsion from the floor plate or gain of responsiveness to the midline attractant Netrin-1 had remained unclear. We provide evidence that motor

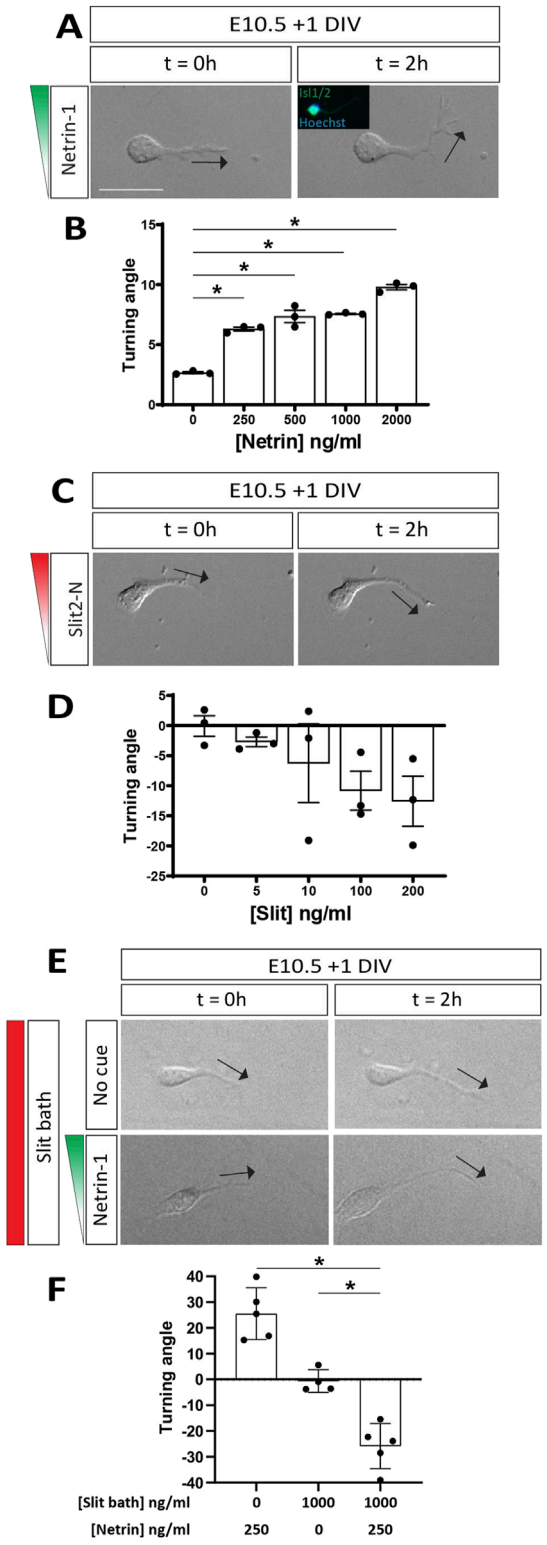

FIGURE 5
Slit2 converts motor axon attraction by Netrin-1 to repulsion. **(A)** DIC images of E10.5 + 1 DIV motor neurons exposed to a Netrin-1 gradient (250 ng/mL) in a Dunn chamber (t = 0 and 2 h). Direction of axon tip at 0 and 2 h indicated by black arrows. Post-hoc immunofluorescent staining for Isl1/2 confirmed molecular identity of analyzed neurons (inset). Axons turn towards the Netrin-1 gradient. **(B)** Quantification of axon turning angles in response to Netrin-1 gradient shows positive turning angles, indicating attraction, at all tested concentrations of Netrin-1 (n = 3 independent experiments, comparison to control: 250 ng/mL, $p< 0.0001$; 500 ng/mL, $p< 0.0001$; 1,000 ng/mL, $p< 0.0001$; 2000 ng/mL, $p< 0.0001$). DIC images of E10.5 + 1 DIV motor neurons exposed to a Slit2-N gradient (100 ng/mL) in a Dunn chamber (t = 0 and 2 h). **(D)** Quantification of turning angles in response to Slit-2N gradients shows negative turning angles, indicating repulsion, albeit not statistically significant (n = 3 independent experiments, comparison to control: 5 ng/mL, $p= 0.6407$; 10 ng/mL, $p= 0.4848$; 100 ng/mL $p= 0.2130$; 200 ng/mL, $p= 0.1662$). **(E)** DIC images of E10.5 + 1 DIV motor neurons exposed to no cue (top panels) or a 250 ng/mL Netrin-1 gradient (bottom panels) with simultaneous bath application of Slit2-N (1,000 ng/mL) in a Dunn chamber (t = 0 and 2 h). Motor axons are repelled by Netrin-1 in the presence of Slit2-N. **(F)** Motor axons exposed to the Netrin-1 gradient in the presence of Slit2-N are strongly repelled, as opposed to the attraction observed in Netrin-1 gradient alone (n = 5 individual experiments, $p< 0.0001$). Additionally, motor axons exposed to Slit-2N (1,000 ng/mL) by bath application alone do not experience attraction or repulsion (n = 4-5 individual experiments; comparison against Netrin-1 gradient and Slit2-N bath: $p= 0.0014$). Data are represented as means ± SEM. Scale bar = 50 μm.

neuron entry into the midline is prevented by repulsion via Slit-Robo signaling and not influenced by DCC-mediated attraction to Netrin-1, whereas motor axon crossing of the ventral commissure in *Robo1/2* mutant mice results from an imbalance between Slit-mediated midline repulsion and Netrin-1 attraction. *In vitro* results indicate that Slits not only repel motor axons but can convert axonal responses to Netrin-1 from attraction to repulsion. These findings support the idea that Robo signaling allows motor axons to avoid the midline through two mechanisms: (1) by directly mediating repulsion from Slits and (2) by preventing DCC-mediated attraction to Netrin-1 and favoring repulsion from this cue.

Robos prevent motor neuron midline entry, which is independent of DCC

Newly generated motor neurons migrate from their birthplace in the ventricular zone into the ventral horn, where they organize into functionally specialized columns and pools through adhesion-driven clustering (Demireva et al., 2011). Several mechanisms ensure that motor neuron cell bodies do not overshoot their settling position and follow their axons into the periphery, including inhibitory interactions with boundary cap cells (Vermeren et al., 2003), perineurial glia (Kucenas et al., 2008; Clark et al., 2014), and radial glia endfeet (Lee and Song, 2013) at MEPs, anchoring by the cell adhesion molecule TAG-1 (Suter et al., 2020), and signaling by secreted Semaphorins and Slits (Lee et al., 2015). Further, dorso-ventral positioning of motor neurons in the spinal cord is influenced by Slit-Robo and Netrin-DCC signaling, as they shift dorsally in *Netrin-1* and *DCC* mutant mice and ventrally in mice lacking Robo1 and Robo2 or all three Slits (Kim et al., 2015). Possibly as a consequence of these phenotypes, MEPs move dorsally or ventrally along with motor neuron cell bodies in these mutants, and these MEP shifts cancel each other out in *Netrin-1/Robo1/Robo2* triple mutants (Kim et al., 2017). Motor neuron positioning, however, in mice with simultaneous disruption of Robo and DCC signaling had not been directly assessed.

We focused on the ventral shifting of motor neurons in *Robo1/2* mutants, specifically the extreme case where motor neurons aberrantly enter the ventral midline. Consistent with previous work indicating that the severity of this phenotype declines with age (Kim et al., 2015), we find that it completely resolves itself between E10.5 and E11.5. We also show that motor neurons that enter the midline are exclusively of MMC identity, likely owing to their proximity to the floor plate, and that the total number of motor neurons in the spinal cord is comparable between wild-type and *Robo1/2*$^{-/-}$ mice. These results argue that, in the absence of Slit-Robo signaling, motor neurons are drawn into the midline by the floor plate, rather than being pushed by overcrowding in the ventral horn. As previously reported (Kim et al., 2015), the number of mispositioned neurons at E10.5 constitutes a very small fraction (<1%) of all motor neurons in the spinal cord. It remains unclear whether the birthdate, molecular profile, or migratory path into the ventral horn underlies the selective vulnerability of certain MMC neurons to aberrant midline entry. Interestingly, we find that motor neurons still enter the midline in *DCC*$^{-/-}$; *Robo1/2*$^{-/-}$ mice, where Netrin-1-mediated floor plate attraction through DCC is abolished. This suggests that floor plate repulsion of motor neurons by Slit-Robo signaling prevents cell body entry into the midline by balancing other attractive signals from the floor plate. The alternative Netrin receptor DSCAM (Ly et al., 2008) might also contribute to motor neuron midline attraction, although the functional importance of this molecule for Netrin signaling in the mouse spinal cord is still unclear (Palmesino et al., 2012). It will also be interesting to understand the transient nature of the *Robo1/2* knockout phenotype, as it implies that motor neurons that enter the commissure undergo cell death, lose expression of motor neuron markers, or exit the commissure to join motor neurons on either side of the midline.

Robo and DCC signaling have opposing effects on motor axon avoidance of the midline

In order to reach their peripheral targets, motor axons first need to leave the spinal cord via MEPs. Motor axons accomplish this feat by responding to peripherally expressed attractants, such as collagen XVIII (Schneider and Granato, 2006) and the chemokine CXCL12 (Lieberam et al., 2005). At the same time, they have to avoid navigating towards inappropriate targets within the central nervous system. Slits are produced by the floor plate and have previously been shown to act as repellants for motor axons *in vitro* (Brose et al., 1999), and deletion of *Robo1* and *Robo2* causes motor neurons to project axons across the midline (Bai et al., 2011; Kim et al., 2017; Gruner et al., 2019), supporting the idea that repulsion from the floor plate via Slit-Robo signaling is required to prevent motor axons from crossing the ventral commissure. It had remained unclear whether motor axon and cell body entry into the midline of *Robo1/2* knockout mice are interdependent and to what extent DCC-mediated attraction to Netrin-1 contributes to aberrant axon crossing of the midline in these mice.

We find that, similar to the cell body positioning defect, only a small subset (≈1-2%) of all motor neurons project axons across the midline in *Robo1/2*$^{-/-}$ mice; however, motor axons persist in the ventral commissure longer than motor neuron cell bodies, indicating that the axon guidance phenotype is not strictly dependent on neuronal mispositioning. The axonal defect does eventually resolve by E13.5, consistent with the idea that misprojecting axons are pruned. Through our analysis of *DCC*$^{-/-}$; *Robo1/2*$^{-/-}$ triple knockout mice, we discovered that, at E10.5, DCC is a strong driver of aberrant motor axon midline crossing when Robo1/2 signaling is abolished, but this does not hold true at E11.5. These finding argue that, initially, Slit-Robo1/2 signaling is primarily required to counteract Netrin-1-dependent motor axon attraction to the floor plate, but, later on, Slit-mediated midline repulsion needs to balance out the attractive effects of other, yet-to-be-identified midline-derived factors. The possibly redundant contributions of Netrin-1 and other floor plate molecules to motor axon midline attraction at E11.5 remain to be determined, but increased responsiveness to these attractants might define the small subset of motor neurons that aberrantly project axons through the commissure in *Robo1/2*$^{-/-}$ mice. Of note, the partial requirement of DCC for motor axon, but not motor neuron, midline crossing in *Robo1/2* knockouts further underscores the independence of these two defects, and it suggests that Netrin-DCC signaling selectively attracts extending motor axons without influencing migrating cell bodies. This might indicate that DCC expression in motor neurons is low until they have settled in the ventral horn and begin to grow axons, or it could be explained by differential deployment and signaling activity of DCC in the axonal and cell body compartments.

Crosstalk between Slit-Robo and Netrin-DCC signaling

The antagonistic relationship between DCC and Robo1/2 in motor axon midline crossing at E10.5 could indicate a balancing act between attraction and repulsion by floor plate-derived Netrin-1 and Slits, respectively; we will refer to this as the balancing model. However, it is also possible that Slit signaling suppresses motor axon attraction to Netrin-1 through Robo-DCC crosstalk, without playing a major direct role in midline repulsion, which we will refer to as the silencing model.

In line with published work (Brose et al., 1999), we found that Slit2 can repel motor axons. While function-blocking experiments using the Robo1 ectodomain *in vitro* had provided evidence against Slits being dominant drivers of motor axon repulsion from the floor plate (Patel et al., 2001), our E11.5 *in vivo* results, where DCC does not promote motor axon midline crossing and the silencing model is excluded, are most readily explained by Robo-dependent motor axon repulsion from midline-derived Slits. This apparent discrepancy might be due to incomplete blocking of Slit activity *in vitro* or higher sensitivity of motor axons to Slits *in vivo*. No matter the explanation, the balancing model, which requires Slit-dependent midline repulsion, could therefore also apply at E10.5. Nonetheless, prior evidence for the silencing model is strong, and our data provide further support. DCC and Robos physically interact, and genetic deletion of the γ-secretase component Presenilin-1 (PS1), which leads to accumulation of intracellular DCC "stubs" that cannot bind Robos and are thought to circumvent silencing, causes motor

axon midline crossing; this phenotype is rescued when *DCC* is knocked out, indicating that DCC drives ectopic midline crossing in mice lacking PS1 (Bai et al., 2011). For the silencing model to remain viable, the *Robo1/2* knockout phenotype had to be similarly DCC-dependent, and this is exactly what we find, at least at E10.5. Further, motor neuron explant experiments at early developmental stages had shown that motor axons fail to grow towards Netrin-1-expressing cells (Varela-Echavarría et al., 1997), but blocking Slit signaling with the Robo1 ectodomain in ventral spinal cord explants allows motor axon attraction by Netrin-1 (Bai et al., 2011). This is readily explained by the fact that motor neurons themselves secrete Slits for autocrine signaling (Jaworski and Tessier-Lavigne, 2012), and motor neuron- and floor plate-derived Slits could therefore both contribute to Netrin silencing. In our axon turning assays, due to the absence of floor plate and the low density of neurons, endogenous Slit levels are likely to be very low, explaining why we observe robust attraction to Netrin-1. This allowed us to directly test whether addition of Slits changes motor axon responses to Netrin, and we found that high concentrations of Slit2 convert Netrin-1 attraction to repulsion. This is the inverse of the Netrin-Slit crosstalk observed in thalamocortical axons, where Netrin-1 can convert repulsive effects of Slit1 to attraction (Bielle et al., 2011). It is possible that, *in vivo*, motor axons respond to Netrin-1 on a continuum that ranges from attraction to repulsion, depending on the level of Slits they are experiencing at any given moment; the range of Slit concentrations that can flip the valence of Netrin chemotactic signaling, as well as the local, physiologically relevant Slit concentrations that extending motor axons are exposed to *in vivo*, remain to be determined. While the mechanism of the observed Slit-Netrin crosstalk remains elusive, it could involve direct binding between the ligands (Brose et al., 1999) and/or their receptors (Bai et al., 2011), or the intersection of downstream signaling pathways. Irrespective of the molecular mechanism, our data are consistent with the idea that Slit-Robo signaling suppresses, or even inverts, attractive motor axon responses to midline-derived Netrin-1, which is a variation of the silencing model. At E10.5, this mechanism might act alone or in parallel to Robo-mediated repulsion from midline-derived Slits to help motor axons steer clear of the floor plate.

At E11.5, DCC is no longer a major contributor to aberrant motor axon midline crossing in *Robo1/2* mutant mice. While this strongly argues for Slit-dependent repulsion becoming the predominant mechanism for Robo function in this context, it also raises the question why DCC silencing by Robos, provided it operates at E10.5, is less important at this age. Interestingly, an intracellular p190RhoGAP-dependent mechanism for inhibiting motor axon attraction to Netrin-1 has been found to prevent motor axon misrouting along the pial surface of the spinal cord (Bonanomi et al., 2019). While disruption of this pathway alone does not cause motor axon midline crossing (Bonanomi et al., 2019), it remains possible that it helps dampen attraction to floor plate-derived Netrin-1, partially relieving Robo1 and Robo2 of this responsibility. Ultimately, our data support roles for Robos in both Slit-mediated midline repulsion and the modulation of Netrin-1 responses in motor axon avoidance of the midline, but the precise relative contributions of these mechanisms, as well as other Netrin silencing mechanisms, at different developmental stages remain to be resolved.

Data availability statement

The original contributions presented in the study are included in the article/Supplementary Material, further inquiries can be directed to the corresponding author.

Ethics statement

The animal study was approved by Brown University's Institutional Animal Care and Use Committee. The study was conducted in accordance with the local legislation and institutional requirements.

Author contributions

KN: Conceptualization, Data curation, Formal Analysis, Funding acquisition, Investigation, Methodology, Project administration, Validation, Visualization, Writing – original draft, Writing – review and editing. FS: Conceptualization, Data curation, Formal Analysis, Investigation, Methodology, Project administration, Validation, Visualization, Writing – original draft, Writing – review and editing. YZ: Data curation, Investigation, Methodology, Validation, Visualization, Writing – original draft. AJ: Conceptualization, Funding acquisition, Methodology, Project administration, Resources, Supervision, Visualization, Writing – original draft, Writing – review and editing.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Generative AI statement

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Publisher's note

Supplementary material

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SUPPLEMENTARY FIGURE S1
Total number of motor neurons in E10.5 wild-type and Robo1/2$^{-/-}$ mice. The total number of motor neurons per hemisection was quantified. There is no significant difference between E10.5 wild-type and Robo1/2$^{-/-}$ mice (n = 5–8 animals/group, p = 0.0723). Data are represented as means ± SEM.

SUPPLEMENTARY FIGURE S2
Mispositioned motor neurons are not observed beyond E10.5. **(A)** E11.5 and E13.5 wild-type, DCC$^{-/-}$, Robo1/2$^{-/-}$, and DCC$^{-/-}$; Robo1/2$^{-/-}$ mouse spinal cord sections were stained for Isl1/2 and Tuj1. Isolated Isl1/2 channel is shown in greyscale. **(B)** Quantification of mispositioned motor neurons in E11.5 and E13.5 wild-type, DCC$^{-/-}$, Robo1/2$^{-/-}$, and DCC$^{-/-}$; Robo1/2$^{-/-}$ mice shows no differences in any mutant genotype compared to age-matched wild type. Data are represented as means ± SEM (n = 3–5 animals/group). Scale bar = 100 μm.

SUPPLEMENTARY FIGURE S3
Motor axons do not aberrantly cross the midline at E13.5. **(A)** E13.5 wild-type, *DCC*$^{-/-}$, *Robo1/2*$^{-/-}$, and *DCC*$^{-/-}$*; Robo1/2*$^{-/-}$ sections were stained for NF and Prph. **(B)** Quantification of Prph^{+} motor axons crossing the midline showed that the previously shown phenotype (Figure 3) is not observed in any mutant genotype or wild type at E13.5. Data are represented as means ± SEM (n = 3–4 animals/group). Scale bar = 100 μm.

SUPPLEMENTARY FIGURE S4
Distribution of individual axon turning angles in Dunn chambers. Quantification of individual axon turning angles measured in Dunn chambers shows spread of data points through attraction (positive angles) and repulsion (negative angles). Data points are color-coded, identifying origin of individual experiments for the conditions. Data are represented as means ± SEM.

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Osteocalcin and GPR158: linking bone and brain function

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Osteocalcin (OCN), a small protein secreted by osteoblasts, has attracted significant attention for its role as an endocrine factor in regulating the central nervous system (CNS) via the bone-brain axis. As a critical receptor for OCN, G protein-coupled receptor 158 (GPR158) facilitates the proliferation, differentiation, and survival of neural cells while directly influencing neurons' structural and functional plasticity, thereby modulating cognitive function. Additionally, GPR158 is involved in cellular energy metabolism and interacts with proteins such as regulators of G protein signaling 7 (RGS7), broadening the understanding of OCN's impact on neural activity. Notably, GPR158 displays region- and cell type-specific bidirectional effects under certain pathological conditions, such as tumor development and mood regulation, adding complexity to its mechanisms of action. Although the precise biological mechanisms underlying the OCN/GPR158 signaling pathway remain incompletely understood, its association with neurodegenerative diseases (NDs), including Alzheimer's disease (AD) and Parkinson's disease (PD), is becoming increasingly evident. Thus, a systematic summary of OCN/GPR158 in CNS regulation and NDs will deepen understanding of its role in brain function and support the development of new therapeutic targets and strategies.

KEYWORDS

osteocalcin, GPR158, neurodegenerative diseases, cellular activity, synaptic plasticity, metabolism

1 Introduction

With the expanding recognition of interorgan crosstalk, such as the liver-brain, muscle-brain, and gut-brain axes, research on biomolecules influencing neurodegenerative diseases (NDs) has transcended traditional boundaries. However, interactions between peripheral organs and the central nervous system (CNS), mainly via the bone-brain axis, remain comparatively underexplored. Traditionally regarded primarily as structural components facilitating support and motor, bones have recently been recognized for their broader physiological roles. Osteocalcin (OCN), a non-collagen matrix protein secreted by osteoblasts, is a critical marker of bone formation and metabolism and functions as an endocrine hormone. Upon entering the circulatory system, OCN modulates peripheral energy metabolism, insulin sensitivity, and muscle function (Ferron et al., 2008; Zhao et al., 2023; Correa Pinto Junior et al., 2024). Additionally, its emerging roles in cognition and emotion have attracted increasing scholarly attention (Oury et al., 2013).

OCN acts through receptors such as GPRC6A, GPR37, and GPR158. The peripheral effects of OCN, including the regulation of glycolipid metabolism and insulin secretion,

TABLE 1 Summary of OCN ligands, distribution, and major research areas.

Receptors	Primary expression	Major research areas
GPR158	Central: neuron-specific expression in the cerebral cortex, hippocampus, and hypothalamus Peripheral: adrenal gland, pancreas, trabecular meshwork cells	Neural plasticity and cognition (Rivagorda et al., 2025), stress response and depression (Sutton et al., 2018), endocrine and metabolic regulation (Fu et al., 2022; Lin et al., 2022), tumor progression (Fu et al., 2022; Sakellakis, 2022)
GPR37	Central: dopaminergic neurons, oligodendrocytes, astrocytes Peripheral: macrophages, smooth muscle cells, cardiomyocytes, alveolar epithelial cells	Oligodendrocyte maturation and myelination (Qian et al., 2021), Parkinson's disease (Marazziti et al., 2004; Zhang et al., 2020b), neuronal function and survival (Owino et al., 2021), inflammation (Bolinger et al., 2023), tumor progression (Xie et al., 2022; Zhou et al., 2024)
GPRC6A	Peripheral: skeletal muscle, immune cells, Leydig cells, anterior pituitary, osteoblasts, pancreatic β-cells, liver, adipose tissue	Metabolic regulation, bone-muscle axis (Sun et al., 2022), male reproduction (Karsenty and Oury, 2014; Taib and Jayusman, 2024), inflammation (Clemmensen et al., 2014), tumor progression (Pi et al., 2018)

are primarily mediated by the GPRC6A, which is exclusively expressed in peripheral tissues. In contrast, GPR37 and GPR158 are predominantly expressed in the CNS and are likely to mediate the effects of OCN on brain function. Although GPR37 supports neuronal migration, glial cell differentiation, and myelination (Bian et al., 2024), its involvement in OCN-mediated synaptic regulation appears limited. GPR37 functions primarily through glial cells and lacks direct regulatory capacity over synaptic plasticity and higher-order neural processes such as emotion and cognition (Bian et al., 2024). Conversely, GPR158 exhibits neuron-specific expression in key brain regions, including the cerebral cortex, hippocampus, and hypothalamus, and is directly involved in modulating synaptic structure and functional plasticity. It has been implicated as a central mediator in neuropsychiatric conditions such as stress, depression, and cognitive impairment. Recent studies further identify GPR158 as a critical receptor mediating OCN's regulation of central energy metabolism, a function in which GPR37 plays only a limited role (Table 1). Moreover, GPR37 activation has been associated with enhanced intracellular stress responses (Imai et al., 2001; Marazziti et al., 2009), which contrasts with the protective effects of OCN against oxidative stress (Wu et al., 2021). These functional divergences suggest that OCN's actions in the brain are not entirely dependent on GPR37, and that GPR158 may play a compensatory or complementary role in brain regions and processes beyond the scope of GPR37.

Therefore, targeting GPR158 may provide novel insights into how OCN regulates brain function and offer new directions for investigating the bone–brain axis in NDs.

2 Physiological functions of OCN in the bone and brain

2.1 OCN and bone

OCN is one of the most abundant proteins in the bone matrix and exists in two distinct forms: carboxylated osteocalcin (cOCN) and undercarboxylated osteocalcin (ucOCN). cOCN primarily contributes to bone mineralization, whereas ucOCN exerts endocrine functions and regulates various physiological processes, including bone metabolism.

2.1.1 Bone mineralization and structural adjustment

Bone formation is a highly dynamic physiological process that progresses through four sequential stages: pre-osteogenesis, matrix synthesis, mineralization, and maturation. During the transition from pre-osteogenesis to matrix synthesis, the expression of osteocalcin OCN gradually increases from a low baseline. Initially, OCN facilitates the differentiation of mesenchymal stem cells and promotes the maturation of osteoblasts (Moriishi et al., 2020). OCN is progressively incorporated into the newly synthesized extracellular matrix as the bone matrix forms, further enhancing matrix deposition and osteoblast maturation (Hosseini et al., 2019). Bone mineralization represents a critical phase that determines bone quality and mechanical strength. Although non-collagenous proteins (NCPs) are present in smaller quantities than collagen within the bone matrix, they play indispensable roles in regulating calcium ion binding, hydroxyapatite nucleation, and crystal growth. Among these, the small integrin-binding ligand N-linked glycoprotein (SIBLING) family—including dentin matrix protein 1 (DMP1), bone sialoprotein (BSP), and osteopontin (OPN)—exerts fine control over mineral deposition via specialized functional domains (Silvent et al., 2013; Vijaykumar et al., 2020). Within this regulatory network, OCN is a critical mediator linking the organic matrix to mineral components. At this stage, OCN is extensively distributed throughout the mineralized matrix and reaches its peak expression level (Xu et al., 2023). Studies have demonstrated that the molecular structure of cOCN contains γ-carboxyglutamic acid (Gla) residues, which exhibit a high binding affinity for calcium ions. Upon binding to Ca^{2+}, OCN functions as a mineralization inducer by promoting the deposition of phosphate PO_4^{3-}, ultimately facilitating hydroxyapatite formation (Tavakol et al., 2024). This

process enhances bone matrix mineralization and contributes to increased bone density.

In addition to its role in mineral deposition, OCN is crucial in optimizing the crystalline organization of bone minerals. By ensuring that mineral particles are systematically aligned along collagen fibers, OCN significantly enhances the mechanical strength of bone (Manolagas, 2020). Despite the presence of mineral deposits in bone following OCN gene knockout, the disorganized arrangement of mineral crystals results in a marked reduction in bone strength (Xu et al., 2023), highlighting the essential role of OCN in regulating bone structure and maintaining its biomechanical properties.

2.1.2 Bone remodeling

Bone remodeling is a dynamic equilibrium process that involves the coordinated regulation of bone formation and resorption. The functions of osteocalcin OCN are multifaceted. First, OCN promotes bone formation by stimulating osteoblasts to synthesize bone matrix proteins. Second, OCN influences the differentiation and activity of osteoclasts and regulates bone resorption through its interaction with specific receptors, such as GPRC6A (Wang H. et al., 2021). Additionally, OCN modulates the secretion of key regulatory factors, including transforming growth factor beta, fibroblast growth factor 23, and osteopontin, by osteoblasts. Through these mechanisms, OCN indirectly influences osteoclast activity and contributes to the regulation of bone resorption (Lee et al., 2007).

OCN not only directly regulates the activity of bone cells but also interacts with other hormones through an intricate endocrine network to collectively modulate bone metabolism. Among these hormones, testosterone is closely associated with OCN function. Studies have demonstrated a significant positive correlation between circulating OCN levels and serum testosterone concentrations (Kanazawa et al., 2013; Zhong et al., 2016). OCN enhances testosterone synthesis by upregulating key steroidogenic enzymes, including cytochrome P450 family 11 subfamily A member 1 (CYP11A1, CYP17A1), and hydroxy-delta-5-steroid dehydrogenase three beta-and steroid delta-isomerase 1 (HSD3β1 and HSD3β6), in a cyclic AMP response element-binding protein (CREB)-dependent manner. This regulatory mechanism is mediated through the binding of OCN to the GPRC6A in testicular interstitial cells, leading to a significant increase in testosterone secretion (Bharath Kumar et al., 2024) and promoting germ cell survival (Oury et al., 2011; Oury et al., 2015; Jawich et al., 2022).

Comparative studies in OCN-deficient male mice have revealed decreased sperm counts and lower circulating testosterone levels, resulting in reduced reproductive capacity (Li and Li, 2014). In addition to its role in reproductive function, testosterone exerts anabolic effects on bone metabolism by stimulating osteoblast activity, promoting bone matrix synthesis, and inhibiting osteoclast function, thereby reducing the risk of bone loss. Furthermore, testosterone undergoes aromatization to estrogen, a process that further enhances bone mineral density (Kanazawa et al., 2013; Zhong et al., 2016).

2.1.3 Osteocytic feedback regulation of OCN secretion by osteoblasts

During bone formation, portions of osteoblasts become embedded within the self-secreted bone matrix and gradually differentiate into osteocytes, thereby establishing the osteocyte network within bone tissue. Osteocytes exert regulatory feedback on osteoblast activity through the secretion of sclerostin, which binds to low-density lipoprotein receptor-related proteins 5 and 6 (LRP5/6) receptors on osteoblast membranes (Delgado-Calle and Bellido, 2022). This interaction inhibits Wnt/β-catenin signaling and downregulates the expression of OCN. Conversely, sclerostin inhibition enhances Wnt/β-catenin signaling, increasing bone formation and elevated OCN expression (Hu et al., 2024).

Osteocytes also play a central role in the regulation of osteoclastogenesis and bone resorption via the secretion of receptor activator of nuclear factor-κB ligand (RANKL) and osteoprotegerin (OPG) (Delgado-Calle and Bellido, 2022). RANKL binds to its receptor RANK on osteoclast precursors, inducing their differentiation into mature osteoclasts and promoting bone matrix resorption. The degradation of the bone matrix releases OCN into circulation, where it functions in various endocrine and paracrine signaling pathways (Wang J. S. et al., 2021). Additionally, moderate bone resorption facilitates the release of growth factors sequestered in the matrix, stimulating new bone formation and supporting the redeposition of OCN.

Both osteoblasts and osteocytes are capable of secreting fibroblast growth factor 23 (FGF23), which negatively regulates OCN synthesis indirectly by suppressing circulating levels of 1,25-dihydroxyvitamin D_3 [$1,25(OH)_2D_3$] (Zhang et al., 1997). Chronically elevated FGF23 levels, as observed in disorders such as tumor-induced osteomalacia and X-linked hypophosphatemic rickets, can lead to hypophosphatemia and impaired bone mineralization (Dallas et al., 2013). Under these conditions, OCN deposition within the bone matrix is diminished, potentially compromising its functional integration into the mineralized structure.

2.2 OCN and brain function

OCN circulates through the bloodstream and reaches various tissues and organs, exerting various endocrine hormone-like effects. Beyond its well-established role in bone metabolism, OCN is critical in regulating brain function.

2.2.1 Cognitive function

A significant positive correlation has been observed between OCN levels and cognitive function. Reduced OCN concentrations in cerebrospinal fluid have been documented in various neurodegenerative disorders, including Alzheimer's disease (AD) and Parkinson's disease (PD) (Hou et al., 2021; Liu et al., 2023). Mice deficient in OCN exhibited impaired spatial learning and memory dependent on the hippocampus (Oury et al., 2013).

Furthermore, OCN supplementation has enhanced cognitive function by reducing amyloid-beta (Aβ) accumulation and gliosis in the hippocampus and cortex. Additionally, OCN increases monoamine neurotransmitters, brain-derived neurotrophic factor (BDNF), and other synaptic plasticity-associated proteins, thereby promoting neuronal plasticity (Shan et al., 2023).

Notably, OCN regulates brain function throughout the life cycle. OCN crosses the placenta during fetal development to facilitate nervous system development, and maternal OCN deficiency has

been linked to abnormal brain development (Oury et al., 2013). In aging populations, the age-related decline in OCN levels has been associated with cognitive deterioration, while exogenous OCN supplementation has been shown to reverse age-related cognitive decline (Oury et al., 2013; Correa Pinto Junior et al., 2024).

2.2.2 Mood and stress response

Beyond its role in cognitive function, OCN is integral to mood regulation. OCN stimulates the synthesis of monoamine neurotransmitters—including serotonin (5-HT), dopamine (DA), and norepinephrine (NE)—thereby directly influencing mood states (Berger et al., 2019). OCN-deficient mice exhibit anxiety-like and depression-like behaviors, which are alleviated by exogenous OCN supplementation (Oury et al., 2013).

Furthermore, OCN is essential for acute stress responses. Notably, acute stress reactions can occur independently of adrenal gland function or even in cases of adrenal insufficiency, and these responses are closely associated with a rapid surge in circulating OCN levels (Berger et al., 2019). Research indicates that exposure to stressors results in increased OCN levels within minutes. This response is directly linked to bone activity, as osteoblasts facilitate the release of bioactive OCN via glutamate uptake (Berger et al., 2019). Unlike conventional stress responses, this mechanism operates independently of classical stress hormone pathways, such as corticosterone and catecholamines (Berger et al., 2019). These findings underscore the pivotal role of OCN in acute stress adaptation.

In conclusion, OCN is essential for maintaining bone health by contributing to bone mineralization, structural remodeling, and regulating bone metabolism by balancing bone formation and resorption. Beyond its bone functions, OCN also plays a pivotal role in brain function, primarily influencing cognition, mood regulation, and stress response (Figure 1).

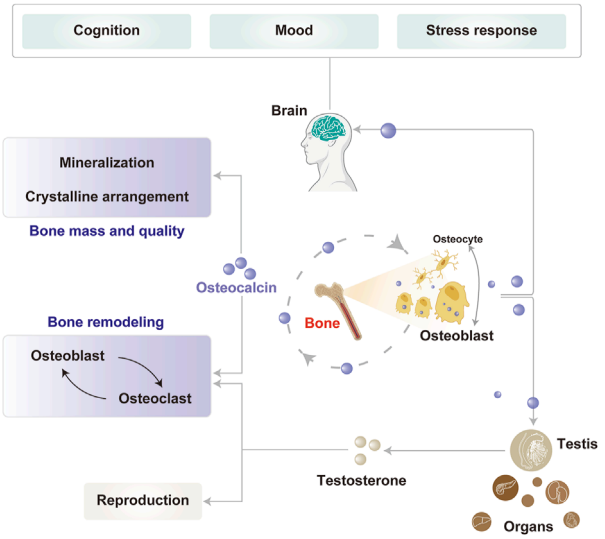

FIGURE 1
Physiological functions of OCN in the bone and brain. OCN, primarily secreted by osteoblasts, plays key roles in bone mineralization, remodeling, and reproduction. Its expression is modulated in part by osteocytes through feedback regulation. Circulating OCN also acts on the brain to influence cognition, mood, and stress responses.

3 Multiple roles of OCN/GPR158 in the CNS

Reduced expression of osteoblast markers, including OCN and osteopontin, has been observed in spinal muscular atrophy (SMA) (Shanmugarajan et al., 2009), while GPR158 knockout impairs novelty preference in autism spectrum disorders (ASD) (Wei et al., 2024). Overexpression of OCN elevates hippocampal BDNF levels, enhancing spatial learning and memory via GPR158 while also reducing anxiety, Aβ accumulation, and glial proliferation in AD (Sun et al., 2021; Shan et al., 2023). These findings establish a connection between OCN/GPR158 and bone health with NDs through their mediating roles in spatial memory and emotional regulation (Cetereisi et al., 2019). This relationship underscores the importance of further investigating the physiological mechanisms underlying the function of OCN/GPR158 in the CNS.

3.1 OCN/GPR158 regulates neuronal proliferation and cell survival

Maternal OCN crosses the placenta during pregnancy, preventing neuronal apoptosis before the embryo produces OCN autonomously, thereby supporting fetal brain development (Oury et al., 2013). Additionally, OCN at various concentrations significantly enhances the proliferation of PC12 cells, promotes neurite outgrowth, and facilitates nerve growth factor (NGF)-induced cell differentiation (Ando et al., 2021). GPR158 may mediate the promotive effects of OCN, as its knockdown suppresses the cell cycle regulator Cyclin D1 (Patel et al., 2013). Furthermore, the eighth helix of GPR158 is an α-helical region containing a nuclear localization signal (NLS). Mutations in this region result in the loss of GPR158-mediated pro-proliferative effects (Patel et al., 2013), indicating that nuclear localization of GPR158 is critical for its pro-proliferative function. Additionally, GPR158 negatively regulates genes associated with the unfolded protein response (UPR) during endoplasmic reticulum stress (ERS), including heat shock protein family A (Hsp70) member 5 (HSPA5), X-box binding protein 1 (XBP1), activating transcription factor 4 (ATF4) and C/EBP homologous protein (CHOP) (Suarez et al., 2023). The alleviation of ERS concurrently contributes to the protection of cell survival (Patel et al., 2013; Itakura et al., 2019; Suarez et al., 2023).

However, the role of GPR158 in brain tumor cells appears to be multifaced. On the one hand, the overexpression of GPR158 in brain tumor stem-like cells (BTSCs) has inhibited cell proliferation and migration while promoting cell differentiation and apoptosis. Conversely, GPR158 downregulation, such as by miR-449a, directly targets its 3′UTR, promoting the proliferation, migration, and self-renewal capacity of BTSCs while inhibiting their differentiation and apoptosis (Li et al., 2018). These effects may be associated with GPR158-mediated activation of the tumor protein 53 (TP53), a transcription factor that responds to cellular stress and halts cell replication by maintaining the cell cycle at the G1/S checkpoints (Suarez et al., 2023). On the other hand, in low-grade neurodifferentiated gliomas and neuroendocrine tumors,

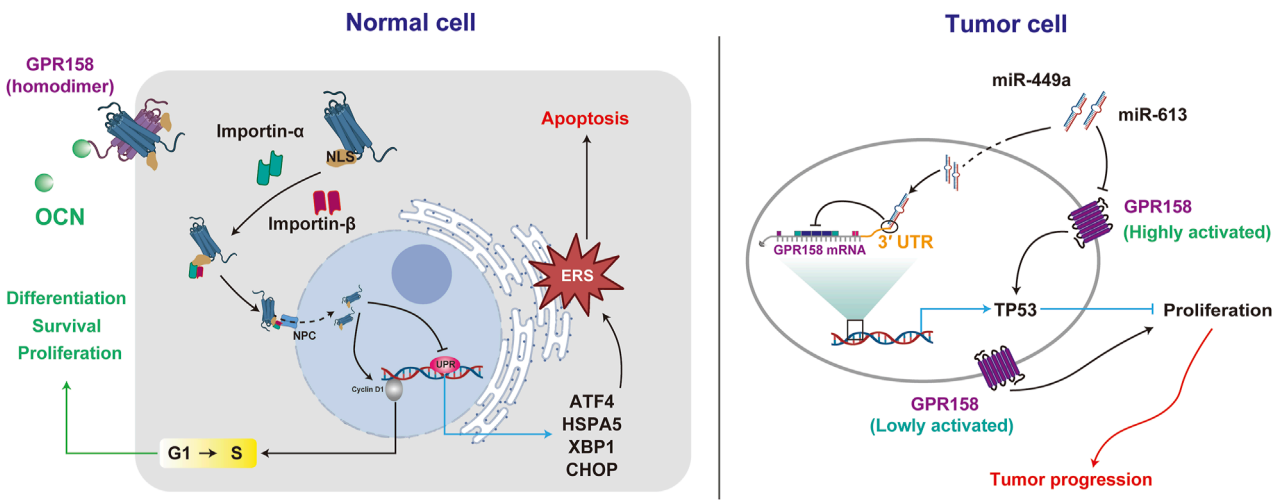

FIGURE 2
GPR158 exhibits different roles in regulating cellular activities in normal and tumor cells. GPR158 facilitates cell proliferation, differentiation, and survival in normal cells by modulating the G1/S checkpoint. Additionally, it suppresses genes associated with the unfolded protein response (UPR). In tumor cells, GPR158 demonstrates dual effects on proliferation, exhibiting context-dependent behavior under high or low activation states.

such as pheochromocytoma and paraganglioma, GPR158 is highly expressed (Wei et al., 2024). Additionally, GPR158 promotes tumor cell proliferation and angiogenesis and may be negatively regulated by miR-613 (Wang et al., 2022).

The diverse effects of GPR158 in tumor cells may stem from its spatiotemporal expression patterns and expression levels. Studies suggest that GPR158 overexpression differentially modulates UPR marker expression depending on the dosage. Notably, transient transfection of GPR158 promotes proliferation in prostate cancer cells. However, in a lentiviral stable transfection model, low doses of GPR158 enhance cell proliferation, whereas high doses exert an inhibitory effect. The bidirectional and complex nature of OCN/GPR158 may provide novel insights into preventing and treating NDs (Figure 2).

3.2 OCN/GPR158 promotes synaptic plasticity

Synaptic plasticity is the ability of synapses to undergo structural and functional modifications, forming the biological basis for learning, adaptation, and recovery in the CNS (Martin et al., 2000; Bin Ibrahim et al., 2022). This process includes both short-term plasticity, such as paired-pulse facilitation driven by presynaptic neurotransmitter release, and long-term plasticity, exemplified by long-term potentiation (LTP) and long-term depression (LTD), which entail alterations of postsynaptic receptors. Notably, OCN/GPR158 signaling is critical in modulating synaptic plasticity. Specifically, OCN supplementation enhances the action potentials (APs) frequency of cornu ammonis 3 (CA3) pyramidal neurons and promotes LTP in the mossy fiber (MF)-CA3, leading to improved hippocampal-dependent memory. The generation of APs originates from the release of neurotransmitters. OCN knockout mice show reduced NE, 5-HT, and DA levels, increased GABA, and exhibit anxiety, depressive-like behavior, and cognitive impairments. OCN supplementation enhances key neurotransmitter-synthesizing enzymes, including Glutamate Decarboxylase 1/2 (GAD1/2), Tryptophan Hydroxylase 2 (TPH2), and Tyrosine Hydroxylase (TH) (Oury et al., 2013). The plasticity changes driven by GPR158 modulation align with those observed for OCN. Activation of GPR158 markedly enhances APs frequency and reduces the threshold current necessary to elicit the initial APs (Laboute et al., 2023). In GPR158 knockout models, these enhancements are abolished, along with a marked reduction in synaptic structure and complexity in hippocampal CA1 and CA3 neurons (Condomitti et al., 2018).

Regional differences in synaptic plasticity regulation by OCN/GPR158 are evident. In GPR158−/− mice, hippocampal CA1 pyramidal neurons predominantly exhibit weakened postsynaptic functions characterized by reduced postsynaptic currents. In contrast, the CA3 region demonstrates impairments in both presynaptic and postsynaptic structures and functions, including reduced paired-pulse facilitation (PPF), shortened synaptic active zone (AZ), and postsynaptic density (PSD) lengths, as well as decreased frequency and amplitude of spontaneous excitatory postsynaptic currents (sEPSCs) (Condomitti et al., 2018). Furthermore, GPR158 demonstrates a distinct expression pattern at the cellular level, being enriched in excitatory neurons while limited in inhibitory interneurons (Chang et al., 2023). This differential expression pattern serves as the structural basis for the varying effects of GPR158 on excitatory and inhibitory neurons. In the mPFC of GPR158−/− mice, a reduction in synaptic vesicles at excitatory synapses was observed, accompanied by decreased expression and phosphorylation of GluN2B, resulting in a marked impairment of synaptic transmission. Notably, inhibitory synapses remained unaffected (Wei et al., 2024).

GPR158 modulates synaptic plasticity through multiple signaling pathways. Its activation downregulates the Kv7.2/KCNQ potassium channel via PKA and ERK pathways, decreasing M current amplitude and increasing the excitability of medium spiny

neurons (MSNs) (Aceto et al., 2024). OCN binds to GPR158, activating the IP3R and retinoblastoma-associated protein 48 (RbAp48) pathways to upregulate BDNF expression, enhance BDNF-enriched vesicle transport, and increase action potential frequency and LTP in the MF pathway, thereby improving cognitive deficits in aged mice (Khrimian et al., 2017; Kosmidis et al., 2018). Transcriptomic data from the mouse cerebral cortex reveal that GPR158 influences the expression of synaptosome-associated protein 25 (Snap25), a key component of the soluble N-ethylmaleimide-sensitive factor attachment protein receptors (SNARE) complex. Snap25 plays a critical role in coordinating calcium signaling to regulate exocytosis-endocytosis coupling. Inhibition of the Gβγ subunit signaling pathway, upon which Snap25 depends, disrupts the GPCR (G protein-coupled receptor)-SNARE interaction, leading to suppressed glutamatergic neurotransmitter release, impaired LTP, and deficits in learning and memory, accompanied by other behavioral abnormalities (Manz et al., 2023).

Contrary to the prevailing view that GPR158 promotes synaptic plasticity, GPR158 knockout enhances glutamatergic neuron plasticity in the mouse mPFC, increasing BDNF expression, dendritic spine density, sEPSCs frequency, and AMPA/NMDA ratio, leading to antidepressant and anti-stress behaviors (Sutton et al., 2018). Elevated baseline levels of GPR158 observed in the stress model may partly explain the contrasting results, as another study identified GPR158 as promoting cell proliferation at low concentrations while exerting inhibitory effects at higher concentrations (Suarez et al., 2023). Additionally, while GPR158 knockout reduced overall synaptic plasticity in the hippocampus, dendritic spine density in the apical stratum lucidum of CA3 increased by 37% compared to wild-type (WT) mice (Condomitti et al., 2018). These findings highlight the complex and context-dependent role of GPR158 in synaptic plasticity, emphasizing the need for analyses tailored to specific cell types, tissue regions, and disease models (Figure 3).

3.3 OCN/GPR158 influences central glucose metabolism to ameliorate NDs

Beyond its effects on neuronal activity, maternal OCN deficiency disrupts gene expression across multiple tissues and organs in offspring, impairing the development of pancreatic islets, testes, and other organs. These disruptions result in progressive metabolic abnormalities, including impaired insulin secretion, dysregulated glucose metabolism, and altered hepatic gluconeogenesis (Ferron et al., 2008; Ferron et al., 2012; Zhang X. L. et al., 2020; Correa Pinto Junior et al., 2024; Paracha et al., 2024). Disruption of peripheral glucose metabolism significantly impacts CNS function (Guo et al., 2020). Metabolomic analyses have revealed substantial impairments in hippocampal glucose metabolism in diabetic rats, characterized by reduced aerobic oxidation and increased reliance on glycolysis (Li et al., 2019a). These metabolic disturbances are closely associated with decreased expression of proteins critical for synaptic plasticity, alongside deficits in working memory (Li et al., 2019b). Notably, these cognitive impairments coincide with reduced serum levels of OCN (Zhao et al., 2024).

In NDs such as AD, PD, and Huntington's disease (HD), reduced OCN levels are frequently observed, often accompanied by widespread disruptions in CNS glucose metabolism. These alterations in glucose metabolism across multiple brain regions contribute to the accumulation of Aβ and tau proteins, abnormal distribution of alpha-synuclein, and motor deficits (Duan et al., 2003; Patassini et al., 2016; Scholefield et al., 2023; Shan et al., 2023). These findings suggest that OCN plays a critical role in modulating cognition associated with aging and NDs, potentially via its regulation of glucose metabolism. OCN supplementation dose-dependently improves metabolic and diabetes-related cognitive impairments (Zhao et al., 2024). The cognitive benefits of OCN are mediated through its regulation of the insulin signaling pathway, particularly the IRS/PI3K/Akt pathway (Dewanjee et al., 2022). Akt inhibition partially abolishes OCN's protective effects on cognitive deficits, underscoring OCN's critical role in regulating insulin signaling to mediate NDs (Zhao et al., 2024).

The interaction between OCN/GPR158 and cell metabolism occurs during the progression of NDs. OCN regulates circulating fasting glucose and total cholesterol levels, indirectly protecting against AD (Guo et al., 2024). Additionally, GPR158 enhances glial aerobic glycolysis, reduces Aβ accumulation, and directly improves cognitive function in AD (Shan et al., 2023). Conversely, chronic hyperglycemia can induce upregulation of the DNA-modifying enzymes (Dnmt1/3b) in the rat hippocampus, which inhibits the expression of GPR158 through epigenetic mechanisms such as methylation (Patricia da Silva et al., 2023). This alteration disrupts the bone-brain axis interactions, adversely affecting cognitive function.

3.4 Protein interaction network of OCN/GPR158

GPR158 facilitates presynaptic differentiation in CA3 pyramidal neurons through its interaction with heparan sulfate proteoglycans (HSPGs) and the coreceptor leukocyte common antigen-related (LAR) family receptors (Kamimura and Maeda, 2021). Unlike the canonical structure of GPCRs, GPR158 predominantly forms a dimer stabilized by interactions with phospholipids and cholesterol molecules. Its N-terminal region contains a distinctive Cache domain and a cysteine-rich region (Laboute et al., 2023), which endows the receptor with diverse ligand-binding capabilities and enhanced structural stability.

Though relatively short, the C-terminal region of GPR158 contains a CT-CC domain that interacts with the Regulator of G-protein Signaling 7 (RGS7)-Gβ5 complex (Laboute et al., 2023). RGS7 is broadly expressed in neurons across multiple brain regions, including the cerebral cortex, hippocampus, thalamus, basal ganglia, and cerebellum, and serves as a key modulator of GPCR signaling in the nervous system (Tayou et al., 2016; Jeong et al., 2021). As a G protein regulatory protein, RGS7 negatively regulates GPCR signaling by accelerating the GTP hydrolysis of Gi/o-class G proteins, thereby promoting their inactivation (Patil et al., 2022). Upon complex formation with RGS7-Gβ5, GPR158 translocates from the cytoplasm to the cell membrane, enabling its function in signal recognition (Orlandi et al., 2015). Under stress conditions, GPR158 enhances GTPase activity by binding to the RGS7 complex, thereby establishing a negative feedback pathway that modulates mPFC neuronal activity (Darira and Sutton, 2022). GPR158 has

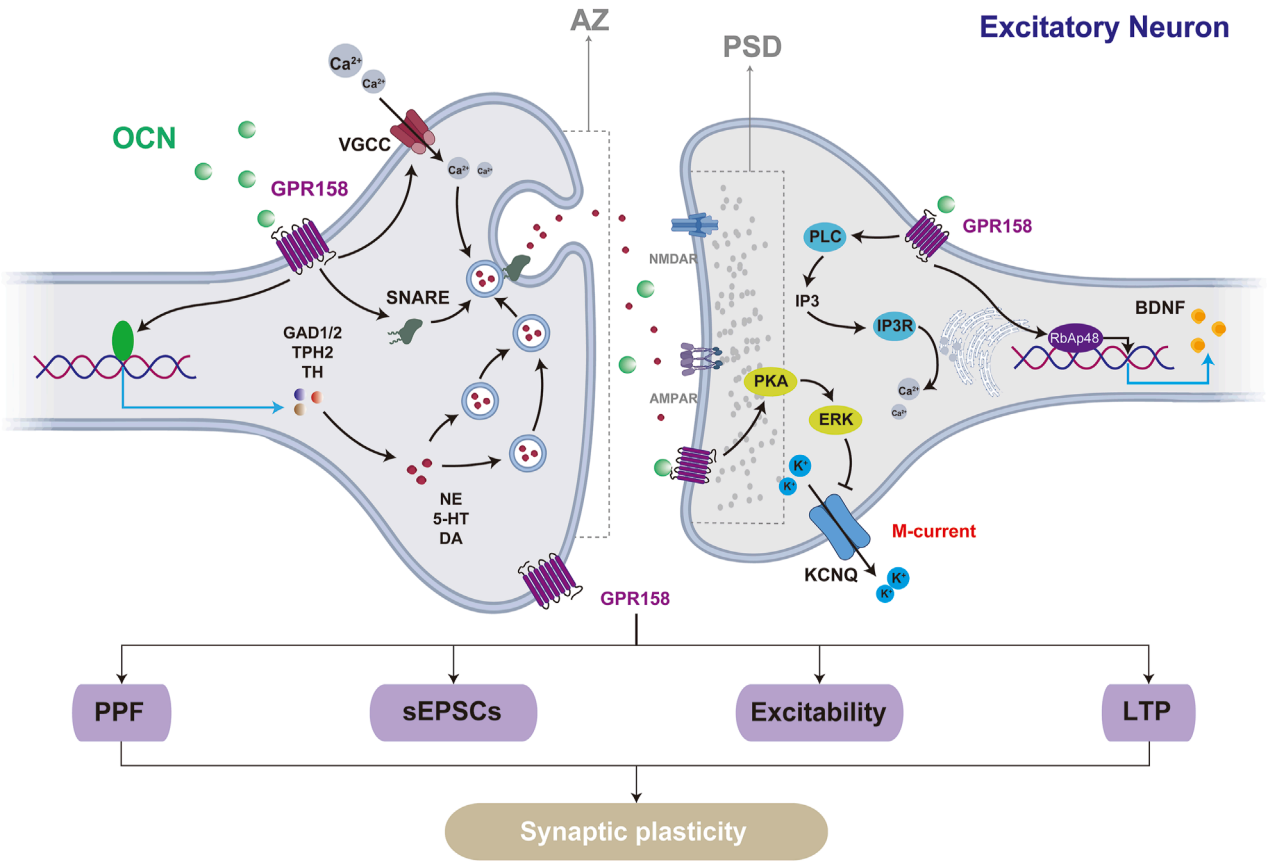

FIGURE 3
GPR158 influences synaptic plasticity in excitatory neurons through multiple mechanisms. Structurally, the activation of GPR158 enhances the lengths of both the AZ and the PSD. Functionally, GPR158 activation promotes short-term plasticity, such as PPF driven by presynaptic neurotransmitter release, and long-term plasticity, as demonstrated by LTP. The underlying mechanisms may involve GPR158-mediated inhibition of the M-current and increased expression of BDNF. VGCC, Voltage-gated calcium channels; KCNQ, Potassium voltage-gated channel, subfamily Q.

also been identified as a membrane anchor for the RGS7-Gβ5 complex, facilitating its stabilization and localization at the neuronal membrane, enhancing RGS7's regulatory efficiency on GPCR signaling (Patil et al., 2022).

RbAp48 is a pivotal regulator of chromatin organization and gene expression in the hippocampus, with its elevated expression levels strongly associated with improved cognitive performance. It has been recognized as a critical downstream effector of GPR158. Perturbations in the OCN/GPR158 signaling pathway lead to a significant reduction in RbAp48. The interaction between RbAp48 and GPR158 is fundamental for maintaining cognitive integrity, as hippocampal inhibition of RbAp48 negates the cognitive benefits mediated by OCN, resulting in pronounced deficits in discriminative memory (Kosmidis et al., 2018).

Analysis of AD samples across Braak stages reveals significant downregulation of GPR158 in the cerebral cortex, with an inverse correlation between GPR158 levels and β-secretase activity. β-secretase is a key enzyme in the amyloid precursor protein degradation pathway that generates Aβ, the primary component of amyloid plaques (Zhu et al., 2020). In PD, the pathological aggregation of α-synuclein from its monomeric form into fibrils disrupts synaptic transmission and represents a hallmark of the disease (Ruiperez et al., 2010). GPR158 suppresses α-synuclein fibril formation by interacting with high mobility group box-1 protein (HMGB1) (Mallah et al., 2019). Consequently, reduced GPR158 levels may aggravate PD pathology by facilitating α-synuclein aggregation (Mallah et al., 2019).

Therefore, the interaction between GPR158 and related proteins underscores OCN's potential role in developing NDs (Figure 4).

4 Strategies for targeting NDs via OCN/GPR158

Central neuropathies, particularly NDs, present substantial treatment challenges due to two primary factors. First, diagnosis based on behavioral phenotypes is inherently subjective and often delayed. Second, the development of therapeutics for NDs is impeded by limited advancements and significant side effects (Bian et al., 2023). Prior discussions have highlighted the neuronal alterations induced by OCN via GPR158 and their potential mechanisms in developing NDs. Consequently, modulation of OCN and GPR158 may play a pivotal role in influencing both the onset and progression of these diseases.

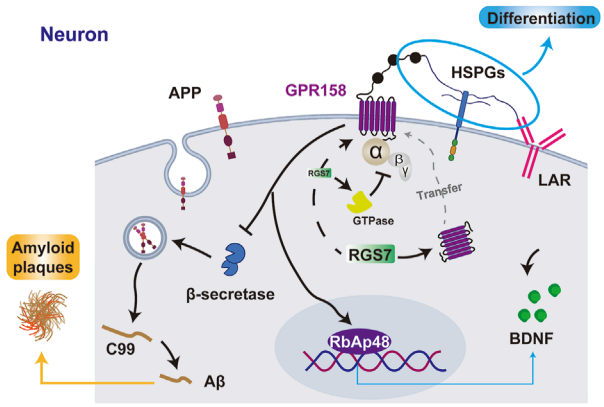

FIGURE 4
The interaction between GPR158 and related proteins. GPR158 interacts with HSPGs and LAR to promote cell differentiation, inhibits β-secretase to reduce Aβ and amyloid plaque formation, and enhances BDNF expression via RbAp48. Additionally, GPR158 binds RGS7, facilitating its membrane localization and forming a negative feedback loop by promoting GTPase activity. GPR158 also interacts with RGS7 to inhibit the GTPase. APP, amyloid precursor protein; C99, β-CTF fragment of APP.

Exercise is valued for its cost-effectiveness and neuroprotective effects. It is increasingly acknowledged as a potential therapeutic approach, partially exerting its effects through the OCN/GPR158 signaling axis.

This section aims to explore the potential of OCN as a disease biomarker and review the impact of exercise on OCN levels, thereby providing a theoretical basis for advancing the diagnosis and treatment of NDs.

4.1 OCN/GPR158 as potential risk markers for NDs

A clinical study has demonstrated a correlation between reduced OCN levels, changes in brain microstructure, and cognitive decline (Puig et al., 2016). Runt-related transcription factor 2 (RUNX2), a pivotal transcription factor regulating OCN expression, may exert its effects by directly binding to multiple recognition elements within the OCN promoter and interacting with transcriptional cofactors such as the vitamin D receptor (VDR) to enhance transcriptional activity (Paredes et al., 2004a; Paredes et al., 2004b). Notably, mutations in RUNX2 are linked to cleidocranial dysplasia, a skeletal disorder frequently accompanied by cognitive deficits, suggesting that RUNX2 and its downstream target OCN may have broader roles beyond bone development (Takenouchi et al., 2014). Furthermore, Mendelian randomization established a causal relationship between OCN and various forms of dementia, including AD, PD, Lewy body dementia (LBD), and vascular dementia (VD), with OCN exhibiting a powerful protective effect against AD (Liu et al., 2023). These findings indicate that OCN-related gene expression may be a promising early biomarker for NDs during developmental stages.

The characteristics of GPR158 regarding its brain region and cellular distribution provide a physiological basis for the observed variations in OCN. Overexpression of GPR158 inhibits the proliferation and migration of BTSCs, whereas knockdown of GPR158 enhances these processes (Li et al., 2018). In contrast, GPR158 is highly expressed in oligodendrogliomas and IDH-mutant astrocytomas (Li et al., 2018). Although these studies indicate that GPR158 may exhibit contrasting roles in different types of neurocytomas, either promoting or inhibiting tumor progression, this does not diminish the potential of the OCN/GPR158 axis as a crucial biomarker for diagnosing neurological diseases. On the contrary, it may even enhance its diagnostic sensitivity. Additionally, the post-translational modification profile of GPR158 holds promise as a potential factor associated with diseases, particularly concerning diabetes-related cognitive impairment. As discussed in Section 2.3, chronic hyperglycemia results in increased methylation of GPR158 in the rat hippocampus, adversely affecting learning and memory (Patricia da Silva et al., 2023).

The expression of GPR158 is significantly upregulated in prostate cancer, neuroendocrine tumors of the digestive tract, mucinous ovarian cancer, and various other malignancies (Fu et al., 2022). Moreover, alterations in GPR158 methylation have been observed in esophageal squamous cell carcinoma and melanoma (Oka et al., 2009; Koroknai et al., 2020; Fu et al., 2022), indicating that GPR158 may serve as a potential risk marker beyond NDs.

4.2 The impact of exercise on OCN levels

Bone functions as a significant mechanosensitive organ. The presence of mechanosensory resident cells enables mechanical stimulation to trigger metabolic responses in osteoblasts and osteoclasts, thereby promoting bone adaptation to a dynamic environment (Qin et al., 2020). Osteocytes are the primary mechanosensory in bone, capable of detecting fluid shear stress generated by mechanical loading through their extensive dendritic processes (Bonewald, 2011). Mechanical stimulation activates various mechanosensitive structures on the osteocyte membrane, including ion channels such as Piezo1, integrin complexes, and primary cilia (Qin et al., 2020; Li et al., 2025). These activations, in turn, trigger downstream signaling pathways such as Wnt/β-catenin, focal adhesion kinase (FAK), and cyclic AMP (cAMP) signaling (Bonewald and Johnson, 2008; Cuevas et al., 2023; Papaioannou et al., 2024). As described in Section 2.1.3, several of these pathways can directly or indirectly influence the OCN expression in osteoblasts.

After stimulation, bone expresses and secrete a range of osteokines (biologically active molecules secreted by bone tissue with endocrine functions), including OCN, lipocalin-2, sclerostin, Dickkopf-1, and FGF23 (Han et al., 2018). Most osteokines can traverse the blood-brain barrier, establishing the brain as an important target organ (Han et al., 2018) and influencing the development and progression of NDs.

Bone is an integral component of the motor system, constantly subjected to mechanical stress during exercise. As an economical and effective intervention for NDs, the beneficial effects of exercise may be linked to alterations in OCN levels. In recent years, studies have increasingly highlighted the impact of exercise on OCN (Table 2). While the findings are not entirely

TABLE 2 Effects of exercise on OCN levels.

Exercise duration	Exercise type	Physiological state	OCN level	Reference
Short term	Aerobic exercise	Health	-	Dror et al. (2022)
	Resistance exercise	Health	↑	Koltun et al. (2024)
Long term	Aerobic exercise	Health	↑	Bergquist (1988), Zhang et al. (2020a), Yang et al. (2021), Davidovic Cvetko et al. (2022), Adilakshmi et al. (2024), Hatakeyama et al. (2025)
		Obesity		Jamka et al. (2022)
		Obesity	-	Guzel et al. (2024)
	Resistance exercise/Endurance-strength training/Interval training	Obesity	-	Jamka et al. (2022), Kurgan et al. (2022), Salus et al. (2023)
		Health	↑	Cheng et al. (2020), Honda et al. (2020), Boudenot et al. (2021), Adilakshmi et al. (2024), Hatakeyama et al. (2025)

consistent, several key trends have emerged. First, resistance exercise seems more effective than aerobic exercise in elevating OCN levels during short-term exercise. This phenomenon could be attributed to the more substantial mechanical loading on bone cells during resistance training. Second, serum OCN levels in individuals with obesity appear to be less responsive to exercise, suggesting that individuals with metabolic disorders, such as obesity, may face more significant challenges in deriving benefits from exercise, particularly in terms of OCN regulation.

Currently, research on the effects of exercise on OCN predominantly focuses on serum analyses, with a notable paucity of studies investigating its role in the brain and its relation to GPR158. There is a critical need for rigorous evidence to identify exercise regimens that can effectively optimize OCN/GPR158-mediated pathways to enhance brain health.

5 Conclusion and perspective

As a critical receptor for OCN, GPR158 regulates cognitive function by modulating cellular activity, glucose metabolism, synaptic plasticity, and interacting with proteins. However, GPR158 has a dual role in contexts such as tumor development and anxiety/depression. Despite this complexity, it primarily supports cognitive regulation. Additionally, OCN and GPR158 are emerging as potential risk markers for NDs.

The role of GPR158 in the CNS extends beyond its current understanding, particularly in its potential involvement in immune regulation. Mutations in GPR158 have been shown to facilitate the clearance of the hepatitis C virus in patients of European and African descent, thereby reducing the risk of liver damage and related complications (Vergara et al., 2019). Furthermore, single nucleotide polymorphisms (SNPs) in GPR158 are associated with antibody levels in African Americans, and GPR158 (rs12775535) has been identified as a critical candidate gene for immune function (Ovsyannikova et al., 2012). Although the specific mechanisms require further investigation, the insights provided by these studies suggest an additional avenue for enhancing the understanding of the central mechanisms underlying OCN/GPR158.

Future studies on the role of OCN/GPR158 should focus on its multi-ligand and multi-receptor properties. GPR37, another receptor for OCN, is widely expressed in the CNS and shares similarities with GPR158 in regulating neuronal activity. Although GPRC6A is predominantly expressed in peripheral tissues, its connection to metabolic processes offers valuable insights into how GPR158 may regulate cognitive dysfunction linked to glucose metabolism. Thus, when targeting GPR158 for NDs, it is crucial to investigate its interaction with other receptors. Moreover, the ligands of GPR158 are diverse, including glycine, peptides, intracellular binding proteins, steroid hormones, glycosaminoglycans, and miRNA (Lin et al., 2022; Laboute et al., 2023; Rosenkilde and Mathiesen, 2023). This diversity adds complexity to its regulation of cognitive function but may also explain the dual role of GPR158 in different physiological and pathological contexts.

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Conflict of interest

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Glossary

5-HT	Serotonin
AD	Alzheimer's disease
APs	Action potentials
ASD	Autism spectrum disorders
ATF4	Activating transcription factor 4
AZ	Active zone
Aβ	Amyloid-beta
BDNF	Brain-derived neurotrophic factor
BSP	Bone sialoprotein
BTSCs	Brain tumor stem-like cells
CA3	Cornu ammonis 3
CHOP	C/EBP homologous protein
CNS	Central nervous system
DA	Dopamine
DMP1	Dentin matrix protein 1
ERS	Endoplasmic reticulum stress
FAK	focal adhesion kinase
FGF23	Fibroblast growth factor 23
GAD1/2	Glutamate decarboxylase 1/2
GPR158	G protein-coupled receptor 158
HD	Huntington's disease
HMGB1	High mobility group box-1 protein
HSPA5	Heat shock protein family A (Hsp70) member 5
HSPGs	Heparan sulfate proteoglycans
LAR	Leukocyte common antigen-related
LBD	Lewy body dementia
LRP5/6	Low-density lipoprotein receptor-related proteins 5 and 6
LTD	Long-term depression
LTP	Long-term potentiation
MF	Mossy fiber
MSNs	Medium spiny neurons
NDs	Neurodegenerative diseases
NE	Norepinephrine
NGF	Nerve growth factor
NLS	Nuclear localization signal
OCN	Osteocalcin
OPG	Osteoprotegerin
OPN	Osteopontin
PD	Parkinson's disease
PPF	Paired-pulse facilitation
RANKL	Receptor activator of nuclear factor-κB ligand
RGS7	Regulator of G protein signaling 7
sEPSCs	Spontaneous excitatory postsynaptic currents
SIBLING	Small integrin-binding ligand N-linked glycoprotein
SMA	Spinal muscular atrophy
Snap25	Synaptosome associated protein 25
SNARE	Soluble N-ethylmaleimide-sensitive factor attachment protein receptors
TH	Tyrosine hydroxylase
TP53	Tumor protein 53
TPH2	Tryptophan hydroxylase 2
UPR	Unfolded protein response
VD	Vascular dementia
VDR	Vitamin D receptor
WT	Wild-type
XBP1	X-box binding protein 1

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Roles of LRRK2 and its orthologs in protecting against neurodegeneration and neurodevelopmental defects

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In humans, variants in the *LRRK2* gene are the most prevalent risk factors for Parkinson's disease (PD). Whereas studies in model organisms have long indicated that the orthologs of the wild-type LRRK proteins protect against neurodegeneration, newer findings indicate that they also protect against neurodevelopmental defects. This normal role of the LRRK proteins can be disrupted by either gain-of-function (GOF) or loss-of-function (LOF) mutations, leading to neurodegeneration and neurodevelopmental defects. Here, we review the roles of the LRRK proteins and their orthologs in these processes, with a focus on autophagy as a common factor that may mediate both of these roles. We also highlight the potential for experiments in vertebrate and invertebrate model systems to synergistically inform our understanding of the role of LRRK proteins in protecting against neurological disorders.

KEYWORDS

LRRK2, Parkinson's disease, autism, intellectual disability, neurodegeneration

Introduction

Variants in the *LRRK2* gene have been associated with Parkinson's disease (PD) in humans and studies of model organisms suggest that orthologs of this gene protect against both age-related neurodegeneration and defects in neurodevelopment. For example, in mice, neurodegeneration can be caused by either a gain-of-function variant in *LRRK2* or by a double mutation that deletes both *LRRK2* and its functional homolog *LRRK1* (Dusonchet et al., 2011; Ramonet et al., 2011; Kang et al., 2024). More recently, it has become apparent that *LRRK2* and its orthologs also protect against neurodevelopmental defects. For example, gain-of-function and loss-of-function mutations in *LRRK2* cause axon guidance defects in mice (Onishi et al., 2020). Likewise, loss of function mutations in the *lrk-1* ortholog of the *LRRK* genes also causes axon guidance defects in *Caenorhabditis elegans* (Kuwahara et al., 2016; Drozd et al., 2024). These observations suggest that the normal role of the LRRK proteins (Human LRRK1, Human LRRK2, *C. elegans* LRK-1, and *Drosophila* dLRRK) is to protect against both neurodegeneration and defects in neurodevelopment. Moreover, these normal roles of the LRRK proteins can be disrupted by either gain-of-function or loss-of-function mutations. Here, we review the roles of the LRRK proteins in protecting against neurodegeneration and neurodevelopmental

defects and consider the regulation of autophagy as a common factor for both of these functions.

Overview of the LRRK2 and LRRK1 proteins

LRRK2 is a large (286 kDa), multidomain, homodimeric protein that is ubiquitously expressed, with the highest levels detected in the kidneys, lungs, and brain. As a member of the Roco protein family, LRRK2's structure includes several functional domains (Figure 1A): armadillo (ARM) repeats, ankyrin (ANK) repeats, leucine-rich repeats (LRR), a GTP-binding Ras of complex (ROC) domain coupled to C-terminal of ROC (COR), a catalytic kinase (KIN) domain, a WD40 domain, and an extended C-terminal αC-helix (Myasnikov et al., 2021). Notably, LRRK2 exhibits two enzymatic activities: a Ras-like GTPase and a kinase, a unique feature of certain Roco family proteins (Alessi and Pfeffer, 2024).

Within the Roco protein family, LRRK1 is a functional homolog of LRRK2, sharing similar LRR, ROC, COR, and kinase domains (Figure 1B) (Marin, 2008). Despite structural similarities, LRRK1 exhibits distinct mechanisms of autoinhibition/activation and physiological functions compared to LRRK2 (Metcalfe et al., 2023; Reimer et al., 2023). Autosomal recessive variants in the *LRRK1* gene that cause frameshift or truncating mutations in the C-terminal domain of the LRRK1 protein, likely lead to loss of function and are associated with osteosclerotic metaphyseal dysplasia, a severe metabolic bone disorder (Alessi and Pfeffer, 2024). Functionally, LRRK1 efficiently phosphorylates Rab7A at Ser72 but does not target Rab8A or Rab10, the primary LRRK2 substrates in cells (Malik et al., 2021).

Pathogenic variants in the LRRK2 protein can cause Parkinson's disease in humans

Mutations in the *LRRK2* gene are the most common genetic cause of familial autosomal dominant Parkinson's disease (PD), accounting for 2%–40% of cases depending on the population studied (Mata et al., 2023). Clinically, the progression of symptoms and neuropathology in patients with LRRK2-associated PD (LRRK2-PD) are indistinguishable from those observed in sporadic PD cases (Aasly et al., 2005; Healy et al., 2008). Thus, investigations of LRRK2 are thought to be a platform for understanding the molecular mechanisms that underlie all forms of Parkinson's.

Seven pathogenic missense mutations have been identified in LRRK2 (Figure 1A), located in the ROC-GTPase domain (N1347H, R1441 C/G/H), COR domain (Y1699C), and kinase domain (G2019S, I2020T). These mutations highlight the critical role of enzymatic activity in LRRK2 function. Mutations in the kinase domain (G2019S and I2020T) enhance LRRK2 kinase activity *in vitro*, while those in the ROC-COR domain (R1441 C/G/H and Y1699C) disrupt dimer stability and reduce GTPase activity (Nguyen and Moore, 2017). LRRK2 kinase phosphorylates various substrates, including a group of ~14 Rab-GTPases (LRRK2-Rabs), implicating LRRK2 in endosomal and vesicle trafficking pathways (Steger et al., 2016). All seven pathogenic mutations increase LRRK2-Rab phosphorylation, suggesting a gain-of-function mechanism through enhanced kinase activity (Steger et al., 2016).

LRRK2 GOF proteins cause age-related neurodegeneration in model organisms

Pathogenic LRRK2 missense mutations that cause increased kinase activity consistently cause axonal degeneration and neuronal cell death across various model systems. In *Drosophila*, expression of the common pathogenic LRRK2 mutant protein G2019S causes severe retinal degeneration, selective dopaminergic neuron loss, reduced climbing ability, and early mortality (Liu et al., 2008; Lin et al., 2010). Additionally, G2019S LRRK2 expression exacerbates tau-induced dendritic degeneration, microtubule fragmentation, and inclusion formation in fly neurons (Lin et al., 2010). In *C. elegans*, dopaminergic neuron-specific expression of pathogenic LRRK2 mutant proteins R1441C and G2019S induces age-dependent locomotor impairments, axonal degeneration, and dopaminergic neuronal loss (Yao et al., 2010; Cooper et al., 2015; Senchuk et al., 2021).

In mammalian models, overexpression of G2019S LRRK2 in mice using the PDGFβ promoter leads to progressive loss of dopaminergic neurons in the substantia nigra pars compacta (SNpc) by 19–20 months of age (Ramonet et al., 2011). Similarly, in rats, overexpression of G2019S LRRK2 via recombinant human adenoviral vectors (Ad5) in the nigrostriatal pathway results in progressive dopaminergic neuron loss in the SNpc (Dusonchet et al., 2011). Remarkably, neurodegenerative phenotypes associated with G2019S LRRK2 are kinase-dependent, as shown by the suppression of these phenotypes through expression of the kinase-dead mutant G2019S/K1906M or treatment with LRRK2 kinase inhibitors (Nguyen et al., 2020). Common pathological features observed in transgenic and adenoviral LRRK2 animal models include axonal abnormalities such as hyperphosphorylated tau accumulation, fragmented axons with spheroids and dystrophic neurites, increased Gallyas silver deposits, and APP-positive inclusions (Li et al., 2009; Li et al., 2010; Melrose et al., 2010; Dusonchet et al., 2011; Tsika et al., 2015; Yue et al., 2015; Nguyen et al., 2020).

LOF mutations in *LRRK* genes cause age-related neurodegeneration in model organisms

While gain-of-function mutations in LRRK2 proteins can cause axonal degeneration and neuronal death, evidence suggests that loss of LRRK proteins can also result in similar pathologies. *LRRK* loss-of-function mutations in *Drosophila* exhibit severe locomotor deficits, reduced tyrosine hydroxylase immunoreactivity, and atrophic dopaminergic neurons (Lee et al., 2007). In mice, deletion of the *LRRK2* gene alone does not cause brain phenotypes (Tong et al., 2010; Herzig et al., 2011). The lack of a pronounced brain phenotype in *LRRK2* knockout mice may be due to compensatory effects by LRRK1. Supporting this, deletion of both *LRRK1* and *LRRK2* leads to age-dependent, progressive loss of dopaminergic neurons in the SNpc and dopaminergic terminals in the striatum starting at 14 months of age (Giaime et al., 2017; Huang et al., 2022). Recently, Kang and colleagues demonstrated that specific deletion of both *LRRK1* and *LRRK2* in mouse dopaminergic neurons causes age-dependent progressive loss of SNpc dopaminergic neurons at 20–24 months of age (Kang et al., 2024). These findings underscore

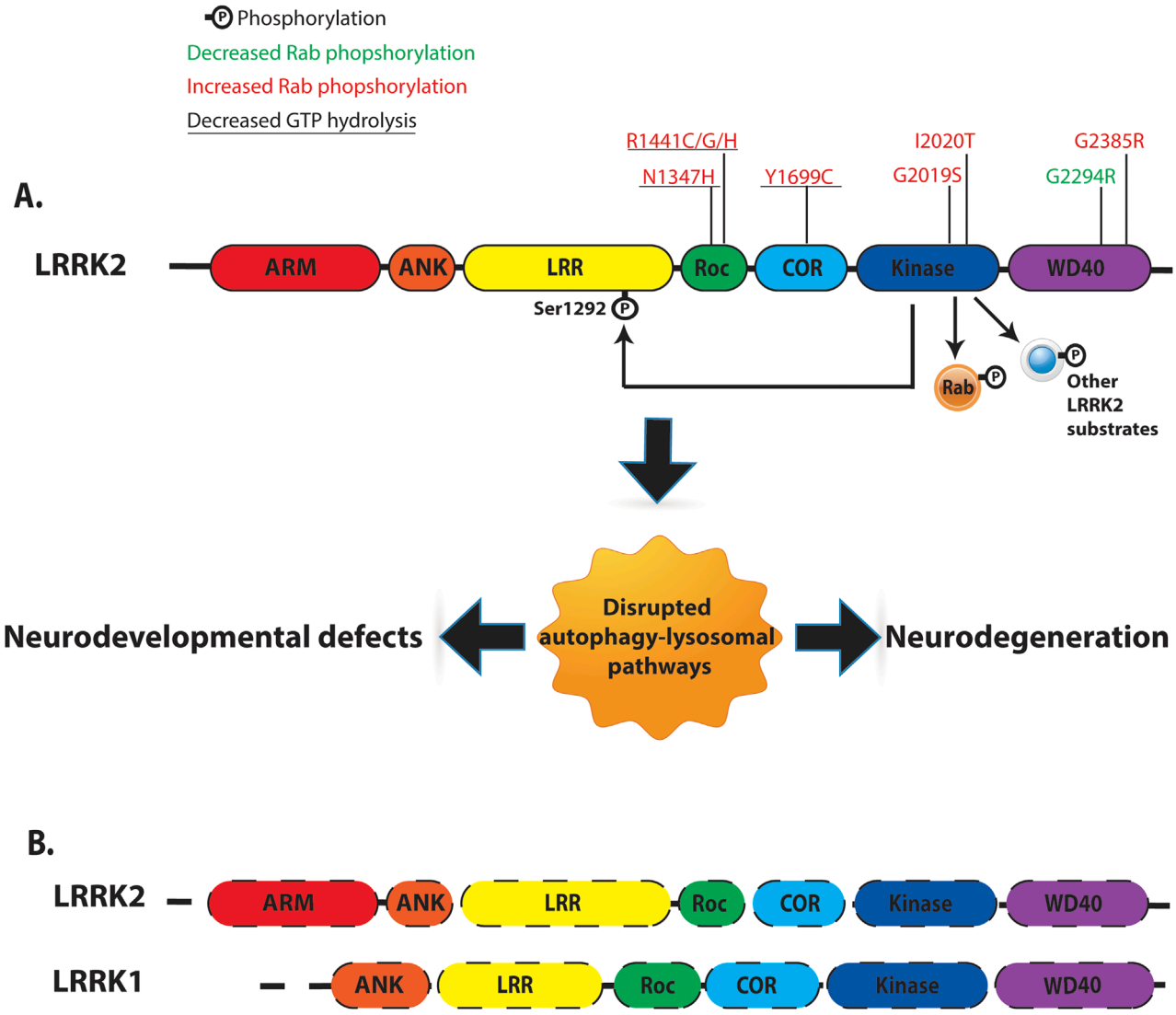

FIGURE 1
Overview of human LRRK2/LRRK1 structure and function. **(A)** LRRK2 is a multi-domain protein containing Armadillo domain (ARM, Red), Ankyrin repeat (ANK, Orange), Leucine rich repeat (LRR, Yellow), Ras-of-complex (Roc, Green), C-terminal of ROC (COR, Blue), Kinase (KIN, Navy), and WD40 (Purple). LRRK2 contains two key enzymatic domains: a Roc-GTPase domain and a kinase domain. PD-linked LRRK2 variants with reported kinase and GTPase activities were indicated. LRRK2 kinase can phosphorylate LRRK2 itself at Ser1292 residue (auto-phosphorylation). Several intracellular substrates have been identified for LRRK2 kinase, including a subset of Rab-GTPases. **(B)** Comparison of domain organization of LRRK1 and LRRK2. LRRK1 and LRRK2 contain similar domain organization: Ankyrin repeat, Leucine rich repeat (LRR), Ras-of-complex, C-terminal of ROC, Kinase and WD40 domain. Dash lines represent loss of LRRK1 and LRRK2 expression.

the critical roles of both LRRK1 and LRRK2 in maintaining dopaminergic neuron homeostasis in animal models.

Whereas LRRK family loss-of-function can cause neurodegeneration in animal models, the role of LRRK2 loss-of-function in humans remains uncertain. On one hand, analysis of predicted loss-of-function variants in the *LRRK1* and *LRRK2* genes failed to find any association with Parkinson's disease (Blauwendraat et al., 2018). On the other hand, two *LRRK2* risk variants have been reported that may have loss-of-function effects. For example, LRRK2 G2385R is one of the most prevalent risk variants worldwide and is reported to cause a reduction in LRRK2 kinase activity *in vitro* and a reduction in LRRK2 stability in cells (Rudenko et al., 2012). However, some studies have reported that the LRRK2 G2385R variant increases LRRK2-Rab phosphorylation (Steger et al., 2016; Zhang et al., 2019; Kalogeropulou et al., 2022). Recently, another *LRRK2* loss-of-function variant, G2294R, has been identified in a patient with familial PD. Consistent with a loss-of-function mechanism, this variant reduces LRRK2 protein levels and LRRK2-mediated Rab10 phosphorylation in cells (Ogata et al., 2021). Together, these observations suggest the hypothesis that *LRRK2* loss-of-function variants can contribute to PD in humans. Nonetheless, more research will be needed to determine if and how LRRK2 loss of function contributes to PD.

The LRRK proteins protect against defects in neurodevelopment in model organisms

In mice, wild-type LRRK proteins protect against defects in axon guidance, and this process is disrupted by either gain-of-function or loss-of-function alleles in the *LRRK* genes (Onishi et al., 2020). For example, knockout of either *LRRK1* or *LRRK2* causes axon guidance defects in the commissural axons of the spinal cord. Likewise, the double knockout of *LRRK1* and *LRRK2* causes axon guidance defects in the midbrain dopamine neurons. The LRRK2 G2019S gain-of-function mutant protein causes axon guidance defects in both spinal cord commissural neurons and mid brain dopamine neurons. These observations indicate that neurodevelopment can be disrupted by either GOF and LOF alleles of *LRRK2*, suggesting that precise regulation of LRRK2 activity is required for normal development.

Recent work has begun to reveal the mechanisms through which the LRRK proteins promote axon guidance. For example, LRRK proteins promote axon guidance by phosphorylating Frizzled3, thereby promoting its interaction with the planer cell polarity pathway. Moreover, observations of cultured neurons suggest that LRRK2 and the planer cell polarity pathway promote axon guidance by regulating the interaction between growth cones. Together, these observations suggest that LRRK2 promotes axon guidance by regulating the planer cell polarity protein, thereby influencing the interactions between growth cones.

Additional mechanistic insight for the role of the LRRK proteins in neuronal development comes from studies of the *C. elegans* LRK-1 ortholog of the LRRK1 and LRRK2 proteins. First, LRK-1 is required for termination of the growth of the PLM and ALM axons. These axons normally extend along the body wall and terminate at defined locations. Loss of LRK-1 function causes these axons to overshoot their normal termination sites (Kuwahara et al., 2016; Drozd et al., 2024). Second, LRK-1 is required for the polarized distribution of synaptic vesicle proteins within neurons. For example, the SNB-1 synaptic vesicle protein is normally localized to axons and excluded from dendrites. Loss of LRK-1 function causes SNB-1 to be localized in both axons and dendrites, suggesting that LRK-1 helps to exclude synaptic vesicle localization in dendrites (Sakaguchi-Nakashima et al., 2007). Moreover, LRK-1 can function with the UNC-16 (JIP3) adaptor protein and the SYD-2 active zone protein to regulate the protein composition and trafficking of synaptic vesicles precursors (Choudhary et al., 2017; Nadiminti et al., 2024).

In humans, defects in neurodevelopment are associated with neurodevelopmental disorders such as autism (ASD) and intellectual disability (ID). In this regard, it is interesting to note that growing evidence suggests a potential association between Parkinson's disease and ASD/ID. For example, a small study has reported a high incidence of Parkinson's disease in autistic individuals (Starkstein et al., 2015). Moreover, although unpublished, a recent large study has suggested that diagnosis of ASD and/or ID is a risk factor for Parkinson's disease (Naddaf, 2024). Although this association is still not well understood, it could reflect the dual roles of LRRK proteins in protecting against both neurodegeneration and neurodevelopment.

Regulation of autophagy may underlie the role of LRRK proteins in PD and neurodevelopment

There is growing evidence suggesting that abnormal LRRK2 activity disturbs the autophagy/lysosomal pathways, including mitophagy, the process of specific elimination of mitochondria by autophagy (Erb and Moore, 2020; Singh and Ganley, 2021). In cultured neurons, expression of G2019S and R1441C/H LRRK2 decreased autophagic flux or autolysosome maturation, possibly through disruption of axonal autophagosome transport (Schapansky et al., 2018; Wallings et al., 2019; Boecker et al., 2021; Dou et al., 2023). In *C. elegans*, G2019S or R1441C LRRK2 expression causes accumulation of LC3-homolog lgg-1:RFP, suggesting a reduction of autophagy flux (Saha et al., 2014). In mice, expression of G2019S or R1441C LRRK2 display increased numbers of large intra-axonal autophagic vacuoles (Ramonet et al., 2011). Mechanistically, the increase of LRRK2 kinase activity was shown to enhance the recruitment of JIP4, a motor adaptor known to bind to LRRK2-phosphorylated Rab proteins, to the autophagosomal membrane. Increased JIP4 levels induce abnormal recruitment and activation of kinesin-1, resulting in an unproductive tug-of-war between anterograde and retrograde motors bound to autophagosomes (Boecker and Holzbaur, 2021).

In contrast to the LRRK2 GOF variants, deletion of the LRRK2 gene caused an increase in autophagic flux in neurons cultured from postnatal day 1 rats, although this did not reach statistical significance (Wallings et al., 2019). Nonetheless, this LRRK2 deletion did cause a statistically significant increase in lysosomal protein degradation. The opposite effect was observed in the brains of ageing mice, where deletion of both LRRK2 and LRRK1 leads to anaccelerated decline of autophagic clearance and accumulation of large autophagic vacuoles in surviving dopaminergic neurons (Giaime et al., 2017; Huang et al., 2022). Taken together, these observations suggest that the deletion of the LRRK genes might have opposite effects on autophagy in young and old neurons. Consistent with this idea, loss of LRRK2 enhances autophagy in young rat kidneys and decreases autophagy in old rat kidneys (Tong et al., 2012).

Work in multiple systems has implicated LRRK2 mutations in the dysregulation of mitophagy, a selective form of autophagy that is critical for the homeostasis of mitochondria. Studies of fibroblasts and neurons derived from patients carrying the G2019S or R1441C LRRK2 mutations revealed abnormalities in mitochondrial morphology, and an increase of mitochondrial DNA damage (Mortiboys et al., 2010; Sanders et al., 2014; Wauters et al., 2020). In *C.elegans*, G2019S or R1441C LRRK2 expression increased the response of the mitochondrial hsp6 reporter to stress (Saha et al., 2014). In mice, G2019S LRRK2 expression was shown to induce progressive mitochondrial morphology changes and reduce basal mitophagy as indicated by the reduction of fluorescent reporter for mitophagy ("mito-QC") (Yue et al., 2015; Singh et al., 2021). Mechanistically, LRRK2 was shown to form a complex with Miro, which is required for its efficient removal during PINK1/Parkin-dependent mitophagy (Hsieh et al., 2016). Expression of LRRK2

G2019S disrupted Parkin-dependent mitophagy, potentially via reducing Parkin's interaction with outer mitochondrial membrane proteins, including the fission regulating GTPase DRP-1 (Bonello et al., 2019). Additionally, LRRK2 mutations impair depolarization-induced mitophagy through inhibition of mitochondrial accumulation of Rab10, a downstream substrate of LRRK2 (Wauters et al., 2020).

Emerging evidence suggests that the role of the LRRK proteins in axon development is also mediated through dysregulation of autophagy. This idea is supported by interactions between mutations in the genes that encode the UNC-16 (JIP3) adaptor protein, the LRK-1 ortholog of LRRK2, and the WDFY-3 selective autophagy protein (Drozd et al., 2024). UNC-16 is required for the retrograde transport of late endosomes and autophagosomes and its loss of function causes axonal accumulation of late endosomes and autophagosomes, which contain LRK-1 protein (Hill et al., 2019; Celestino et al., 2022; Drozd et al., 2024). Moreover, loss of *unc-16* causes overextension of the PLM axon and this phenotype can be suppressed by loss of *lrk-1* function (Drozd et al., 2024). The PLM axon overextension phenotype can also be suppressed by loss of *wdfy-3*, which encodes a selective autophagy protein. These observations suggest that excessive activity of LRK-1 and WDFY-3 might cause axon overgrowth in *unc-16* mutants. Furthermore, no additional suppression of this phenotype is observed in in *lrk-1*;*wdfy-3*;*unc-16* triple mutants, suggesting that *wdfy-3* and *unc-16* function in a genetic pathway with each other.

Based on these observations, we hypothesize that LRK-1 and WDFY-3 function within a pathway that can promote axon extension and that excessive accumulation of these proteins in the axon can cause axon termination defects. Moreover, it is interesting to note that that the *C. elegans* WDFY-3 protein is an ortholog of the human WDFY3 selective autophagy protein, which is encoded by a gene that has been associated with ASD and ID (Fu et al., 2022). Therefore, we hypothesize that the WDFY3 and LRRK proteins could function together to protect against autism.

Studies of cultured mammalian neurons also support the idea that the role of the LRRK family in axon growth is mediated through the dysregulation of autophagy. Multiple studies have indicated that the LRRK2 G2019S mutation reduces the growth of axons and dendrites in cultured primary neurons (Stafa et al., 2012; Sepulveda et al., 2013; Stafa et al., 2014; Kang et al., 2024). One study of the SH-SY5Y neuroblastoma cell line has also found that the LRRK2 G2019S mutation causes an accumulation of autophagosomes within neurites along with a decrease in neurite length (Plowey et al., 2008). Moreover, both of these phenotypes can be suppressed by knockdown of either the ATG7 or LC3 autophagy proteins. These observations suggest that LRRK2 G2019S disrupts axon growth through the dysregulation of autophagy. These observations are also consistent with the hypothesis that wildtype LRRK2 has a role in regulating axon growth through the regulation of autophagy.

Discussion

Here, we have reviewed the roles of the LRRK proteins in protecting against neurodegeneration and promoting axon development in multiple model organisms. We have also considered evidence that the LRRK family regulates autophagy, and that disruption of autophagy is likely to underlie the neurodegenerative and neurodevelopmental phenotypes of *LRRK* gene variants. Moreover, we have discussed genetic interactions suggesting that the LRK-1 ortholog of LRRK2 regulates axon development by functioning in a pathway with the ortholog of the WDFY3 selective autophagy protein (aka Alfy), which is encoded by an autism-associated gene. Taken together, these observations suggest the hypothesis that the role of the LRRK proteins in regulating autophagy could underlie their roles in protecting against neurodegeneration and neurodevelopmental defects. We also hypothesize that these dual roles for LRRK proteins could explain the association between ASD and PD. Further investigation of this hypothesis will require additional work in model organisms and further human genetic analysis.

A key question for future investigation is the potential involvement of *LRRK2* in protecting against neurodevelopmental disorders. Given the role of *LRRK* genes in protecting against neurodevelopmental defects in mice, *Drosophila* and *C. elegans*, we propose that they might protect against neurodevelopmental disorders in humans. Thus far, investigations of *LRRK2* association with neurodevelopmental disorders have been inconclusive. On one hand, comparative genomic mapping with microdeletions has suggested that deletion of *LRRK2* can cause a syndrome that presents as intellectual disability and autism (Labonne et al., 2020). On the other hand, a large study of human *LRRK2* loss of function variants failed to identify an association with any disorders (Whiffin et al., 2020). One possible reason for this discrepancy is that autism may occur as a result of a genetic interaction between *LRRK2(LOF)* and variants in other neurodevelopmental genes. Thus, the microdeletions could cause autism by synergizing with variants in one or more other autism-associated genes. Therefore, we propose that an important goal for future research with model organisms will be to identify synergistic genetic interactions between mutations in *LRRK* genes and neurodevelopmental disorder-associated genes. With regards to human genetic analysis, it may be useful to investigate a potential association between *LRRK2(GOF)* variants and neurodevelopmental disorders.

Another key question for future investigation is the potential involvement of the *WDFY3* gene in protecting against Parkinson's disease and other neurodegenerative disorders. Considering the genetic interactions between *wdfy-3* and *lrk-1* in *C. elegans*, we propose that the *WDFY3* gene could be involved in protecting against Parkinson's disease. Although *WDFY3* gene has not been associated with Parkinson's, the WDFY3 protein has been implicated in mitophagy, which is thought to be involved in Parkinson's (Gao et al., 2017; Napoli et al., 2018). In addition, *WDFY3* has been implicated in protecting against Huntington's disease, suggesting that it can protect against neurodegeneration (Fox et al., 2020). To further investigate the role of WDFY3 in neurodegeneration, future investigations may seek to explore genetic interactions between variants in *WDFY3* and *LRRK2* in animal models of Parkinson's disease.

Data availability statement

The original contributions presented in the study are included in the article/supplementary material, further inquiries can be directed to the corresponding authors.

Author contributions

AT: Writing – original draft, Writing – review and editing. LN: Writing – original draft, Writing – review and editing. BS: Writing – original draft, Writing – review and editing. CQ: Writing – original draft, Writing – review and editing.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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The author(s) declare that no Generative AI was used in the creation of this manuscript.

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Emerging roles for E3 ubiquitin ligases in neural development and disease

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Neurodevelopment is an intricate process with highly regulated, overlapping stages including neuronal differentiation and axon guidance. Aberrations during these and other stages are tied to the etiology of neurodevelopmental disorders like Autism Spectrum Disorder, Angelman Syndrome, and X-linked Intellectual Disability. Ubiquitination is a dynamic and highly reversible post-translational modification conferred by E3 ubiquitin ligases. Recent discoveries have advanced the understanding of how substrate ubiquitination can guide protein localization, drive protein degradation, and alter protein post translational modifications. In this review, we highlight members of the RING and HECT E3 ligase families to discuss their novel roles in the molecular mechanisms regulating neurodevelopment. These findings are both instrumental for informing the future directions of neurodevelopmental research, and in expanding knowledge of intracellular mechanisms of protein trafficking. In addition, a deeper understanding of the molecular mechanisms of E3 ligase function in development promises to offer new insights into the pathogenesis of neurodevelopmental disorders.

KEYWORDS

neural differentiation, axon guidance, neural developmental disorders, E3 ubiquitin ligase, commissureless, Ndfip, slit

Introduction

Neurodevelopment begins with the specification of neural tissue and the differentiation of neural cells. Newborn neurons are then influenced by spatial and temporal hierarchies of extrinsic and intrinsic patterning signals that give rise to diverse neuronal populations. These neurons then migrate and extend axons and dendrites that contact target cells to form functional synapses. This process concludes with synapse maturation and the establishment of plastic circuits throughout the peripheral and central nervous systems (Alberts et al., 2002). These overlapping and tightly choreographed stages of neurodevelopment require extensive and highly dynamic changes in protein expression levels and localization. One versatile way to mediate these changes is through post-translational modifications of proteins.

Ubiquitination is an essential post-translational modification generated by the covalent linking of ubiquitin, a highly conserved 76 amino acid protein, to a protein target (Hershko and Ciechanover, 1998; Weissman, 2001; Akutsu et al., 2016). The process of ubiquitination is stepwise and requires three separate enzymes for

the transfer of the ubiquitin onto a substrate. First the E1 enzyme (E1) activates the ubiquitin in an ATP-dependent reaction that creates a thioester-linked ubiquitin. Through this linkage, the E1 can then transfer the ubiquitin to the cysteine residue of an E2 enzyme (E2) (Haas et al., 1982; Kerscher et al., 2006). Then, the E2 coordinates with the E3 ligase to attach the ubiquitin group(s) through an isopeptide bond to substrate proteins (Johnson et al., 1995; Hershko and Ciechanover, 1998; Clague et al., 2015). The E3 ligase is also responsible for substrate recruitment, either through direct binding to the substrate (Cowan and Ciulli, 2022) or through binding to an adaptor protein (Mund and Pelham, 2009; Zheng and Shabek, 2017). All together there are around 600 E3 ligases in humans, which is orders of magnitude more than the one to two ubiquitin-modifying E1 enzymes and around 40 E2 enzymes encoded in the human genome (Schulman and Harper, 2009; Stewart et al., 2016; Jevtić et al., 2021). This vast diversity of E3 ligases and their myriad functions have generated sustained interest in understanding their roles in biological processes.

The three most characterized families of E3 ligases are distinguished by their catalytic mechanism of ubiquitin ligation (Figure 1). Really Interesting New Gene (RING) E3 ligases act as scaffolds for E2s by either forming a Zn^{2+} ion cross brace or through binding of the U-box and facilitating direct ubiquitin transfer to proximal substrates. Homologous to E6-AP C-terminus (HECT) family E3 ligases use a two-step process in which the HECT E3 first acts as a linker to accept the ubiquitin from the E2 onto a catalytic cysteine residue in the HECT domain and later catalyzes the transfer of the ubiquitin to the substrate lysine through a thioester bond (Kim et al., 2011; Metzger et al., 2014). In some cases, this requires a conformational change to expose the accepting cysteine. Lastly, RING-between-RING (RBR) family mechanism of catalysis shares elements of both the RING and HECT families; the RING domain binds the E2 similarly to the RING E3s, but this binding is in turn used to stabilize the transfer of the ubiquitin from the E2 to the catalytic domain of the RBR, which then transfers the ubiquitin to the substrate in an aminolysis reaction reminiscent of that of HECT E3 ligases (Wang et al., 2023). Each of these large families of E3s can be further stratified into subfamilies based on differences in substrate binding domains and catalytic domains. In addition to the major families, the recent discovery of the RING-Cysteine-Relay (Pao et al., 2018), ATP-dependent RZ finger (Ahel et al., 2021; Otten et al., 2021), and CRL-RBR-E3 (Horn-Ghetko et al., 2021) classes of E3 ligases have expanded understanding of ubiquitination mechanisms.

The inducible and reversible transfer of ubiquitin canonically occurs at single or multiple available lysine residues, but can also occur at cysteine, serine, and threonine residues of a protein substrate, as well as non-proteinaceous lipids (Zheng and Shabek, 2017; Pao et al., 2018; McClellan et al., 2019; Mabbitt et al., 2020; Otten et al., 2021). These modifications can be classified as either mono or multi-mono ubiquitination, characterized by the conjugation of one molecule of ubiquitin (Dikic et al., 2009), or as poly-ubiquitination, characterized by the linkage of a polymerized ubiquitin chain(Figure 2). Other layers of complexity include the potential to ligate ubiquitin groups to N-terminal methionine (M1) residues, the selection of ubiquitin lysine residues (K6, K11, K27, K29, K33, K48, or K63) for chain elongation, and the subsequent types of homogeneous or heterogeneous poly-ubiquitin linkages (Komander and Rape, 2012; Swatek and Komander, 2016; Musaus et al., 2020).

Due to the various possible combinations of these ubiquitin modifications, the function of many linkages is still poorly understood. Of those that are better characterized, poly-ubiquitination at M1 is primarily implicated in immune signaling. Further, poly-ubiquitination at K63 is linked to a constellation of processes, including DNA damage repair, immune signaling, kinase activation, endocytosis, and entry into the endo-lysosomal pathway (Madiraju et al., 2022). Alternatively, poly-ubiquitination at K11 or K48 are associated with proteasomal degradation. Lastly, mono and multi-mono ubiquitination are associated with protein interactions, localization, and endocytosis (Suryadinata et al., 2014; Zinngrebe et al., 2014) (Figure 2). Generally, ubiquitin-induced endocytosis directs proteins to the endo-lysosomal degradation pathway, resulting in a range of fates from recycling to degradation in the lysosome. Ubiquitination is also a vital cue for the initiation of autophagy and binding of autophagy adaptors to proteins and organelles destined for degradation (Mizushima, 2024) (Figure 2). While the linkage-dependent outcomes for some proteins are well reported, the linkages conferred by each E3 ligase are not as well documented. For this reason, many E3 ligases are studied in the context of substrate interaction and downstream effects within a given signaling pathway. In this review, we highlight E3 ligases from the RING and HECT families with non-degradative and degradative functions in several neurodevelopmental processes and further discuss their implications in specific neurodevelopmental disorders (NDDs). Since the role of E3 ligases in synapse formation, function, and plasticity has been extensively studied and is the focus on several recent reviews (Widagdo et al., 2017; Mabb and Ehlers, 2018; Kawabe and Stegmüller, 2021; Mabb, 2021), our discussion will focus instead on the contribution of specific E3 ligase functions to neural differentiation, axon guidance, and dendrite morphogenesis. In the context of NDDs, we will highlight select instances where connections between E3 ligases and the regulation of specific substrate proteins have offered mechanistic insight into these disorders.

Section 1: E3 ligases in neural specification

Specification of the neural plate from embryonic stem cells (ESCs) is coordinated by the spatiotemporal balance of secreted inhibitory factors and neural-promoting autocrine signaling (Gaspard and Vanderhaeghen, 2010). After neural plate formation, neurulation, and the specification of neural progenitor cells (NPCs), neural diversity is established through a series of lineage-dependent responses to spatiotemporal inputs. Morphogenic gradients and other external factors contribute spatial information for differentiation, and act as switches for cell-autonomous mechanisms.

Sonic hedgehog (Shh) is an important morphogen in neural specification. After neurulation, Shh is expressed in both the notochord and the floorplate of the emerging spinal cord, producing a gradient along the dorsal-ventral axis, with Shh expression highest ventrally (Dessaud et al., 2008). Shh signaling and the dynamic activation and repression of its targets by Gli transcription factors (TFs), contributes to the expression of distinct and restricted

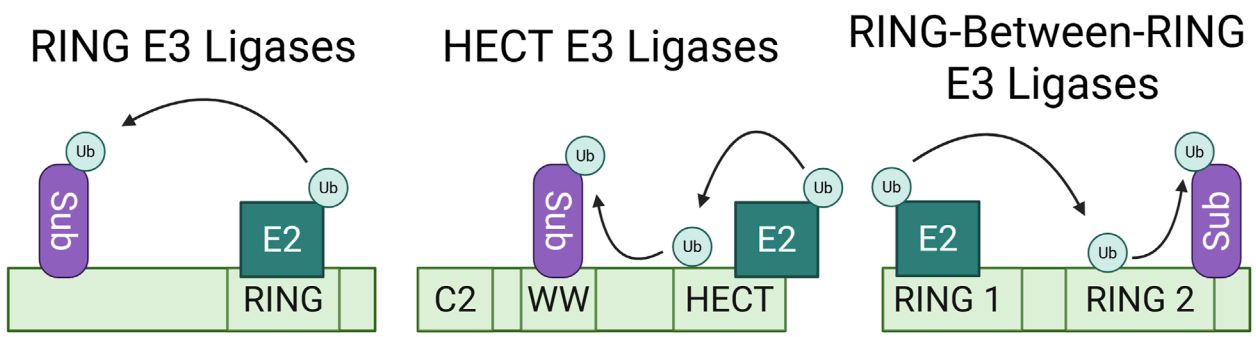

FIGURE 1
Most characterized E3 ubiquitin ligase families. Ubiquitination mechanisms of the RING, HECT, and RING-between-RING E3 ligase families. This simplified schematic shows direct E3 ligase-substrate binding, but each family can also employ one or more adaptors to bind substrates and bring them into proximity for ubiquitination. RING E3 ligases act as scaffolds for E2 enzymes, facilitating the direct transfer of ubiquitin to their proximal substrates. HECT family E3 ligases function as linkers between the E2 enzyme and their substrate. They temporarily accept the ubiquitin onto an available cysteine residue and the HECT domain later catalyzes the transfer of the ubiquitin onto the substrate. RING-between-RING E3 ligases share aspects of both RING and HECT catalytic mechanisms, wherein the RING1 domain binds the E2 enzyme and the RING2 domain temporarily accepts the ubiquitin, to then transfer the ubiquitin to the proximal substrate.

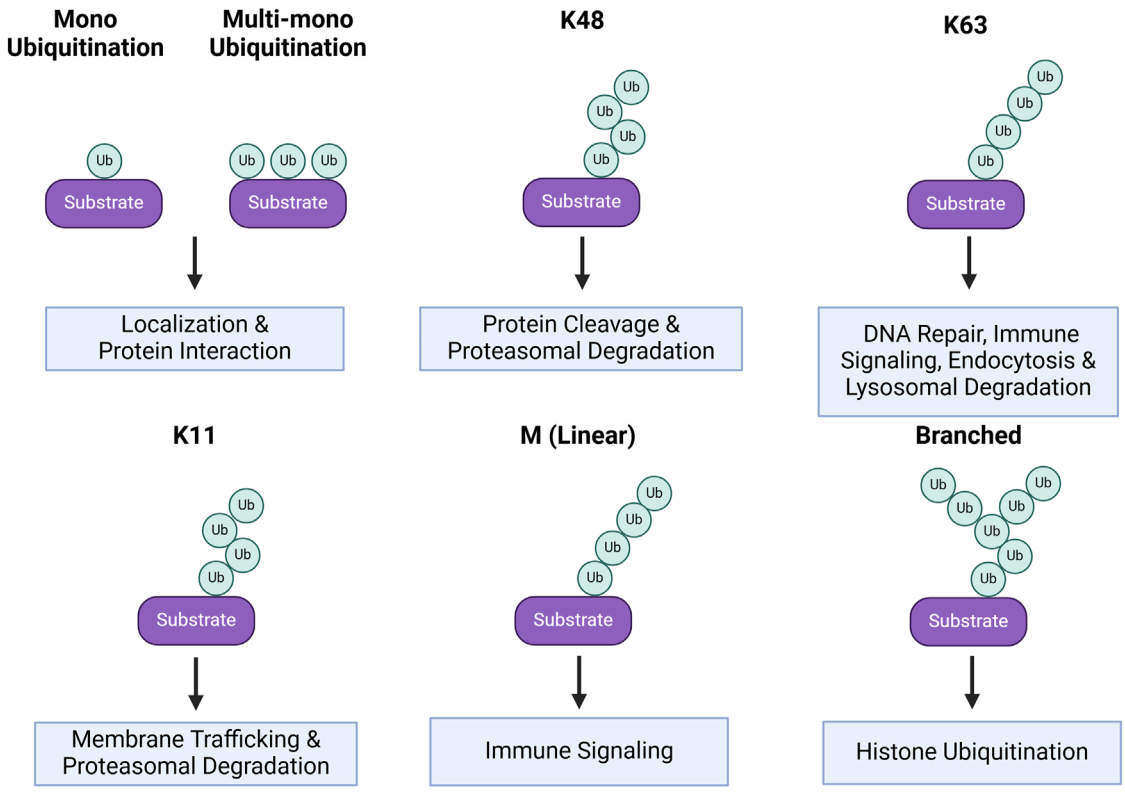

FIGURE 2
Ubiquitin linkages and substrate protein fates. Protein fates based on their ubiquitin linkage. Mono and multi-mono ubiquitination is when a single ubiquitin is conjugated to the substrate, rather than a chain. This form of ubiquitination generally alters the substrate localization or protein-protein interactions. These are also more transient post-translational modifications. K48, K63, K11, M, and Branched are all linkages in which chains of ubiquitin are conjugated onto the substrate. Chains linked at K48 result in protein cleavage and/or target the protein for proteasomal degradation. K63 ubiquitin chains have myriad effects including roles in DNA repair, immunity, endocytosis, and lysosomal degradation. K11 linkages result in changes in protein membrane trafficking and proteasomal degradation. M linkages occur at the N-terminal methionine of the protein and are associated with immune signaling. Lastly, branched ubiquitin chains are associated with histone ubiquitination.

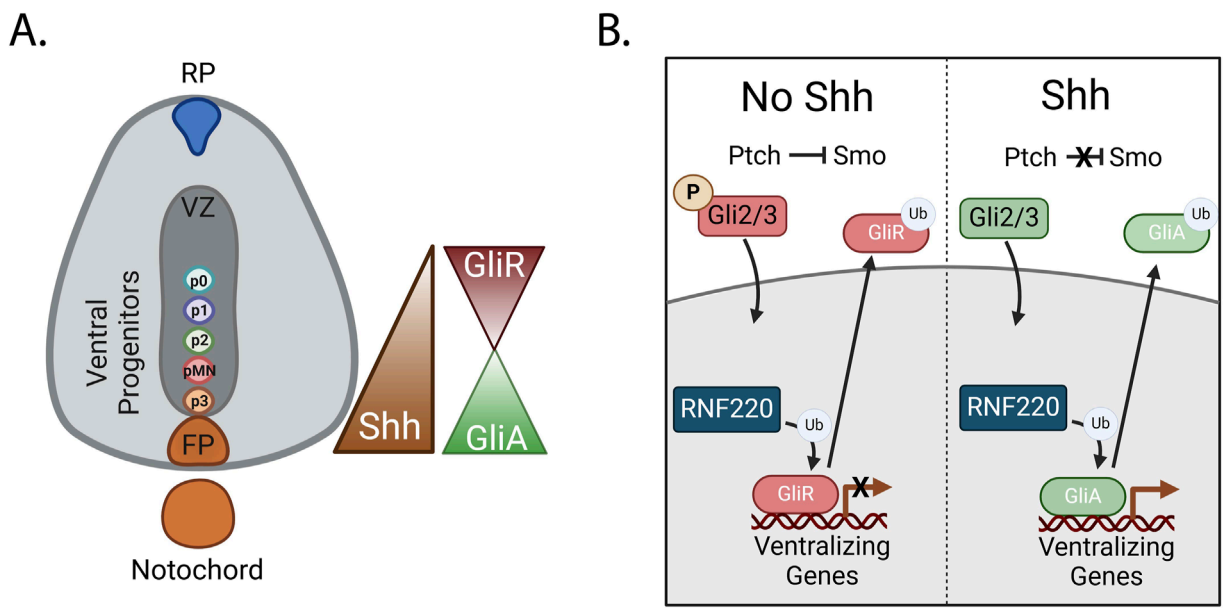

FIGURE 3
RNF220 regulates Shh signaling by ubiquitinating Gli proteins **(A)** Shh is secreted from the notochord and the floorplate. It then diffuses dorsally, creating a dorsal-ventral concentration gradient. Gli proteins are TFs expressed in the developing spinal cord. The repressive or activating function of Gli2 and Gli3 proteins is controlled by the expression levels of Shh. **(B)** Dorsally, where there are low levels of Shh, Gli2/3 proteins are phosphorylated, promoting their cleavage and resulting in a repressive function (GliR). Nuclear translocation and genomic binding of GliR results in the repression of ventralizing genes. Alternatively, when Shh is present, Gli2/3 are not phosphorylated and remain in an activating form (GliA). GliA translocation to the nucleus and genomic binding results in the transcription of ventralizing genes however, expression of the E3 ligase RNF220 ubiquitinates GliR and GliA, resulting in their transport out of the nucleus.

patterns of ventralizing genes defining medial populations of ventral neuronal progenitors including the most ventral floorplate, p3, pMN, and least ventral, p2-p0 domains (Figure 3A). In later stages of spinal cord development, these progenitors give rise to interneurons, glial cells, and motor neurons (Lu et al., 2015; Ravanelli and Appel, 2015). Recent data implicates Ring Finger Protein 220 (RNF220), a highly conserved RING E3 ligase, in the tuning of Shh signaling and subsequent specification of ventral progenitor fates in the neural tube (Ma and Mao, 2022).

RNF220, a cytosolic protein, is expressed within the neural tube beginning at E8.5. RNF220 interacts with and ubiquitinates the Gli TFs (Ma et al., 2019). In mammals, three Gli TFs play key roles in the cellular response to the Shh gradient. Gli1, a direct target of Shh, functions exclusively as a transcriptional activator, contributing to a positive-feedback loop of Shh target gene expression. In the presence of Shh, Gli2/3 promote ventral fates by activating Shh target genes. On the other hand, in the absence of Shh, Gli2/3 are phosphorylated, enabling recognition for cleavage. Gli2/3 cleavage removes the Shh activating domain, resulting in repression of Shh target genes upon translocation of these TFs into the nucleus, and less ventralized cell fates (Hui et al., 1994; Ruiz i Altaba, 1998; Persson et al., 2002) (Figure 3B). In the absence of RNF220, mouse embryos display aberrant differentiation of ventral progenitor populations, with substantial increases in the p3 and p0 populations on the extreme ends of the Shh gradient and decreases in the p1 and p2 populations (Ma et al., 2019).

Interestingly, RNF220-mediated ubiquitination of both active and repressive forms of the Gli proteins results in decreased nuclear localization *in vitro* by improving the accessibility of a zinc-finger domain in the Gli proteins. This enables recruitment of CRM1 to drive nuclear export, ultimately modulating the expression of Shh target genes. The expansion of the p3 and p0 populations in RNF220 deficient embryos is likely due to an aberrant increase in activating Gli (GliA) TF binding in locations of high Shh availability and a reciprocal increase in repressive Gli (GliR) TF binding in more dorsal locations of low Shh availability (Ma et al., 2019) (Figure 3B).

Conditional knockout of RNF220 later in embryonic development also leads to alteration of the progenitor regions and their post-mitotic lineages in the hindbrain. By E12.5, the p0 domain and its daughter V0 interneurons remain expanded; however, loss of RNF220 exacerbates the subsequent decreases in V1 and V2 regions. Notably, while the pMN domain is still expanded, the p3 is also broadened. Given that Shh signaling is known to pattern both the embryonic spinal cord and the hindbrain, it is interesting that the alterations in progenitor domains due to the loss of RNF220 in the hindbrain are distinct from those in the spinal cord. In addition to the resulting differences in sMN/oligodendrocyte progenitors, there is also a significant increase of the serotonergic (5-HT) neuron population of the hindbrain, corresponding with p3 domain expansion. These findings may indicate a broader role for RNF220-mediated regulation in neuronal differentiation and psychiatric disorders associated with dysregulation of 5-HT circuitry (Wang et al., 2022).

In addition to regulating TF localization, E3 ligases and their adaptors also directly downregulate TF protein expression and play important roles in fine-tuning gene expression during

neural specification in the cortex. For example, the Sox2 TF is expressed in neural stem cells (NSCs) and NPCs during early central nervous system (CNS) development, where it is required for NSC maintenance. *In vitro* models of ESCs also identified Sox2 as a TF for Shh, further linking it to known differentiation pathways (Favaro et al., 2009). *In ovo* inhibition of Sox2 leads to delamination of the ventricular zone and exit of the progenitors from the cell cycle, while constitutive expression of Sox2 inhibits neuronal differentiation and maintains progenitor characteristics through Oct3/4 (Graham et al., 2003; Masui et al., 2007). Accordingly, downregulation of Sox2 is crucial for the modulation of NPC fate and recent data implicates Cullin-RING finger ligase 4 (CRL4) complex in this process.

In one form of CRL4, Cullin4A (CUL4A) serves as a core scaffold for a RING finger binding protein, ROC1, that recruits E2 ligases. CUL4A also binds to one or more of the adaptor proteins, DDB1, DET1, and COP1, to interact with its target substrates and allow for their ubiquitination (Cheng et al., 2024). For example, Sox2 interacts with COP1 and is ubiquitinated by the CUL4A complex in NPCs. This ubiquitination and subsequent degradation of Sox2 increases over the course of development, resulting in neuronal differentiation of NPCs. Loss of DET1 and COP1 also abolishes the interaction between Cul4a and Sox2, thereby stabilizing Sox2 expression, further supporting the importance of CUL4A in Sox2 regulation. The novel Sox2 deubiquitinase, OTUD7B, is sufficient to prevent neuronal differentiation and maintain the NPC population, further reinforcing the importance of Sox2 ubiquitination and degradation by the CUL4 complex for timely NPC differentiation (Cui et al., 2018).

Interestingly, early studies of mouse ESC differentiation reported that Sox2 is ubiquitinated by WWP2, a HECT family E3 ligase, and subsequently degraded (Buckley et al., 2012; Fang et al., 2014); however, recent data report low levels of WWP2 expression in NPCs. This raises the question of how Sox2 is regulated in these NPCs. Additionally, Sox2 K119 mono-methylation causes a conformational change that facilitates its ubiquitination by WWP2, but CUL4 complex-mediated ubiquitination is independent of Sox2 K119 mono-methylation, indicating that despite regulating the same protein, WWP2 and the CUL4A complex likely utilize a different Sox2 ubiquitination site. This could be due to differences in substrate recognition and/or enzymatic activity inherent to RING E3s and HECT family E3s. This difference in binding combined with low levels of WWP2 expression in NPCs could be evidence of a cell-specific Sox2 mechanism of ubiquitination and regulation found in NPCs, but not in the ESC pool (Cui et al., 2018). Data revealing critical roles for RNF220 in Shh signaling in the spinal cord and hindbrain, and CUL4A in Sox2 regulation in the cortex, exemplify the importance of E3 ligases in neural differentiation.

Section 2: E3 ligases in axon guidance

Newly differentiated neurons project their axons toward synaptic targets to form functional circuits. Guidance of these axons is mediated by the spatiotemporal regulation of attractant and repellant receptors on the membrane of the growth cone, a highly motile structure at their axon terminal (Evans and Bashaw, 2010). Binding of secreted and membrane-tethered axon guidance cues to these trans-membrane receptors leads to downstream signaling. This binding which remodels the growth cone plasma membrane and cytoskeleton to allow for directional growth responses (Chédotal, 2019). Ligand binding frequently leads to receptor internalization and receptor cleavage events that are intimately associated with receptor regulation and signaling. Endocytosis of receptors alters growth cone responsiveness by tuning the surface levels of receptors and can also play a vital role in initiating downstream signaling (O'Donnell et al., 2009). Receptor cleavage can regulate local signaling to the cytoskeleton and allow for nuclear translocation of intracellular domains (ICD) fragments that can regulate transcription. The ability of receptor ICDs to regulate transcription adds another layer of regulation to the process of axon guidance and suggests that guidance receptor signaling may also control additional aspects of neuronal maturation and function (Zang et al., 2021). Cytoskeletal rearrangement, endocytosis, and cleavage all facilitate the dynamic gradient- and receptor-dependent directionality of growth cone extension (Evans and Bashaw, 2010; Zang et al., 2021). In this section, we will discuss some of the roles of E3 ligases in the process of axon guidance with a particular emphasis on recent studies of Netrin-dependent axon attraction and Slit-dependent axon repulsion.

Netrin-mediated attraction

During axon guidance, Netrin is secreted from the floor plate and ventricular zone in the spinal cord, and in multiple cortical and subcortical regions (Wu et al., 2019). Netrin binding to *Drosophila* Frazzled (Fra) or vertebrate deleted in colorectal cancer (DCC) induces canonical chemoattractant signaling resulting in cytoskeletal rearrangement (Harris et al., 1996; Moore et al., 2007). Some downstream targets of Netrin-Fra/DCC signaling include the WAVE regulatory complex (WRC) which activates Arp2/3 to promote branched actin network assembly and Mena/VASP family of actin-regulatory protein which prevent actin capping and facilitate the formation of long unbranched actin filaments (Drees and Gertler, 2008; Chaudhari et al., 2024). In the context of Netrin-DCC signaling, Ena interacts with the barbed end of F-actin, increasing protrusion and extension of filopodia for growth cone attraction (Lebrand et al., 2004).

Recent data links two RING family E3 ligases, Trim9 and Trim67, with the regulation of Mena and filopodial extension (Figure 4) (Menon et al., 2015; Plooster et al., 2017; Boyer et al., 2018; Boyer et al., 2020). Trim9 is expressed in the growth cone of cortical neurons during embryonic mouse development and endogenous Trim9 interacts with Mena, VASP, and EVL. *In vitro*, Trim9-Mena/VASP interaction leads to VASP ubiquitination. Notably, VASP ubiquitination does not decrease VASP protein expression but instead alters VASP protein localization at filopodial tips. Interestingly, a ubiquitin group can be ligated to three separate lysines of VASP. This suggests that VASP could be multi-monoubiquitinated, a linkage associated with altered protein localization and interaction dynamics, further supporting that Trim9 ubiquitination regulates VASP outside of a degradative pathway (Dikic et al., 2009) (Figure 4). Trim9 ubiquitination of VASP may be important for regulating filopodial stability as *in vitro* knockout of *TRIM9* increases growth cone area and increases the duration of filopodial extension, and the number of

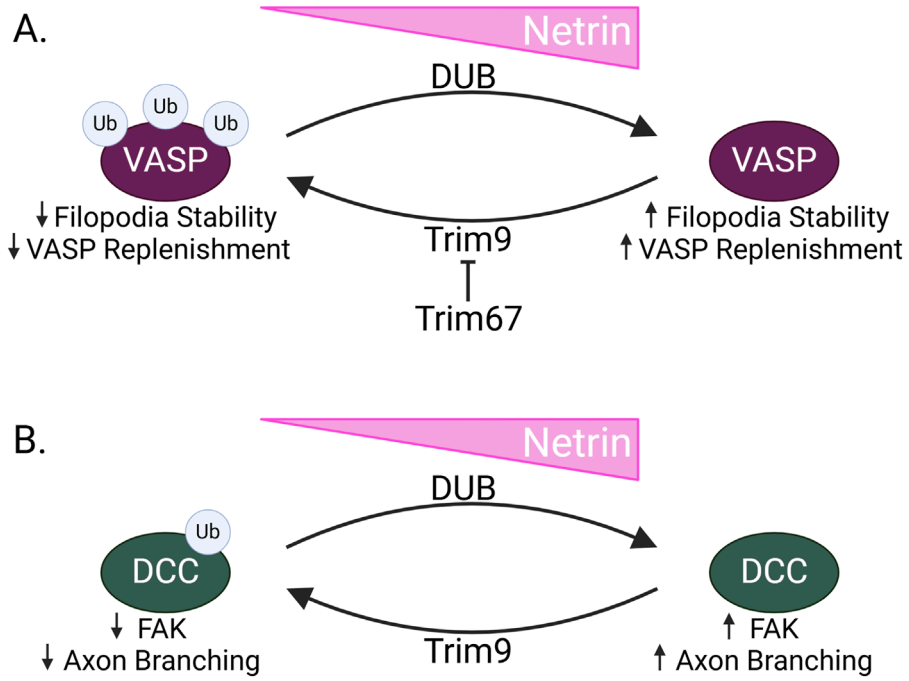

FIGURE 4
TRIM9 and TRIM67 in Netrin signaling **(A)** When Netrin expression is low in neurons during axon guidance, the RING E3 ligase Trim9 ubiquitinates VASP. This results in decreased filopodial stability and decreased replenishment of VASP within filopodia. Conversely, when Netrin levels are high, VASP is deubiquitinated by a deubiquitinating enzyme, resulting in increased filopodial stability and VASP replenishment. Trim67, another RING E3 ligase, inhibits Trim9, acting as a switch to allow for altered filopodial dynamics in response to Netrin. **(B)** Trim9 also ubiquitinates DCC when Netrin is low. This decreases FAK binding and prevents FAK-induced axon branching. In the presence of Netrin, DCC is deubiquitinated, allowing for increased FAK signaling and increased axon branching.

filopodia. This effect requires the presence of the VASP protein, as well as the Trim9 domains that are responsible for interaction with VASP (Menon et al., 2015).

Despite the propensity of Trim9$^{-/-}$ primary neurons to grow more filopodia, addition of Netrin does not potentiate this increase. Interestingly, switching between the ubiquitinated and un-ubiquitinated VASP may be required for Netrin response as there is no *in vitro* response to Netrin in the presence of either non-ubiquitinatable VASP mutants or in conditions preventing VASP deubiquitination. This supports a model in which Trim9 ubiquitinates VASP, altering its localization at filopodial tips. It is also possible that recruitment of Trim9 to filopodia by Mena/VASP/EVL facilitates the ubiquitination of many VASP proteins, maintaining a less-stable and more motile state of the filopodia; however, upon Netrin stimulation, VASP is deubiquitinated, allowing for increased filopodial stability and Netrin-induced attraction (Menon et al., 2015) (Figure 4A).

Trim9 also plays a role in Netrin signaling though its ubiquitination of DCC in neurons. Akin to the ubiquitination of VASP, Trim9-mediated DCC ubiquitination in primary cortical neurons does not decrease protein expression but appears to promote DCC multimerization and aggregation in the absence of Netrin (Menon et al., 2015). This is significant because the DCC crystal structure and DCC-Netrin binding affinity suggest that the cytosolic domain of DCC must dimerize for Netrin-induced attraction (Finci et al., 2014).

Within the cytoplasmic tail of DCC, there are FAK and SFK binding sites with two of the potential ubiquitin-binding lysines flanking the FAK binding site. These FAK and SFK binding sites recruit nonreceptor tyrosine kinases to DCC and are implicated in axon outgrowth in response to Netrin (Li et al., 2004; Ren et al., 2004). Both the loss of *Trim9* and mutation of the ubiquitin-accepting lysines result in increased interaction with and activation of FAK, suggesting that DCC ubiquitination sterically hinders binding of FAK, preventing downstream FAK/SFK signaling. In accordance with increased Trim9 substrate ubiquitination in the absence of Netrin, the loss of *Trim9* abolishes the Netrin response. The *in vivo* importance of Trim9 in the regulation of FAK-induced axon branching was investigated in the mouse corpus callosum, where loss of *Trim9* increased branching, in line with the purported effect of decreased DCC ubiquitination and subsequent increases in FAK signaling. In line with this, the branching phenotype is rescued by removing FAK (Figure 4B). Together, this suggests that Trim9 is not only impacting filopodial stability but may also inhibit axon branching by ubiquitinating DCC (Plooster et al., 2017).

Trim67 is also connected to filopodial stability through its regulation of VASP activity. Similarly to Trim9, Trim67 is highly expressed in the embryonic cortex and localizes to the growth cone (Boyer et al., 2018). It also colocalizes and interacts with VASP at growth cone filopodia *in vitro* and knockout of *Trim67* increases growth cone area; however, the direct comparisons to Trim9 end here. In contrast, Trim67 decreases VASP ubiquitination, through

an undefined mechanism. As an E3 ligase, it is possible that Trim67 ubiquitinates Trim9, promoting its degradation and preventing VASP ubiquitination. Alternatively, it could downregulate a protein within the deubiquitination pathway, promoting deubiquitinase activity that antagonizes VASP ubiquitination. Additionally, Trim67 affects filopodial dynamics like protrusion and retraction in primary cortical neurons. In the corpus callosum, TRIM67 affects axon guidance and tract formation rather than axon branching as observed for TRIM9. Trim67 is also required for growth cone turning in response to Netrin (Boyer et al., 2020).

The opposing functions of Trim9 and Trim67 support a mechanism wherein TRIM67 inhibits the ubiquitination of VASP by Trim9. Through this, and the function of the Netrin-induced deubiquitinase suggested in previous work (Menon et al., 2015), these proteins alter filopodial stability to regulate Netrin-induced attraction (Figure 4A). Of interest, loss of *TRIM67* results in additional defects in adult mice brain. This includes thinning of the hippocampal commissure, as well as decreased brain weight, and decreased area of the hippocampus, the lateral ventricles, and the amygdala. These neurodevelopmental differences may underly decreased learning and altered social novelty behaviors observed in *Trim67* knockout mice (Boyer et al., 2018). In addition to playing a part in axon guidance, these phenotypes may suggest a role for these E3 ligases in additional processes like neuronal migration, proliferation, or survival. This data reveals TRIM9 and TRIM67 as crucial proteins for the fine-tuning of signaling pathways that guide netrin-mediated attraction.

Finally, a more recent study supports a role for Trim9 in regulating axon repulsion in response to Netrin through the Unc-5 receptor. Specifically, high concentrations of Netrin *in vitro* can trigger Unc-5 dependent axon repulsion, and these effects are inhibited in the absence of *trim9* (Mutalik et al., 2025). The precise mechanism through which Trim9 impinges on Unc-5 activity awaits future exploration; however, it is interesting to note that *trim9 and Unc-5C* mutant mice share similar axonal phenotypes in the internal capsule of the brain (Srivatsa et al., 2014; Menon et al., 2015).

Slit-mediated repulsion

Slit binding to its receptor Roundabout (Robo) induces repulsion in projecting neurons. In both invertebrates and vertebrates there are three Robo family proteins—Robo1, Robo2, and Robo3— involved in axon guidance (Iversen et al., 2020). While the distinct and overlapping functions of the respective Robo proteins in vertebrates and invertebrates have been reviewed elsewhere, here we will focus exclusively on Robo1 function at the midline (Blockus and Chédotal, 2016). These proteins are well characterized for their function in midline crossing and commissure formation in the invertebrate ventral nerve cord and the vertebrate spinal cord of bilaterally symmetrical organisms. In these structures, Slit is expressed at the midline and the ventral floorplate respectively; however Slit expression coincides with Netrin expression. Therefore, for the crossing commissural neuron (CN) to be selectively permissive to attractive Netrin signaling, CNs must downregulate growth cone expression of Robo1 receptors to prohibit premature Slit-induced repellant signaling. During CN exit of the midline or floorplate, Robo1 surface expression increases, promoting repulsion and preventing re-entry into these regions.

In *Drosophila*, Commissureless (Comm) downregulates Robo1. This occurs through a shunting mechanism in which Comm is expressed in pre-crossing CNs and targets nascent Robo1 for endosomal degradation, preventing its expression at the growth cone membrane (Keleman et al., 2002; Keleman et al., 2005). Loss of Comm leads to a complete loss of commissures and increased Robo1 surface expression (Keleman et al., 2002; Myat et al., 2002). While the requirement of Comm for Robo1 downregulation is accepted, there is conflicting data about how Comm performs this function. One model proposes that Comm downregulates Robo through conserved PY motifs. These motifs would presumably interact with the WW motifs on HECT family E3 ubiquitin ligases, resulting in Comm ubiquitination, and subsequent degradation of the Comm-Robo1 complex. Since expression of Comm variants where these motifs are mutated abolishes Robo1 localization in the late endosome *in vitro* and reduced ectopic midline crossing *in vivo* the importance of the PY motifs is not disputed; however, initial findings determined this to be independent of the HECT E3 ligase Nedd4 (Keleman et al., 2005). In contrast, another report maintains that PY motif-dependent binding of Comm to Nedd4 and Comm ubiquitination are necessary for Robo1 downregulation (Myat et al., 2002).

More recently, additional *in vivo* experiments support the requirement of Comm PY motifs for midline crossing. *In vitro* and *in vivo* data demonstrate that Comm PY motifs are required for Robo1 ubiquitination and subsequent downregulation in the lysosome. Comm's PY motifs are then linked to Comm-mediated Robo1 localization in the late endosome and decreased Robo1 expression at the cell surface both *in vitro* and *in vivo*. Additional data establishes that Comm-dependent Robo1 downregulation is mediated by the formation of a Nedd4/Comm/Robo1 ternary complex. Finally, *in vivo* genetic evidence supports a requirement for Nedd4 in midline crossing. These findings establish a midground between the two previously proposed mechanisms implicating the PY motifs of Comm and Nedd4 in the downregulation of Robo1. In addition to resolving the mechanism of Comm-dependent Robo1 downregulation, this study also puts forth additional information about the role of these PY motifs in the endogenous late endosomal localization of Comm, as Comm colocalization with a late endosomal marker is decreased in PY mutants (Sullivan and Bashaw, 2024) (Figure 5A). It also indicates a PY dose-dependent Comm stabilization, suggesting that the Comm/Nedd4 interaction may be important for Comm downregulation. This is in-line with previous data detailing PY-dependent Comm ubiquitination (Myat et al., 2002). The potential ubiquitination and degradation of Comm by Nedd4 could provide a mechanism to explain the rapid downregulation of Comm in post-crossing axons that triggers increased Robo1 surface expression. This is all the more intriguing given that the mechanism of Comm downregulation remains undefined.

Unlike Slit and its receptor Robo1, Comm is apparently not conserved outside of dipterans, raising the question of how Robo1 receptors are maintained at low levels in pre-crossing commissural axons in the mammalian spinal cord. Interestingly, a similar E3 ubiquitin ligase adaptor-based mechanism for the degradation of mammalian Robo1 receptors was recently discovered (Gorla et al., 2019). Like Comm, Nedd4 Family Interacting Proteins 1 and 2 (Ndfip1/2) are also expressed in commissural

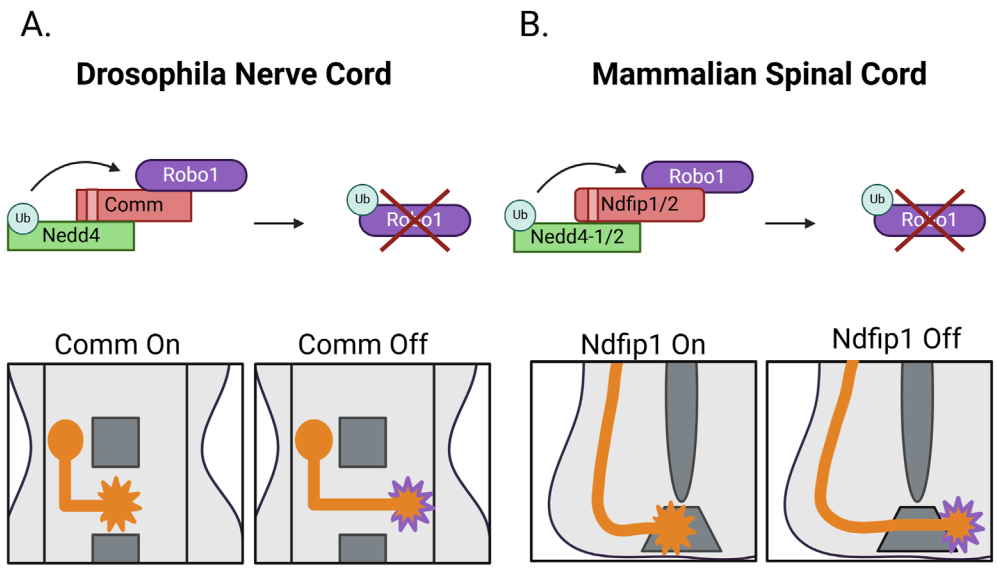

FIGURE 5
Nedd4-induced Robo1 degradation **(A)** During commissure formation in the *Drosophila* embryonic nerve cord, Comm binds Robo1 and acts as an adaptor to bring Robo1 into proximity with the HECT E3 ligase Nedd4. Through this ternary complex formation, Nedd4 ubiquitinates Robo1, resulting in its endo-lysosomal degradation. Robo1 downregulation prevents nascent Robo1 from reaching the growth cone membrane and impedes premature repulsive signaling in crossing commissural neurons. **(B)** The mammalian spinal cord leverages a similar adaptor-based mechanism during formation of the ventral commissure in which Robo1 binds the adaptors Ndfip1 and/or Ndfip2. These adaptors bind the HECT E3 ligases Nedd4-1 and Nedd4-2. Upon Robo1-Ndfip-Nedd4 complex formation, Robo1 is ubiquitinated and degraded via the endo-lysosomal degradative pathway, preventing Robo1 expression at the growth cone membrane. Post-crossing, Robo1 levels increase at the growth cone to prevent re-entry into the floorplate.

neurons of the murine embryonic spinal cord during commissure formation. Ndfip1/2 are known to act as adaptors for HECT family E3 ubiquitin ligases to assist in substrate recruitment via their WW-interacting PY and LPSY motifs. This interaction relieves the autoinhibitory conformation of the E3 ligase, promoting catalytic activity (Mund and Pelham, 2009). Notably, Ndfip proteins interact with Robo1, decrease Robo1 protein levels, and decrease Robo1 surface expression *in vitro*. Expression of Ndfip1/2 also increases Robo1 ubiquitination and degradation in a PY-dependent fashion (Gorla et al., 2019).

In vivo, the constitutive knockout of *Ndfip1/2* leads to dose-dependent decreases in commissure thickness at the floor plate in E11.5 mouse embryos. Dye-fill experiments in open-book preparations of the embryonic spinal cord provide more resolution to this reduction in commissure thickness and show that the loss of *Ndfip1/2* leads to increased CN stalling at the floor plate and aberrant ipsilateral turning both pre- and post-crossing. Interestingly, Robo1 protein levels increase in the spinal cord, the brain, and in the ventral commissure of these Ndfip mutant mice during crossing stages. This is in striking contrast to wildtype conditions, where Robo1 protein levels are downregulated until after E12.5 to promote CN crossing. Additionally, Robo1 expression is typically restricted to post-crossing CNs, creating a distinct absence of Robo1 protein at the ventral commissure (Gorla et al., 2019). This elevated expression of Robo1 prior to CN crossing could explain the CN stalling and ventral commissure thinning phenotypes.

After establishing Ndfip1/2 as Comm-like regulators of Robo1 during commissure formation of the mammalian spinal cord, subsequent work connected Ndfip1/2 to an E3 ligase-dependent mechanism of Robo1 lysosomal degradation. As their names indicate, Ndfip1/2 interact with many HECT family E3 ligases, and similarly to Comm, this interaction is dependent on their PY and LPSY motifs. Co-expression of E3 ligases with Ndfip proteins also increases Robo1 ubiquitination and degradation *in vitro*. This effect is dependent on the catalytic activity of E3 ligases as treatment with Heclin, a small molecule inhibitor of the catalytic HECT domain, prevents Nedd4-1/2 mediated Robo1 ubiquitination and degradation. Biochemical data showing that Robo1 ubiquitination is strongly attenuated in mammalian cells expressing both Robo1 and Nedd4 proteins but not Ndfip, reveals that Robo1 ubiquitination relies on the Ndfip1/2-dependent formation of the Robo/Ndfip/Nedd4 ternary complex. In addition, heclin-induced inhibition of HECT E3 ligases in primary CNs increases Slit-induced repulsion, indicating increased Slit responsiveness. *In vivo* Nedd4-1/2 are expressed during stages when CNs are crossing the floor plate, and the loss of *Nedd4-1/2* in commissural neurons results in thinning of the ventral commissure. The conditional knockdown of *Nedd4-1/2* also increased CN stalling and failure to reach the floorplate, although to a smaller extent than in *Ndfip1/2* knockout animals (Gorla et al., 2022). The pre-mature repulsion implied by the *in vivo* data, combined with the increased Slit response in heclin-inhibited primary CNs bolsters the model of Ndfip1/2-mediated Robo1 downregulation by Nedd4-1/2 during mammalian commissure formation (Figure 5B). Interestingly, in addition to Ndfip proteins, the PRRG4 protein has also been implicated in the regulation of Robo1 receptors *in vitro*, and in the context of breast cancer tumor metastases, PRRG4 has been shown to regulate Robo1 degradation through recruitment of Nedd4

(Justice et al., 2017; Zhang et al., 2020). Whether PRRG4 or other PRRG proteins regulate Robo1 in the context of axon guidance, in the mammalian spinal cord has not been explored.

Notably, for both Comm and Ndfip1/2, the ability to interact with multiple members of the HECT E3 ligases family does not translate to a role for all binding partners in the regulation of Robo1. In the case of Comm neither Smurf nor Su(dx), the other *Drosophila* HECT E3s, affect commissure formation *in vivo* (Sullivan and Bashaw, 2024). Similarly, only Nedd4-1, Nedd4-2, and WWP1 promote the *in vitro* ubiquitination and degradation of Robo1, despite the fact that other E3 ligases such as Smurf can form a ternary complex with Robo1 and Ndfip proteins (Gorla et al., 2022). These findings suggest there may be an additional layer of regulation between substrate recognition/recruitment and E3 ligase-mediated ubiquitination. These findings reveal the importance of Nedd4 proteins and their adaptors in the regulation of Slit-induced repulsion during midline crossing. Together with their importance in growth-cone attraction, this data identifies E3 ligases as important regulators of axon guidance.

Section 3: E3 ligases in neurodevelopmental disorders

Neurodevelopmental disorders (NDDs) constitute a diverse group of conditions with NDD patients exhibiting a wide range of neurological and psychological symptoms. According to the most recent edition of the Diagnostic and Statistical Manual of Mental Disorders there are seven categories of NDDs: Autism Spectrum Disorders, Attention-Deficit/Hyperactivity Disorder, Communication Disorders, Intellectual Disorders, Motor Disorders, Specific Learning Disorders, and Tic Disorders (American Psychiatric Association, 2013). These conditions often share common symptoms like cognitive impairment, seizures, mood disorders, social deficits, and varying degrees of motor dysfunction.

The neurodevelopmental field has undertaken the daunting task of attempting to link the genome wide association data derived from patient samples back to basic science to gain insight into the pathological mechanisms behind these disorders. Over time, one of the common themes that has emerged from this research is the important role of E3 ubiquitin ligases and the disruption of ubiquitin-induced protein degradation in the pathogenesis of NDDs (Wang Y. et al., 2020; Mabb, 2021; Krzeski et al., 2024). In this section, we will connect our discussion of the broader neurodevelopmental functions of E3 ligases like neuronal differentiation, axon guidance, and dendrite morphogenesis, with recent discoveries that shed light on the neurodevelopmental root of some NDDs (Table 1). This discussion is not intended to be exhaustive and only serves to highlight a few RING and HECTE3 ligases with well-defined mechanisms of specific substrate regulation in the context of NDDs.

Angelman Syndrome

Angelman Syndrome (AS) is a neuro-genetic disorder affecting 1 in 15,000 individuals that becomes apparent within the first year of life. Symptoms of AS include developmental delay, recurring seizures, movement disorders, sleep problems, and severe speech impairment. AS patient studies have revealed some of the underlying molecular mechanisms for the pathogenesis of AS that implicate mutations in the gene encoding UBE3A, a HECT E3 ligase. Some loss of function mutations decrease UBE3A expression and result in impaired dendritic spine development, while other variants are instead reported to decrease the E3 ligase activity of UBE3A (Kishino et al., 1997; Cooper et al., 2004; Dindot et al., 2007; Margolis et al., 2015; Beasley et al., 2020).

Interestingly, *in vitro* data supports an interaction between UBE3A and Huntingtin-associated protein 1 (HAP1), a protein expressed in the brain that has primarily been studied in the context of neurodegenerative disorders. In neurodegeneration, HAP1 is implicated in retrograde autophagosome transport and subsequent fusion with competent lysosomes (Maday et al., 2012; Wong and Holzbaur, 2014). Selective autophagy is a homeostatic process in which the autophagosome degrades organelles and other protein cargoes through fusion with the lysosome. In mice modelling the neurodevelopmental loss of function caused by *UBE3A* patient mutations, there is an increase in HAP1 protein expression, a decrease in HAP1 ubiquitination, and an increase in autophagy (Wang T. et al., 2019). *In vitro* assays in cells derived from *UBE3A* mutants and in cell lines expressing inactive forms of UBE3A affirmed that similar increases in autophagy were due to decreased HAP1 ubiquitination and its subsequent over-expression. The aberrant dendritic spine morphology seen in AS models, may also be linked to increased autophagy since pharmacological inhibition of autophagy rescues morphological and some behavioral phenotypes associated with these models; however, there is currently no direct connection between the HAP1 over-expression observed in AS neurons, and AS pathology (Wang T. et al., 2019).

By determining how HAP1 increases autophagy it may be possible to establish a causal link between AS and UBE3A loss of function. In the early stages of autophagy, autophagic receptors (ARs) bind membrane-bound autophagy-related (ATG) proteins that are important for the formation of the phagophore and the initiation of autophagy. Specifically, ATG14 is important for the formation of the PtdIns3-kinase (PtdIns3K) complex, and upon binding, targets the complex to the pre-autophagosome (Obara and Ohsumi, 2011). This targeting results in the formation of PtdIns3P, a lipid essential for the recruitment of additional autophagy machinery to the autophagosome (Brier et al., 2019). ARs also bind ubiquitinated cargo to mediate their incorporation into the autophagosome. (Münch and Dikic, 2018; Liénard et al., 2024). Data reporting HAP1-ATG14 associations also connects UBE3A loss of function with recruitment of the PtdIns3K complex, which enhances PtdIns3P formation, and increases autophagosome assembly (Wang T. et al., 2019; Nishimura and Tooze, 2020). These findings both expand the role of HAP1 in autophagy to neurodevelopment and provide insights into AS pathology. They also implicate HAP1 in autophagosome assembly, rather than its function in autophagosome transport and motor association that are linked to neurodegeneration. Additionally, PtdIns3P on the autophagosome recruits Tectonin domain-containing protein 1 (TECPR1), a protein required to induce autophagosome-lysosome fusion (Chen et al., 2012; Terawaki et al., 2015). HAP1 facilitating PtdIns3P formation and potentially recruitment of TECPR1 could also place HAP1 upstream of an autophagosome-lysosome fusion pathway. Given the recent discovery of various neural UBE3A

TABLE 1 Summary of discussed E3 ligases, substrates, and functions in Neurodevelopment.

Neurodevelopmental Process	E3 Ligase	Substrate	Neurodevelopmental Role	Associated NDD	References
Neural Differentiation	RNF220	Gli2/3	Shh gene transcription in spinal cord and hindbrain		Ma et al. (2019)
	CUL4A	Sox2	Neural progenitor gene transcription		Cui et al. (2018)
	WWP2	Sox2	Neural progenitor gene transcription		Fang et al. (2014)
	HUWE1	p53	Neural progenitor gene transcription	Juberg-Marsidi Syndrome	Aprigliano et al. (2021)
	RNF12/Rlim	Rex1	Embryonic stem cell gene transcription	X-linked Intellectual Disability	Bustos et al. (2018)
Axon Guidance	TRIM9	VASP	Netrin-mediated filopodia extension		Menon et al. (2015)
	TRIM9	Dcc	Netrin-mediated FAK signaling	[a]Congenital Mirror Movement Disorder	Plooster et al. (2017)
	TRIM67	Trim9	Netrin-mediated filopodia extension		Boyer et al. (2020)
	NEDD4	Robo1	Slit-mediated repulsion during midline crossing	[a]Horizontal Gaze Palsy	Gorla et al. (2022), Sullivan and Bashaw (2024)
Dendritic Morphology	CRL4	Dcx	Dendrite & axon outgrowth	X-linked Intellectual Disability	Shim et al. (2024)
	TRIM32	CDYL	Dendrite arborization; BDNF signaling	Autism Spectrum Disorder	Liu et al. (2022)
	UBE3A	HAP1	Autophagy during dendritic spine formation	Angelman Syndrome	Wang et al. (2019a)
	UBE3A	XIAP	Caspase3-mediated dendritic pruning	Autism Spectrum Disorder	Khatri et al. (2018)

[a]The direct involvement of E3 ligase regulation in the pathogenesis of this NDD is unclear

substrates (Krzeski et al., 2024), these insights highlight just one example of UBE3A as a key factor in the dysregulation of autophagy that is associated with the pathophysiology of AS.

Autism spectrum disorders

Autism Spectrum Disorders (ASD) are highly heritable, polygenetic disorders that are frequently characterized by social and language impairments and repetitive behaviors. According to the CDC, 1 in 36 children was diagnosed with ASD in 2020, with males being four times more likely to be diagnosed than females (Maenner et al., 2023).

UBE3A (also known as E6AP) is also linked to ASD susceptibility. While loss of function mutations in *UBE3A* are linked to AS symptoms, duplications and triplications of *UBE3A* are associated with ASD. The expression of only the maternal copy of *UBE3A* in the cerebral cortex and in Purkinje neurons in the cerebellum reinforces the importance of *UBE3A* dosage control in the brain (Albrecht et al., 1997; Hogart et al., 2010; Roy et al., 2023). In addition to increases in UBE3A copy number, a *de novo* autism-linked missense variant that leads to elevated UBE3A activity has also been identified (Yi et al., 2015). This specific mutation renders UBE3A resistant to normal inhibition by protein kinase A (PKA) phosphorylation, resulting in excessive E3 ligase activity. PKA inhibition of UBE3A appears to underly the effect of PKA on cortical neuron dendrite morphogenesis, since the increases in dendritic spine density observed upon chronic inhibition of PKA in primary cortical neurons is lost in UBE3A mutant neurons. This indicates that PKA's negative regulation of UBE3A may normally act to constrain dendritic formation. Interestingly, mis-expression of this "active" variant of UBE3A by *in utero* electroporation leads to a significant increase in dendritic spine density in layer 2/3 pyramidal neurons *in vivo* (Yi et al., 2015); however, the UBE3A substrates that account for the increased spine density remain to be explored.

In direct contrast to these findings, a more recent study reported that over-expression of UBE3A in primary neurons and elevated UBE3A expression in an ASD mouse model that carries three copies of the normal UBE3A gene leads to the opposite effect, a decrease in dendritic spine length and complexity. The effects of UBE3A over-expression coincide with increased levels of active caspase-3 (Khatri et al., 2018), which has previously been shown to promote dendritic pruning. UBE3A leads to the elevation of active caspase-3 by targeting its upstream inhibitor X-linked inhibitor of apoptosis protein (XIAP) for ubiquitination and degradation (Scott et al., 2005; D'Amelio et al., 2010). Consistent with this idea, expression of XIAP rescues the reduction in dendritic spine length and complexity in primary neurons over-expressing UBE3A (Khatri et al., 2018). Curiously, the UBE3A-dependent decrease in dendritic complexity is consistent with the earlier observation that UBE3A over-expression in hippocampal slice culture leads to reduction in synaptic transmission; however, in this study no effects on dendrite morphology were reported (Smith et al., 2011).

While the explanation for these discordant findings on the effects of UBE3A over-expression on cortical dendrite morphogenesis and spine density is unclear, there are many differences in the ways these studies were performed that make direct comparisons difficult. For example, two of these groups used UBE3A mice that carry triplication of the locus to achieve over-expression (Smith et al., 2011; Khatri et al., 2018), while the other used *in utero* electroporation (Yi et al., 2015); thus, the timing and levels of over-expression varied between the studies. In addition, the specific neurons examined differed in layer location and level of maturity, and there were differences in the ways dendritic structures were categorized. Regardless of these apparent discrepancies on the role of UBE3A, these observations indicate that the association of elevated UBE3A with ASD is correlated with changes in dendritic complexity and spine density and/or synaptic function. In addition, key UBE3A substrates that may contribute to these effects have begun to be identified, forming the foundation for future investigation.

In addition to UBE3A, mutations in RING E3 ligase Tripartate motif-containing protein 32 (TRIM32) increase risk for ASD and knockout of TRIM32 in mouse models results in an ASD-like phenotype (Zhu et al., 2021). Recent data proposes a role for TRIM32 in the regulation of Chromodomain Y-like (CDYL), a chromatin-binding protein that recruits histone methyltransferases to inhibit downstream gene transcription (Zhang et al., 2011; Wang M. et al., 2020). Specifically, CDYL interaction with Polycomb Repressive Complex (PRC2) and the subsequent recruitment of H3K27 methyltransferase to the promoter of brain-derived neurotrophic factor (BDNF) inhibits BDNF (Qi et al., 2014). This decreases BDNF binding to TrkB receptor tyrosine kinase and attenuates MAPK signaling important for dendritic growth (Finsterwald et al., 2010).

Biochemical data using proteins purified from rat brains demonstrates that TRIM32 interacts with CDYL through its N and C-termini. *In vitro* data reports that this results in CDYL ubiquitination and proteasomal degradation. TRIM32 over-expression in cultured hippocampal neurons significantly increases dendritic branching in a catalytic domain-dependent fashion, while shRNA-induced knockdown of TRIM32 decreases dendritic branching. This affect is CDYL-dependent, placing TRIM32 upstream of CDYL-mediated dendritic arborization (Liu et al., 2022). Further investigation of the impact of TRIM32 manipulation on BDNF transcription would cement this connection. The high density of dendritic spines in Purkinje neurons, combined with the developmental expression of TRIM32 and CDYL in the cerebellum may imply a generalized function for TRIM32 in dendritic arborization (Wang M. et al., 2020). TRIM32 seems to impact the formation of dendritic spines in the adult brain as well, marking a potential for sustained TRIM32 function (Zhu et al., 2021). These findings indicate an indispensable role for CRL4 and TRIM32 in orchestrating dendritic outgrowth.

X-linked intellectual disability

X-linked intellectual disability (XLID) is a broad term for over 150 different syndromes and more non-syndromic forms. Over 100 genetic mutations account for the syndromic forms alone, making them highly heterogeneous disorders (Lubs et al., 2012; Stevenson et al., 2012). Due to this marked heterogeneity, the clinical features of XLID vary, but they are commonly defined by impairment of mental abilities that alter adaptive conceptual, social, or practical skills (American Psychiatric Association, 2013). XLID is thought to arise from abnormalities in neural differentiation, neurite projection and dendritic spine formation due to the cortical differences observed in patients with XLID (Bassani et al., 2013; Telias and Ben-Yosef, 2014).

RNF12/Rlim is a RING E3 ligase associated with XLID. RNF12/Flim regulates neural gene expression through REX1 degradation and X-chromosome inactivation (Jonkers et al., 2009; Bustos et al., 2018; Frints et al., 2019; Wang and Bach, 2019). XLID-associated mutations in RNF12/Rlim are found in the basic region and the RING domain of the protein. *In vitro* experiments in cultured ESCs expressing the XLID RNF12/Rlim mutations results in decreased ubiquitination of its known substrates, REX1 and Smad7, due to decreased catalytic activity. Based on data recapitulating this decreased catalytic activity, accelerations in neural differentiation, and abnormal ESC differentiation in a knock-in mouse model, alterations in RNF12/Rlim-mediated ubiquitination could be the mechanism of pathology caused by these mutations in XLID patients (Bustos et al., 2018).

The HECT E3 ligase HUWE1 is also genetically linked to XLID and plays an important role in the neuronal and glial differentiation of NPCs in mice (Zhao et al., 2008; Friez et al., 2016; Giles and Grill, 2020; Muthusamy et al., 2020). Since HUWE1 regulates p53 in non-neuronal cells, and p53 is also linked to the NSC metabolic balance and neuronal differentiation, it is postulated that a similar mechanism could be at play in neurodevelopment (Yang et al., 2018; Marin Navarro et al., 2020). Interestingly *de novo* mutations in human patients with XLID have been traced to point mutations in the HECT domain and other regions of HUWE1. These mutations result in the upregulation of members in the p53 signaling pathway. A severe form of XLID called Juberg-Marsidi Syndrome (JMS), is characterized by a G4310R point mutation within the HUWE1 HECT domain (Friez et al., 2016). Despite the location of the mutation implying a possible difference in catalytic activity, the mutation seems to instead alter protein stability, resulting in decreased expression. In this context, it is

interesting to note that previous work on several other HECT family proteins including Itch, WWP1, and WWP2 indicates that HECT-WW domain interactions can confer autoinhibition (Wang Z. et al., 2019). When this intramolecular binding is perturbed, these HECT ligases display increased autoubiquitination and decreases in protein stability (Wang Z. et al., 2019). It remains to be explored whether the G4310R JMS mutant in HUWE1 upregulates the p53 pathway by reducing the binding affinity between HUWE1 and p53, or alternatively by leading to the autoubiquitination and degradation of HUWE1 itself.

Induced pluripotent stem cells cultured from patients with the G4310R point mutation, display an accumulation and excessive activation of p53, increased expression of CDKN1A/p21, and a concordant decrease in neural differentiation. Using patient-derived HUWE1 mutations, these findings support a causal link between the pathological neural differentiation impairment of JMS and aberrant regulation of the p53 signaling pathway caused by decreased HUWE1 stability (Aprigliano et al., 2021). These discoveries reveal functions for RNF12/Rlim and HUWE1 in the atypical neural differentiation found in XLID, and JMS respectively.

Mutations in the CUL4B loci are also linked to XLID (Zou et al., 2007). As previously discussed, Cullin Ring Ligase 4 complex (CRL4) can refer to a Cul4a-containing E3 ligase complex; however, CRL4 can also form with a Cul4b core, creating a similar but distinct complex. Interestingly, gene ontology and interactome analysis on cultured rat cortical neurons show interaction of Cul4a/b with several cytoskeletal proteins, including Doublecortin (Dcx), a microtubule associated protein (MAP) (Shim et al., 2024). Dcx stabilizes microtubules, facilitating their polymerization for the formation of exploratory axonal and dendritic extensions that will eventually synapse with surrounding neurons and form functional circuits (Parato and Bartolini, 2021). The potential importance of this protein's regulation in neurodevelopment are underpinned by the causative link of *Dcx* mutations in X-linked lissencephaly (Fu et al., 2013).

In addition to interaction, CRL4 ubiquitinates and downregulates Dcx *in vitro*. *In vitro* knockout of Cul4a and Cul4b resulted in longer, more complex neurites and dendrites, presumably through increased microtubule stability from sustained Dcx expression and activity. In cortical neuron cultures, activation of Cul4a/b is initiated by neddylation, and occurs early in neurodevelopment. Over-expression of Cul4a/b variants that cannot bind to their RING finger subunit or be activated by neddylation only increased neurite outgrowth in the Cul4a condition and increased dendritic branching in both conditions. Furthermore, *in vitro* over-expression of Cul4a alone decreases axonal and dendritic outgrowth, while Cul4b over-expression has no effect. This supports a mechanism in which CRL4a and CRL4b regulation of Dcx may differentially regulate axonal and dendritic outgrowth (Shim et al., 2024).

DCX is also ubiquitinated and degraded by Kelch-like 15 (KLHL15), a substrate-adaptor of the CRL3 complex. *In vitro* data indicate that DCX-KLHL15 ubiquitination depends on the DCX FRY domain. Like CRL4, the expression of KLHL15 antagonizes dendritic outgrowth in the presence of DCX (Song et al., 2021). Despite the previously identified mutations in DCX that are associated with X-linked intellectual disability seeming to be outside of its FRY domain, the similarity of key players and phenotypes might suggest that further investigation of potential link between key regulators of DCX and X-linked intellectual disability (Matsumoto et al., 2001).

Section 4: Future directions

Over the last several years, research has expanded our understanding of E3 ligases, implicating them in diverse neurodevelopmental processes. Advances in genetic tools and access to patient genomic data have also revealed roles for E3 ligases in the etiology of neurodevelopmental disorders. Nevertheless, many questions remain. In the case of Nedd4 and the regulation of the Robo1 receptor, Ndfip-dependent recruitment of HECT ligases to the receptor is necessary but not sufficient to trigger Robo1 ubiquitination. Specifically, both Smurf and Nedd4 can form a ternary complex with Robo1 and Ndfip proteins *in vitro*, but only Nedd4 can drive Robo1 degradation. This raises the important question of what distinguishes the substrate specificity of an E3 ligase from its functional specificity? Better understanding of this area may also provide structural information, enabling modulation of E3 ligase substrate interaction and E3 ligase function. Another important area for future investigation is the mechanism underlying differential E3 ligase expression and activation that can confer cell-type or temporal control of target protein activities, as exemplified by differential Sox2 regulation in NPCs versus ESCs.

Moreover, many of the E3 ligases discussed here have multiple functions throughout neurodevelopment; however, it remains to be seen if these proteins have important neuronal functions throughout life or in processes like neurodegeneration. This could inform whether NDD phenotypes in adults, like decreased synapse number in adults with ASD, primarily arise from developmental deficits, or if E3 ligase mutations continue to cause aberrations into adulthood, due to sustained requirements for these proteins in neuronal homeostasis (Matuskey et al., 2024). Continuing to leverage genomic data to direct the mechanistic analysis of NDD-associated E3 ligase mutations is also an important area for future work. Approaching the mechanism from the perspective of known NDD-associated proteins or pathways and determining their ability to interact with additional E3 ligases could also yield new insights.

Lastly, with so many E3 ligases encoded in the human genome, and many of them having more than one name, it will be beneficial to construct a consolidated interactive repository of E3 ligase substrates and spatiotemporal expression patterns in the central nervous system. Currently, expression and substrate data are divided between databases like ELiAH and UbiNet2.0, with a notable absence of temporal expression and all CNS expression data on ELiAH (Li et al., 2021; Paik et al., 2024). In all, continued exploration of novel E3 ligase substrates will improve understanding of how substrate ubiquitination leads to protein degradation, guides localization, and regulates alternative post-translational modification. This understanding will undoubtedly elucidate mechanisms important for neurodevelopment and the molecular basis of NDDs, but also in biological contexts outside of the nervous system.

Author contributions

MH: Conceptualization, Writing – original draft, Writing – review and editing. GB: Conceptualization, Funding acquisition, Writing – review and editing.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Generative AI statement

The author(s) declare that no Generative AI was used in the creation of this manuscript.

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Neuronal guidance behaviours: the primary cilium perspective

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The establishment of functional neuronal circuits critically relies on the ability of developing neurons to accurately sense and integrate a variety of guidance signals from their surrounding environment. Such signals are indeed crucial during key steps of neuronal circuit wiring, including neuronal migration and axon guidance, to guide developing neurons or extending axons towards their target destination in the developing brain. The growth cone, located at the tip of developing neurons, is a key subcellular structure in this process, that concentrates many different guidance receptors and signalling molecules and specialises in the probing and integration of extracellular signals into various guidance behaviours. Interestingly, the small primary cilium, long considered as a vestigial organelle, has progressively emerged as a cellular antenna specialised in cell signalling, and has been reported, just like the growth cone, to harbour a variety of guidance receptors. How primary cilium-elicited signals are then transduced into specific cellular processes to guide developing neurons and axons remains however obscure. In this review, we will summarise our emerging understanding of the role of primary cilium-elicited signalling pathways on neuronal guidance processes, by focusing on neuronal migration and axon guidance. We will highlight the primary cilium molecular diversity, and how it shapes the primary cilium functional versatility, allowing the ciliary compartment to instruct various guidance behaviours through the regulation of different cellular processes. We will moreover discuss current and future avenues of research, to unravel the different molecular effectors activated downstream of specific ciliary signals, and clues to be gained from studies performed in non-neuronal cells. Rising challenges of the field will also be addressed, such as the technical challenge induced by the dual subcellular localisation (*i.e.*, ciliary and extra-ciliary) of many ciliary guidance receptors, and the importance of the development of new genetic/chemo-genetic/optogenetic tools. Finally, we will highlight the insight such studies will bring for our understanding of the aetiology of different disorders, including ciliopathies, neurodevelopmental and neurodegenerative disorders, but also cancer cell migration/invasion, which are associated with defective primary cilium formation and function.

KEYWORDS

neuronal guidance, primary cilium, neuronal migration, axon guidance, signalling pathways

1 Introduction

Neuronal guidance signalling encompasses all the signalling processes that ensure precise neuronal positioning and wiring (Yuasa-Kawada et al., 2022). Neuronal migration and axon pathfinding are two major steps of this guidance process. Newly generated neurons indeed migrate from their birthplace to their final destination in the developing brain and extend their growing axons towards the right synaptic targets. The neuron's environment is a key ally in this developmental journey, as it provides different spatiotemporally-controlled guidance signals that enable developing neurons to ultimately integrate functional neuronal circuits. Depending on the neuronal subtype and/or the developmental stage, migration and axon navigation can occur either sequentially or concomitantly. Adding to this complexity, a same guidance signal can steer different populations of neurons and/or elicit different types of guidance behaviours (*e.g.*, neuronal migration or axon guidance), highlighting the importance for developing neurons to accurately sense and integrate multiple extracellular signals in order for accurate neural circuit wiring to occur.

Extracellular guidance cues are sensed by receptors/channels expressed at the surface of developing neurons and come in many different flavours. They can be chemical, including diffusible extracellular or cell-bound ligands (proteins, lipids, small molecules …), but also mechanical, or even electrical (Gangatharan et al., 2018; Medvedeva and Pierani, 2020; Dorskind and Kolodkin, 2021). The growth cone, that is formed at the tip of extending axons and migrating neurons alike, is known to express many guidance receptors and is extensively studied as a key structure specialised in the probing and integration of the extracellular environment (Lowery and Van Vactor, 2009; Stoeckli, 2018; Nakajima et al., 2024). Interestingly, developing neurons–as almost all vertebrate cells–possess another key subcellular compartment, the primary cilium (PC), that has progressively emerged as a cell antenna specialised in collecting signals from the environment. Indeed, mutations affecting the PC structure and/or function have been found to induce a group of developmental disorders termed ciliopathies. While the clinical manifestations of ciliopathies are multisystemic, and include retinopathy, obesity, diabetes, skeletal malformations, and hepatic disease, ciliopathies are also characterised by a wide range of neurodevelopmental defects, such as in the Joubert (JBTS), Meckel-Grüber (also called Meckel syndrome, MKS) or Bardet–Biedl syndromes (Reiter and Leroux, 2017; Andreu-Cervera et al., 2021; Karalis et al., 2022). These defects include brain malformations, ataxia, epilepsy, mental disability and highlight the importance of primary cilia in neuronal circuit wiring and function. Accordingly, recent studies have located several receptors/effectors of major guidance signalling pathways to the ciliary compartment (Higginbotham et al., 2012; Loukil et al., 2023). However, the precise signalling events elicited in response to guidance signals within the PC and transduced to downstream intracellular effectors in order to regulate neuronal guidance behaviours remain poorly understood.

In this review, we will summarise our current understanding of the role of PC-elicited signalling pathways on neuronal guidance processes, focusing on neuronal migration and axon guidance. We will highlight the importance of the molecular diversity of the ciliary compartment, and how it determines the functional versatility of PC signalling during neuronal guidance, regulating: **(i)** different guidance processes (*i.e.*, neuronal migration and axon navigation) sequentially or concomitantly, and **(ii)** different molecular mechanisms converging on a same guidance process (*e.g.*, neuronal migration). It is indeed important to bear in mind that the generic PC does not exist, and that ciliary composition is highly versatile, at different levels. First, **(i)** the PC protein composition varies throughout the lifespan of the cell: for example, the expression of the ciliary marker, adenylate cyclase 3 (AC3; *i.e.*, enzyme responsible for the cAMP cyclic nucleotide synthesis) is low in the embryonic brain, but increases during the first postnatal weeks, before decreasing again at later stages (Arellano et al., 2012). Ciliary protein composition is moreover **(ii)** highly dependent on the cell type, and depending on the cell type, **(iii)** a same ciliary protein can show different sub-ciliary localisation patterns (Hansen et al., 2022). We will moreover discuss current and future research avenues to unravel the many ramifications of molecular effectors activated downstream of specific PC-elicited guidance signals, and clues to be gained from studies performed in non-neuronal cells. Finally, we will highlight the insight such studies will bring for our understanding of ciliopathies, but also neurodevelopmental and neurodegenerative disorders or cancer cell migration, associated with defective PC formation and function.

2 The neuronal primary cilium: a signalling hub sensing environmental guidance cues

2.1 The primary cilium subcellular compartment

Primary cilia are small, microtubule-based structures that are contiguous with the plasma membrane and bud from the surface of almost all vertebrate cells. Observed as early as 1898 (Zimmermann, 1898), technical limitations have long relegated the PC to a vestigial organelle, until the development of transmission electron microscopy and the association made between primary cilia and ciliopathies gradually boosted our interest for this tiny organelle. Since then, ciliopathies have been reported one after the other, with the discovery of more and more ciliopathy-associated genes (Reiter and Leroux, 2017), the study of which has contributed to considerably increase our knowledge of the PC structure and function.

2.1.1 The primary cilium structure and composition

The architecture of the PC has been extensively studied. The PC is organised by a modified mother centriole, called **the basal body**, from which the ciliary microtubule core, called **the axoneme** (comprising nine microtubule doublets), extends, surrounded by the ciliary membrane (Figure 1). In mammalian neurons, the PC extends 2 to 12 µm from the cell surface, with a diameter ~ 200–500 nm (DeMars et al., 2023; Macarelli et al., 2023). Two main ciliogenesis pathways have been described: the extracellular pathway, and the intracellular one, that is the most studied (Wang and Dynlacht, 2018; Hoffman and Prekeris, 2022; Zhao et al., 2023). While extracellular ciliogenesis occurs in most polarised

epithelial cells, the intracellular pathway appears to be favoured by most other cell types (Sorokin, 1962; 1968; Molla-Herman et al., 2010; Labat-de-Hoz et al., 2021). In the intracellular pathway, ciliogenesis starts in the cytoplasm with the docking of the basal body to a large ciliary vesicle. The axoneme assembles from the basal body beneath this vesicle. As the axoneme extends, the ciliary vesicle expands to encapsulate the axoneme in a double membrane layer, with the ciliary membrane facing the axoneme and the ciliary sheat facing the cytoplasm. PC budding at the cell surface is then enabled by fusion of the ciliary sheat with the plasma membrane. Conversely, extracellular ciliogenesis is initiated by the docking of the basal body to the plasma membrane. As the axoneme extends from the basal body, the ciliary membrane is gradually formed from the plasma membrane. Whether in the extracellular or intracellular pathway, extension of the PC, in which translation does not occur, relies on a ciliary transport system, **the intraflagellar transport** (IFT), that uses the axoneme scaffold to provide all the building material required for membrane and axoneme extension, as well as for protein delivery and exit to and from the PC. IFT (Taschner and Lorentzen, 2016) is powered by the kinesin-II and dynein microtubule-based molecular motors for anterograde and retrograde transport along the axoneme, respectively. Trains of IFT particles, each composed of IFTA and IFTB subcomplexes, are assembled at the ciliary base and couple the molecular motors to the cargoes for ciliary trafficking to and from the PC tip.

2.1.2 The primary cilium: a signalling hub

This IFT system is important not only for ciliogenesis, but also for PC function. Indeed, the wide range of ciliopathy-associated phenotypes and target organs–ranging from skeletal, heart, kidney, renal or retinal malfunction to brain malformations and cognitive defects–highlights the crucial involvement of the highly conserved PC in the regulation of cell signalling and function. The PC is indeed now well established as a signalling hub at the crossroads between various signalling pathways (Christensen et al., 2012; Hilgendorf et al., 2016; Pala et al., 2017; Wheway et al., 2018; Anvarian et al., 2019; Nishimura et al., 2019; Mill et al., 2023). The IFT transport machinery plays an important part in the concentration and trafficking into and out of the tiny ciliary volume of many membrane receptors (*e.g.*, G-protein coupled receptors, ion channels, extracellular matrix receptors, purinergic receptors …) and signalling molecules (*e.g.*, second messengers, soluble proteins …). Of note, the precise molecular mechanisms involved in these various trafficking events remain to be clarified, and an IFT-independent lateral diffusion of certain ciliary membrane receptors along the axoneme has also been proposed (Milenkovic et al., 2009; Ye et al., 2013). Proteomic studies performed in non-neuronal systems have nevertheless contributed to confirm the diversity of proteins concentrated within the ciliary volume and hint at the wide variety of processes in which the PC signalling hub is involved (Ishikawa et al., 2012; Mick et al., 2015; Hansen et al., 2024; Liu et al., 2024).

This dense and diverse protein composition is a key feature of the PC compartment, along with its lipidic composition, that is distinct from that of the plasma membrane (Nakatsu, 2015; Conduit and Vanhaesebroeck, 2020). Different gating mechanisms, based on evolutionarily-conserved domains located at the base of the PC, act in concert with the IFT to strictly restrict the exchanges between the cytoplasm and the cilioplasm (Jensen and Leroux, 2017; Park and Leroux, 2022; Moran et al., 2024).

At the very base of the PC, the **distal appendages (or transition fibres**, see Figure 1**)** of the cell body connect the basal body to the ciliary membrane. IFT particles dock onto transition fibres before cargo trafficking to the ciliary compartment (Deane et al., 2001; Wei et al., 2013). Distal to the transition fibres, the **transition zone** is composed of Y-links that connect the axoneme to the ciliary membrane, and the ciliary necklace, comprising rows of membrane particles that encircle the base of the ciliary shaft. The transition zone appears to apply different gating mechanisms to safeguard the functional specificity of the ciliary compartment. Consistently, many ciliopathy-associated gene mutations affect transition zone proteins (Gonçalves and Pelletier, 2017). First, the transition zone appears to constitute a membrane diffusion barrier, with a ciliary zone of exclusion that prevents non-ciliary membrane proteins from entering the PC, but also maintains ciliary membrane proteins within the PC compartment (Williams et al., 2011; Cevik et al., 2013; Jensen and Leroux, 2017). Additionally, the transition fibres and transition zone appear to establish a soluble diffusion gate, in the way of a molecular sieve. Indeed, studies using a permeabilised system for ciliary trafficking in mammalian cells have reported that proteins of increasing size fused to GFP do not enter the PC with the same dynamics: while proteins below 4.8 nm enter the PC, entry is decreased for proteins between 4.8 and 8.6 nm, and is no longer detectable for larger proteins (Breslow et al., 2013). Similarly, diffusion of fluorescent proteins established a ciliary sieve-like barrier allowing the entry of soluble proteins with a Stokes radius as large as 7.9 nm (Lin et al., 2013). The precise molecular mechanisms involved in this sieve remain however elusive. A similarity with the nuclear pore complex (NPC) has been proposed, with studies revealing the implication of the nuclear transport machinery in ciliary trafficking (Dishinger et al., 2010; Fan et al., 2011; Kee et al., 2012), although some diffusion events may occur independently (Breslow et al., 2013).

This membrane and soluble diffusion barrier at the base of the PC allows the separation between the cytoplasm and the cilioplasm, and is essential for the functional specialisation of the ciliary antenna as an extracellular signal sensor. Consistently, studies have challenged the view that small second messenger signals (*e.g.*, cAMP and cGMP cyclic nucleotides, calcium), locally produced within the PC compartment in response to the activation of ciliary membrane receptors, can freely diffuse between the cytoplasm and cilioplasm (Delling et al., 2016; Jiang et al., 2019), and argue in favour of a ciliary compartmentalisation of second messenger signals, that signal and function independently from the cytoplasmic pool. Indeed, in FRET experiments, Moore and colleagues reported that in inner medullary collecting duct cells (IMCD3), primary cilia have a high basal cAMP concentration with regards to the cytoplasm (~5 times higher; Moore et al., 2016). In another study, pharmacological inhibition of the ciliary-localised vasopressin receptor type-2 in kidney epithelial cells induced increased cilioplasmic, but not cytoplasmic, cAMP levels. Conversely, fluid-shear stress decreased cilioplasmic cAMP levels, without affecting the cytoplasmic pool (Sherpa et al., 2019). In the case of cGMP, studies in *Caenorhabditis elegans* olfactory sensory neurons expressing a genetically encoded cGMP indicator show that, following odour exposure, ciliary cGMP levels transiently

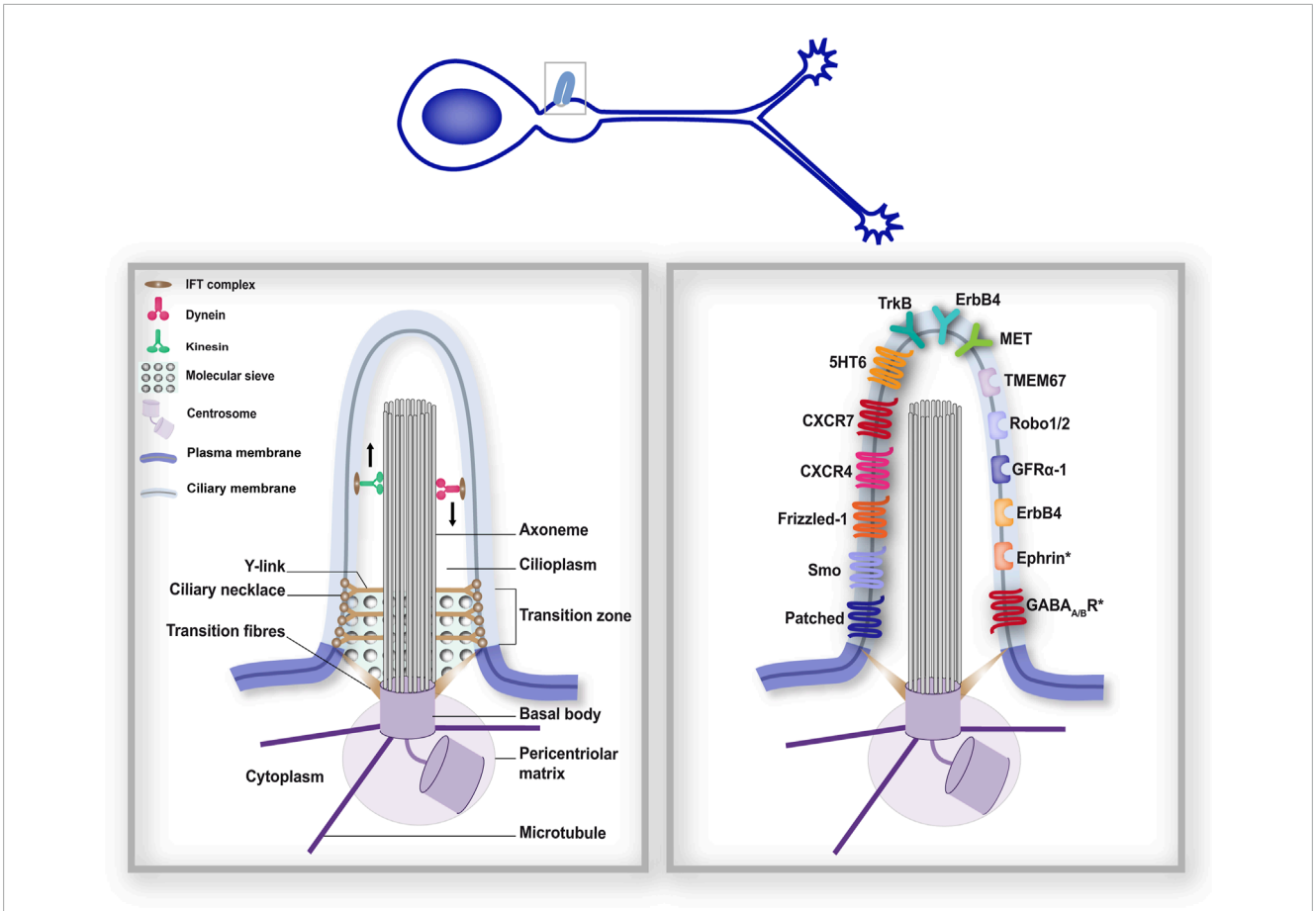

FIGURE 1
The primary cilium forms a distinct subcellular compartment that functions as a signalling hub. The structural organisation of the PC (left-hand boxed region) comprises different gating mechanisms that ensure a distinct protein composition of the ciliary compartment, in addition to its distinct lipidic composition. As a consequence, many membrane receptors have been reported at the surface of the PC. The right-hand boxed region depicts neuronal guidance-related membrane receptors reported at the surface of neuronal primary cilia during development (Rodriguez Gil and Greer, 2008; Williams et al., 2010; Petralia et al., 2011; Higginbotham et al., 2012; Toro-Tapia and Das, 2020). Receptors marked with an (*) were found in neuronal cilia postnatally (Loukil et al., 2023). Left- and right-hand boxed regions correspond to a higher magnification of the PC of the developing neuron depicted above.

decreased, while cGMP levels in dendrites and soma gradually increased (Shidara et al., 2017). Similar observations have also been reported for calcium (Nauli et al., 2008; Delling et al., 2013; Jin et al., 2014; Sanchez et al., 2023; Shim et al., 2023). At the functional level, ciliary *versus* extra-ciliary second messenger signals have been reported to regulate different signalling pathways and mechanisms. For example, optogenetic increase of ciliary cAMP levels in zebrafish developing somites was shown to inhibit Hedgehog signalling, while cytoplasmic cAMP levels did not (Truong et al., 2021). Similarly, in developing zebrafish embryos, ciliary PKA, by contrast to cytosolic PKA, was found to specifically regulate the Hedgehog pathway (Zhang et al., 2024). In line with these observations, Hansen and colleagues unravelled a ciliary cAMP signalosome that is functionally distinct from the cytoplasm and drives kidney cyst formation (Hansen et al., 2022). Moreover, during cortical interneuron migration, ciliary cAMP and cGMP signals were found to antagonise each other to regulate cell polarity, while centrosome-located cAMP and cGMP acted in synergy to control another aspect of migration, which is nucleokinesis (Atkins et al., 2023b). Similar reports have been made concerning calcium, unravelling the PC as a calcium-mediated mechanosensory compartment that is necessary and sufficient to instruct left-right asymmetry during zebrafish development (Djenoune et al., 2023).

2.2 The primary cilium: a key signalling platform for neuronal guidance signalling pathways

Among the variety of signalling pathways and cell functions regulated by the PC signalling hub, receptors for some of the major signalling pathways that are involved in neuronal guidance processes have been found.

The first major evidence establishing the PC as a key signalling compartment in neuronal development arose in 2003 from a forward genetic screen conducted by Huangfu and colleagues in mouse embryos. They discovered that genes encoding intraflagellar transport machinery proteins are essential for embryonic ventral

patterning through the signalling of Sonic hedgehog (Shh; Huangfu et al., 2003), one of the most important morphogens involved in neuronal development (Douceau et al., 2023). Since this pioneer study, the ciliary transduction of the Shh pathway–most commonly referred to as the canonical pathway (Teperino et al., 2014) – has been described (Rohatgi et al., 2007), and its role in neuronal development extensively reviewed (Bangs and Anderson, 2017). Since then, several components of the Shh transduction machinery have been localised to neuronal primary cilia (Figure 1, right-hand), such as the Patched receptor for Shh and the Smoothened (Smo) GPCR (a key signal transducer of the Shh pathway) in the PC of rat hippocampal neurons, or GPR161, which is a negative regulator of Shh canonical signalling (Mukhopadhyay et al., 2013), in the PC of dI1 commissural neurons (Petralia et al., 2011; Toro-Tapia and Das, 2020). Notably, Shh signalling at the PC has been involved in several neuronal guidance processes, including neuronal migration (Baudoin et al., 2012; Pedraza et al., 2024) and axon pathfinding (Dumoulin et al., 2024).

But the role of the PC in neuronal guidance processes is not limited to the transduction of the Shh signalling pathway. Another major guidance molecule, Wnt, primarily identified as a guidance molecule for navigating commissural axons in the mammalian spinal cord (Lyuksyutova et al., 2003) and subsequently involved in neuronal migration (Boitard et al., 2015; Bocchi et al., 2017), has been linked to the PC. The Wnt signalling pathway comprises a network of various signalling molecules, with Wnt ligands often activating frizzled receptors together with an array of different co-receptors. Two main branches of the pathway are classically distinguished: the canonical Wnt/β-catenin pathway and the non-canonical Wnt/PCP pathway. Signalling molecules of the Wnt transduction machinery have been found to localise to the PC of non-neuronal cells (*e.g.*, Dishevelled, β-catenin, LRP5/6). Among these, some have been reported in the primary cilia of neurons. Such is the case, for example, of Frizzled-1, expressed in the PC of developing olfactory sensory neurons (Rodriguez Gil and Greer, 2008). The transmembrane Frizzled-like receptor Tmem67/MKS-3, a transition zone protein that functionally binds Wnt5a (Abdelhamed et al., 2015) and whose mutations are responsible for the MKS and JBTS ciliopathies, has moreover been located to the PC base of the *C. elegans* ciliated sensory neurons (Williams et al., 2010). It has further been shown to regulate canonical Wnt/β-catenin signalling in the developing cerebellum (Abdelhamed et al., 2019). However, the relationship between PC and Wnt signalling is complex. While Wnt signalling can regulate ciliogenesis, the PC can regulate Wnt signalling. Moreover, the question of whether the PC structure is required for the activation and transduction of the Wnt/β-catenin signalling pathways is controversial (Anvarian et al., 2019; Vuong and Mlodzik, 2023; Niehrs et al., 2025). Of note, several ciliary signalling components of the Wnt pathway are not exclusively localised to the PC. Such is the case of Frizzled-1, which has also been found in dendrites and axons of developing olfactory sensory neurons (Rodriguez Gil and Greer, 2008), highlighting the need for further studies to distinguish ciliary from extra ciliary regulations of Wnt-associated processes.

In addition to the Shh and Wnt pathways, extensively studied for their ciliary transduction, key molecular players in neuronal guidance pathways classically studied for their role in growth cones, have also been linked to the PC compartment. Immunohistochemistry experiments performed in migrating cortical interneurons have indeed identified several guidance receptors at the ciliary surface, namely, the TrkB receptor for BDNF (Brain-derived neurotrophic factor), the GFRα-1 receptor for GDNF (glial cell line-derived neurotrophic factor), CXCR4 and CXCR7 receptors for the CXCL12 chemokine, the ErbB4 receptor for Neuregulin1 (NRG-1), serotonin receptor 6 (5HT6), receptors Robo1 and 2 for Slit, and the MET receptor for HGF/SF (hepatocyte growth factor/scatter factor; Higginbotham et al., 2012). In addition to these receptors, an *in vivo* BioID (iBioID) proteomic screen has recently revealed in the PC of adult neurons (Loukil et al., 2023) the presence of Ephrin (involved both in neuronal migration and axon guidance processes) and GABA-A and GABA-B receptors, involved in synaptogenesis (Fiorentino et al., 2009; Sui et al., 2024) and neuronal migration (Heck et al., 2007). Finally, the receptor tyrosine kinase PDGFR-α (Clement et al., 2013), the CD44 hyaluronan receptor (Jones et al., 2012; Lee et al., 2020) and neuropilin 1 (Pinskey et al., 2017), all involved in different neuronal guidance processes (see sections below), have also been localised to the PC of non-neuronal cells. Future studies will be crucial to unravel how this multitude of ciliary signalling receptors regulate specific steps of neuronal guidance, in a cell type and cell stage specific manner.

Together, these studies pinpoint the neuronal PC as a key subcellular signalling compartment in neuronal guidance, integrating a variety of extracellular cues at the crossroads between different guidance processes. The downstream signalling effectors activated by ciliary guidance receptors, and how they regulate guidance processes, remain however obscure. This is mostly due to the technological challenge that represents the dissection of the ciliary-specific functions of guidance signalling receptors/effectors, with dual subcellular localisation (*i.e.*, ciliary and extra-ciliary). Yet, during the past decade, some labs have developed innovative strategies to tackle this issue and provided important new insights into the molecular mechanisms underlying the PC-elicited regulation of neuronal guidance pathways. In the following sections, we will review our current knowledge of PC function in neuronal migration and axon guidance, and discuss future avenues to be explored.

3 Primary cilium signalling in neuronal migration

3.1 The primary cilium compartment in neuronal migration

A role for the PC in the acquisition of cell polarity and directed cell migration has long been established in various non-neuronal systems (Christensen et al., 2013; Veland et al., 2014). In fibroblasts, for example, the PC–together with the centrosome–re-orients prior to the initiation of migration (Katsumoto et al., 1994) and is then oriented parallel to the direction of the movement (Albrecht-Buehler, 1977). Furthermore, the PC genetic ablation abrogates chemical or electrical stimuli-evoked directed cell migration in fibroblasts or mesenchymal stem cells (Schneider et al., 2005; 2010; Pruski et al., 2016; 2019; Lee et al., 2020; Nakazato et al., 2023). Mutation of a ciliopathy-associated gene was also found to

induce neural crest cell migration defects in the zebrafish model (Tobin et al., 2008). Despite such evidence, a role for primary cilia in neuronal migration has remained vaguer and more controversial, with some data reporting PC formation in the neocortex only after neuroblast migration has occurred, and no PC involvement in the establishment of neuronal polarity, neuronal migration or cortical laminar organisation (Arellano et al., 2012). By contrast, other groups have reported a role for primary cilia in the apico-basal polarity of radial glial cells (Higginbotham et al., 2013), in the tangential migration of cortical interneurons (Baudoin et al., 2012; Higginbotham et al., 2012), as well as in neuroblasts migrating postnatally through the rostral migratory stream towards the olfactory bulb (Matsumoto et al., 2019; Stoufflet et al., 2020). Strengthening the decisive role of the PC in neuronal migration, several gene mutations responsible for neurodevelopmental disorders–including ciliopathies or focal malformations of cortical development–and affecting ciliogenesis have been reported to impair radial or tangential neuronal migration in the developing cortex (Guo et al., 2015; Park et al., 2018).

3.2 Guidance cue-evoked primary cilium molecular pathways in neuronal migration

Neuronal migration is a well-documented cyclic saltatory process (Bellion et al., 2005; Schaar and McConnell, 2005; Tsai and Gleeson, 2005). In the first step of the cycle, migrating neurons probe their surroundings by extending and stabilising a leading process in an attractive or permissive environment. The centrosome then moves forwards to a proximal region within this process, called the dilatation or swelling compartment, before the nucleus dynamically translocates towards the centrosome in a process termed nucleokinesis. In 2012, Baudoin and colleagues showed that the PC genetic ablation altered the ability of interneurons migrating *ex vivo* in brain organotypic slices to exit their tangential migration stream and invade their target destination (*i.e.*, the developing cortical plate), in a way that mimics Shh pathway inhibition, suggesting a role for Shh-initiated PC signalling in neuronal migration (Baudoin et al., 2012). The same year, Higginbotham and colleagues identified by immunohistochemistry experiments many guidance cue receptors in the PC of migrating cells (*i.e.*, TrkB, GFRα-1, CXCR4, CXCR7, ErbB4, 5HT6, Robo1 and 2, MET). Using a microfluidic device, they moreover cultured cortical interneurons and dorsal cortical cells in two opposite chambers linked by microlanes, allowing to expose the cortical interneurons of one chamber to a gradient of migration-regulating cues secreted by the dorsal cortical cells of the other chamber. Using this setup, the authors further revealed that PC-ablated cortical interneurons (*i.e.*, interneurons carrying a null-mutation for the small regulatory GTPase Arl13b) exhibit defective migration towards the source of the gradient, compared to wild-type interneurons (Higginbotham et al., 2012). These two pioneer studies have opened the exciting and complex question of how the activation of guidance receptors at the PC may regulate the different steps of neuronal migration: what are the specific downstream signalling events and cellular processes regulated by these PC-dependant guidance signals?

Very few studies have started to tackle this question. In a study performed in tangentially-migrating mouse neurons in the postnatal rostral migratory stream, genetic ablation of the PC led to altered nucleokinesis of migrating neurons, in a mechanism dependent on a centrosome-located cAMP hotspot, thereby linking the PC regulation of migration to a downstream centrosomal component (Stoufflet et al., 2020). Recently, the same group proposed a ciliary pathway involving GPR161 mechanosensitivity as the upstream trigger regulating the centrosomal cAMP hotspot and the organisation of the nuclear cage of microtubules, required for proper nucleokinesis to occur (Paillard et al., 2025). Given the wide range of guidance receptors expressed at the ciliary surface, linking specific PC-elicited guidance signals to specific downstream effectors and migratory behaviours remains however challenging. The fact that many ciliary membrane receptors are also expressed at the extra-ciliary plasma membrane further complexifies the situation, highlighting the need to develop new tools to bypass loss of function approaches and alter PC-elicited signals specifically at the ciliary compartment. Using newly developed genetically encoded molecular tools targeted to the PC to selectively modulate (*i.e.*, increase or buffer) PC-elicited second messenger signals, combined with live cell imaging and pharmacological/genetic approaches, Atkins and colleagues recently added some pieces to the puzzle. They showed that the CXCL12 chemokine controls the cell polarity and branching behaviour of migrating cortical interneurons by decreasing the ciliary cAMP/cGMP ratio upon binding to its CXCR4 receptor (Atkins et al., 2023b; Figure 2, top). Such technological development paves the way towards the dissection of the specific role on migratory behaviours of other guidance receptors present at the ciliary surface, and to the identification of their specific downstream molecular effectors.

Precious clues may be gained from studies already linking ciliary molecular mechanisms to cell migration in non-neuronal cells. Interestingly, in such systems, PC-elicited signals have been reported to impact cell migration through the regulation of various mechanisms.

3.2.1 The primary cilium and the regulation of membrane dynamics

One of those mechanisms concerns **the regulation of membrane dynamics** (Figure 2, middle). In a study conducted by the Christensen lab in fibroblast cells, the Platelet-Derived Growth Factor AA (PDGF-AA) protein activated the PI3K-AKT and MEK1/2-ERK1/2-p90^{RSK} pathways at the PC, and inhibiting these pathways counteracted the ability of PDGF-AA to stimulate migration in scratch-assay experiments (Clement et al., 2013), corroborating previous studies from the group (Schneider et al., 2005; 2010). Moreover, Clement et al. found that PDGF-AA signalling at the PC activates the Na^+/H^+ exchanger NHE1 and is critical for directed migration. More precisely, they show that while AKT inhibition impedes NHE1 vesicles from reaching the plasma membrane, inhibition of MEK1/2 abolishes the preferential localisation of NHE1 to the plasma membrane of the cell front, with cells displaying a broader NHE1 membrane distribution in multiple membrane locations (Clement et al., 2013). This study builds upon a previous study from the group involving NHE1 in directed cell migration downstream of ciliary PDGF-AA signalling (Schneider et al., 2009), and is in agreement with other studies

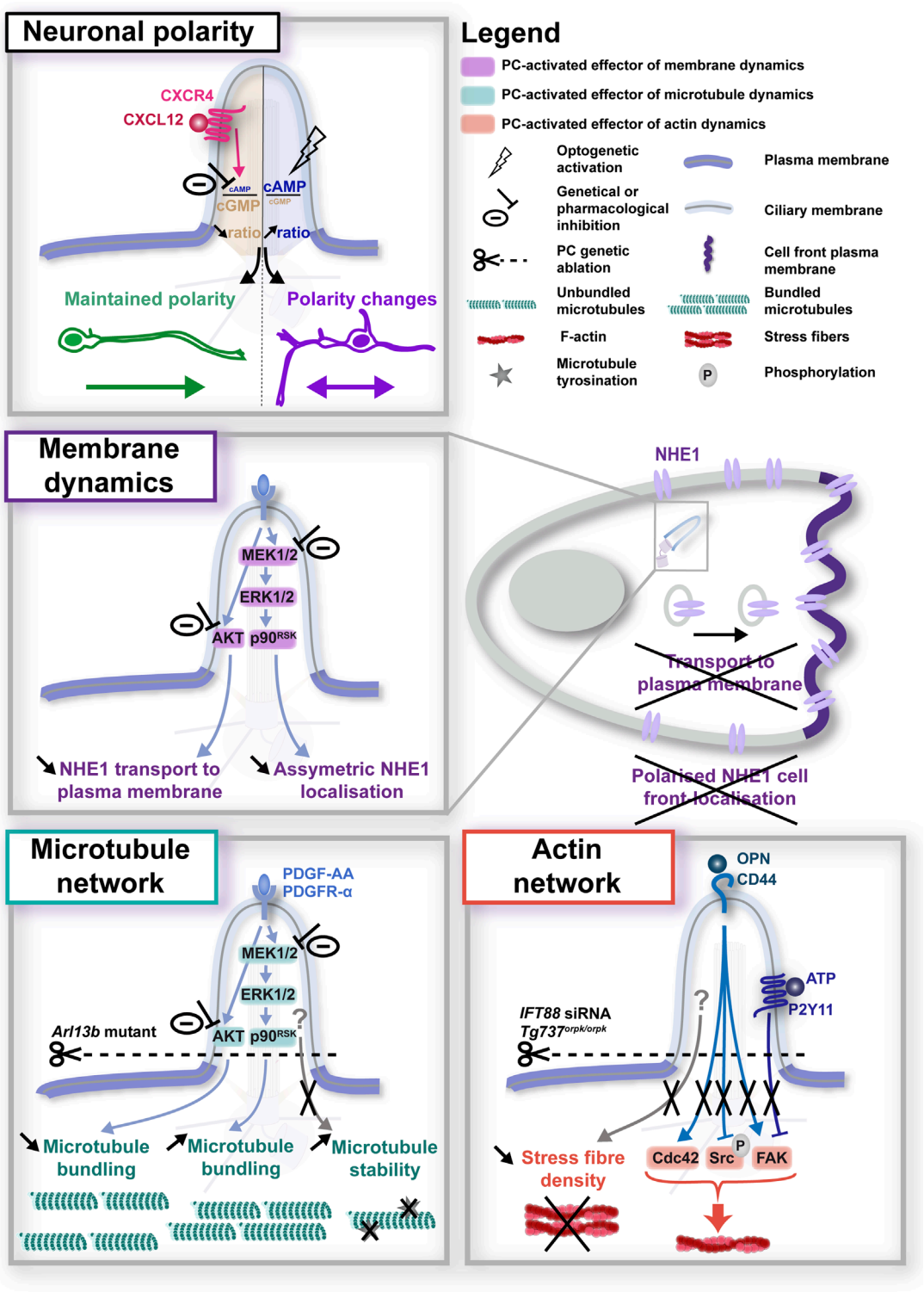

FIGURE 2
Primary cilium-elicited signalling pathways in neuronal migration. Top: in neurons, regulation of the ciliary cAMP/cGMP ratio downstream of CXCL12/CXCR4 activation at the PC surface was found to regulate the cell polarity and direction of migrating cells (top), although the downstream effectors activated in the cytoplasm remain to be identified (Atkins et al., 2023b). Middle and bottom summarise the research on downstream cytoplasmic effectors performed in migrating non-neuronal cells, that converge on the regulation of membrane dynamics (middle; Clement et al., 2013) or the microtubule (left-hand bottom; Clement et al., 2013; Pruski et al., 2016) and actin network (right-hand bottom; Jones et al., 2012; Mansini et al., 2019; Lee et al., 2020). In all panels, experimental manipulations (genetic, optogenetic or pharmacological) performed to alter ciliary signals, together with their phenotypic consequences, are colour-coded in black.

establishing a role for NHE1 in cell migration and invasion (Cardone et al., 2005; Stock and Schwab, 2006; Stock and Pedersen, 2017), through various mechanisms, such as the regulation of cell polarity by anchoring actin filaments to the cell front plasma membrane (Denker and Barber, 2002). Of note, this role for ciliary MEK1/2 activation in NHE1 asymmetric membrane localisation is highly coherent with the well-established role of the PC in cell polarity and directed migration, also reported in migrating neurons (Atkins et al., 2023b). Together, these data open the possibility of a role for the PC in the regulation of the cell front behaviour through the control of membrane dynamics and/or the targeting of specific receptors to the plasma membrane (Figure 2). Interestingly, the PDGFR-α receptor for PDGF-AA has been found expressed in migrating neurons of the external germinal layer (EGL) of the cerebellum (Andrae et al., 2001). However, although it has been involved in the migration of astrocytes (Itoh et al., 2011), its role in neuronal migration remains uncharacterised. On the other hand, NHE1 has been involved in the migration and invasive behaviour of cancer cells in glioblastoma (Cong et al., 2014), as well as in early neurite outgrowth during neuronal development (Sin et al., 2009; 2020). To our knowledge, its regulation of neuronal migration has so far not been described, let alone downstream of neuronal PC activation. Thus, while they appear as attractive candidate players in PC-dependant cell migration, future studies will be required to determine whether PC-elicited guidance pathways, PDGFR-α-NHE1-related or -independent, may regulate membrane dynamics to control cell polarity or plasma membrane composition in a context of neuronal migration.

3.2.2 The primary cilium and the regulation of cytoskeletal dynamics

Another key cell process reported in non-neuronal migrating cells downstream of PC-elicited pathways is the **regulation of cytoskeletal dynamics** (Figure 2, bottom). Very few studies have analysed the effect of PC signalling on microtubule dynamics. The Christensen lab has nevertheless reported defects in extra-ciliary microtubule bundling downstream of PDGF-AA signalling at the PC (Clement et al., 2013), in addition to an effect of an *Arl13b* null mutation on microtubule detyrosination (*i.e.*, a post-translationnal modification that correlates with a more stable state of microtubules) reported by Pruski and colleagues in mouse embryonic fibroblast cells (Pruski et al., 2016). By contrast, more studies have addressed the question of a role for the PC on actin dynamics during cell migration, with the identification of different F-actin regulators activated by PC signalling during cell migration. First, genetic ablation of the PC by siRNA-mediated knockdown of the intraflgellar transport 88 (IFT88) protein was found to abolish the phosphorylation of **focal adhesion kinase** (FAK, a tyrosine kinase that functions as a signalling scaffold for the assembly and maturation of the focal contacts regulating cell adhesion), that occurs in response to osteopontin (OPN) signalling at the PC in wild type migrating mesenchymal stem cells (Lee et al., 2020). A similar decrease in FAK phosphorylation following PC genetic ablation (deletion of intraflagellar transport protein Tg737: $Tg737^{orpk/orpk}$) was observed in endothelial cells, in association with a decreased directionality of migrating cells (Jones et al., 2012). Moreover, in migrating cholangiocytes, ATP stimulation of the ciliary purinergic receptor P2Y11 induced a rapid degradation of FAK in ciliated cells, which was abolished in de-ciliated cells (Mansini et al., 2019). Another F-actin regulator targeted by PC signalling is the **Src kinase**, whose phosphorylation dynamics are disrupted in migrating cells upon PC genetic ablation compared to controls, whether in basal conditions or following OPN signalling (Lee et al., 2020). Of note, the same study reported an increased expression of **the Cdc42 Rho GTPase** in IFT88-silenced cells. Finally, and in addition to these different actin regulators, the PC has been suggested to regulate **the stress fibre network** of migrating endothelial cells, which regulates several functions in migrating cells, such as the generation of traction forces, the maturation of integrin-based adhesions, the establishment of cell polarity (Vicente-Manzanares et al., 2009). Intriguingly, studies report a reduction of the actin stress fibres observed in mutated endothelial cells displaying impaired PC assembly ($Tg737^{orpk/orpk}$), compared to controls (Jones et al., 2012). To our knowledge, the P2Y11 purinergic receptor and the CD44 surface hyaluronan receptor (for OPN) have not been localised to neuronal primary cilia. However, independently of the PC, CD44 has been involved in the migration of neural precursor cells (Deboux et al., 2013). Similarly, purinergic receptors have been involved in neuronal migration or axon guidance (Rodrigues et al., 2019), although the P2Y11 receptor has not been reported so far in such processes.

Importantly, microtubule and F-actin remodelling are well established as key driving forces of neuronal migration (Schaar and McConnell, 2005; Shan et al., 2021) and axon guidance (Sánchez-Huertas and Herrera, 2021; Atkins et al., 2023a). Consistently, several guidance receptors found by the Anton lab in the PC of migrating cortical interneurons (Higginbotham et al., 2012; see Figure 1) are known to regulate membrane or cytoskeletal dynamics in a PC-independent context. These data highlight the need to dissect whether and how guidance signals elicited in neuronal primary cilia regulate cytoskeletal remodelling and/or membrane/receptor trafficking to drive specific migratory or axon steering behaviours.

4 Primary cilium signalling in axon guidance

4.1 The primary cilium compartment in axon guidance

Evidence of a role for the PC in axon navigation processes came from axonal tract defects observed in patients. Indeed, several ciliopathies (*i.e.*, Joubert, Meckel Gruber, Acrocallosal and Orofacial Digital Syndromes) have been associated with a defective development of the corpus callosum (CC; Salonen, 1984; Odent et al., 1998; Holub et al., 2005; Takanashi et al., 2009; Poretti et al., 2011; Putoux et al., 2011), which consists in the largest axonal tract of the brain, formed by millions of axons that connect homologous cortical areas of the two brain cerebral hemispheres. Consistently, in Joubert Syndrome, defects of other major axonal tracts, displaying failure to cross the midline, have also been reported, such as the corticospinal tract (CST; Poretti et al., 2007; Théoret et al., 2013) and the superior cerebellar peduncle (SCP) tract (Spampinato et al., 2008). Of note, the molar tooth sign, characterised by thickened and elongated SCPs that fail to

cross the midline, is one of the hallmarks of Joubert Syndrome and related disorders (Maria et al., 1999; Sattar and Gleeson, 2011; Romani et al., 2013). Defective decussation, fasciculation and/or branching of axonal tracts–including the SCP, CST, CC tracts and developing sensory corneal nerves–has also been reported in mouse models of Joubert syndrome and related disorders (Guo et al., 2019) or following the conditional knockout of the ciliopathy-associated *IFT88* gene (Portal et al., 2019). Additionally, abnormal projection of thalamocortical axons towards the amygdala was reported in two ciliary mouse mutants (Magnani et al., 2015). Similarly, RNAi silencing of the Joubert Syndrome gene *C5orf42* in chick embryos led to pathfinding defects of the commissural dI1 axons (Asadollahi et al., 2018). Corroborating these studies, in a genetic screen based on the *in utero* electroporation of a library of 30 shRNA targeting ciliopathy-linked genes in the cortex of E14,5 mouse embryos, Guo and colleagues identified aberrant axonal trajectory and fasciculation of neurons depleted for BBS5, BBS7, BBS9, BBS11, BBS12 and TMEM216 (Guo et al., 2015). Of note, changes in the adhesion properties of a developing neuron are likely to modify the way its axon will interact with other axons and/or cells from the surrounding environment, in a complex manner that can lead to axon guidance defects. Consistently, in the case of BBS5 and BBS7 knockdown, the authors moreover report defective axonal midline crossing towards the contralateral cortex, with miss-directed axons that, instead of crossing, project aberrantly towards subcortical targets once they have reached the midline.

4.2 Guidance cue-evoked primary cilium molecular pathways in axon guidance

While some of these axonal tract defects have been shown to occur in a non-cell autonomous manner, as a result of the defective distribution of glial and neuronal guide post cells (Benadiba et al., 2012; Laclef et al., 2015; Putoux et al., 2019), studies have also identified a cell autonomous role for the ciliary compartment in the regulation of axon pathfinding, involving different PC-elicited signalling pathways. In a study performed by the Anton lab, the conditional knockdown of the Joubert Syndrome-associated gene *Arl13b* in cultured deep cerebellar nuclei (DCN) neurons led to reduced dynamic axonal branching, aberrant growth cone morphology with altered filopodia-lamellipodia balance (*i.e.*, numerous longer filopodial protrusions), as well as impaired axon-axon adhesion associated with reduced recruitment of the protocadherin-17 (Pcdh17) to axon-axon contacts (Guo et al., 2019; Figure 3, bottom and middle). Interestingly, these axonal and growth cone morphological defects were associated to an increase in the ciliary levels of the PIP3 second messenger. Using elegant tools based on the CIBN/CRY2 dimerization optogenetic system, Guo and colleagues showed that recruiting PIP3 or AKT to the PC of DCN neurons is sufficient to alter growth cone morphology and dynamics by inducing filopodial protrusions. They further use DREAAD chemo-genetic tools to show that modulating the activity of ciliary G-protein coupled receptors GPCRs (that are known to converge onto PIP3) recapitulates the PIP3-AKT-linked growth cone morphological defects (Figure 3, top). Together, these data highlight PIP3-AKT as a PC-elicited signalling pathway involved in growth cone remodelling and behaviour.

Given that the PC, that is organised by the centrosome, is located near the cell soma and consequently at a distance from the axonal growth cone, such results raise the question of the ciliary downstream molecular effectors and mechanisms that propagate the signals down the axon to the exploring growth cone. Interestingly, Guo and colleagues observed a gradual increase in PIP3 activity at the growth cone of DCN neurons following ciliary PIP3 activation (Guo et al., 2019; Figure 3, top), and propose that positive feedback networks involving kinase-dependent cascades may rapidly spread locally-induced PC signalling over long distances. Following RNA-seq analyses in E12.5 *Arl13b*$^{-/-}$ and control embryos, they further propose PC-induced regulation of transcriptional programs as an additional mechanism to regulate axon navigation processes (Figure 3, bottom). Their identification in ciliary mutants of differentially expressed genes involved (among other processes) in cell adhesion opens the possibility that the defective Pcdh17-mediated axon-axon adhesion observed in Arl13b conditional knockout neurons may be due to altered gene transcription. In agreement, the Stoeckli lab has recently identified a role for the PC of developing chick commissural axons in mediating a transcriptional switch of Shh receptors, required to elicit the well-documented behavioural switch (from attraction to repulsion) of commissural axons crossing the midline (Dumoulin et al., 2024). In chick dI1 neurons, the authors indeed showed that *IFT88* silencing impaired dI1 axon midline crossing in a cell autonomous manner. *IFT88* silencing was moreover associated in *in situ* hybridisation experiments with a reduced expression of the Hhip (hedgehog-interacting protein) receptor, which is required for the repulsive response to Shh and the rostral turn of post-crossing commissural axons (Bourikas et al., 2005; Wilson and Stoeckli, 2013). Importantly, preventing Smo entry in the PC in response to Shh activation, pharmacologically or genetically (using a hSmoCLD construct that prevents Smo ciliary localisation after endogenous Smo silencing), led to misprojecting commissural dI1 axons or reduced Hhip expression, respectively, supporting the requirement of Shh signalling at the PC for the induction of *Hhip* transcription and correct dI1 axon guidance (Dumoulin et al., 2024; Figure 3, bottom). This elegant study further opens the question of whether additional mechanisms required for axon guidance may be regulated by the PC, such as the axonal transport or exocytosis of Hhip at the growth cone membrane. In addition to a role for canonical Shh signalling in mediating gene transcription required for axon guidance, a non-canonical Shh pathway (*i.e.*, that is transcription independent) that relies on the PC has been reported in the axonogenesis of chick postmitotic neurons (Toro-Tapia and Das, 2020). In developing chick embryos, neuroepithelial cells undergoing proliferation have been reported to delaminate from the neuroepithelium as they exit the cell cycle. Postmitotic neurons then initiate axon outgrowth and navigation for the formation of functional neuronal circuits. In this study, authors showed that as neuroepithelial cells delaminate, the PC is disassembled through apical abscission, followed by a PC re-assembly at the onset of axonogenesis. Preventing ciliary re-assembly by chromophore-assisted light inactivation impaired the axonogenesis of newborn neurons by inducing axonal collapse. Using a Gli reporter construct, authors further observed that canonical Shh signalling (*i.e.*, Gli activity-dependent) in the PC is lost upon delamination, and is no longer observed in the newly assembled PC. Although this newly-assembled PC gradually displayed Smo accumulation (suggestive of Shh signalling), immunostaining revealed the presence

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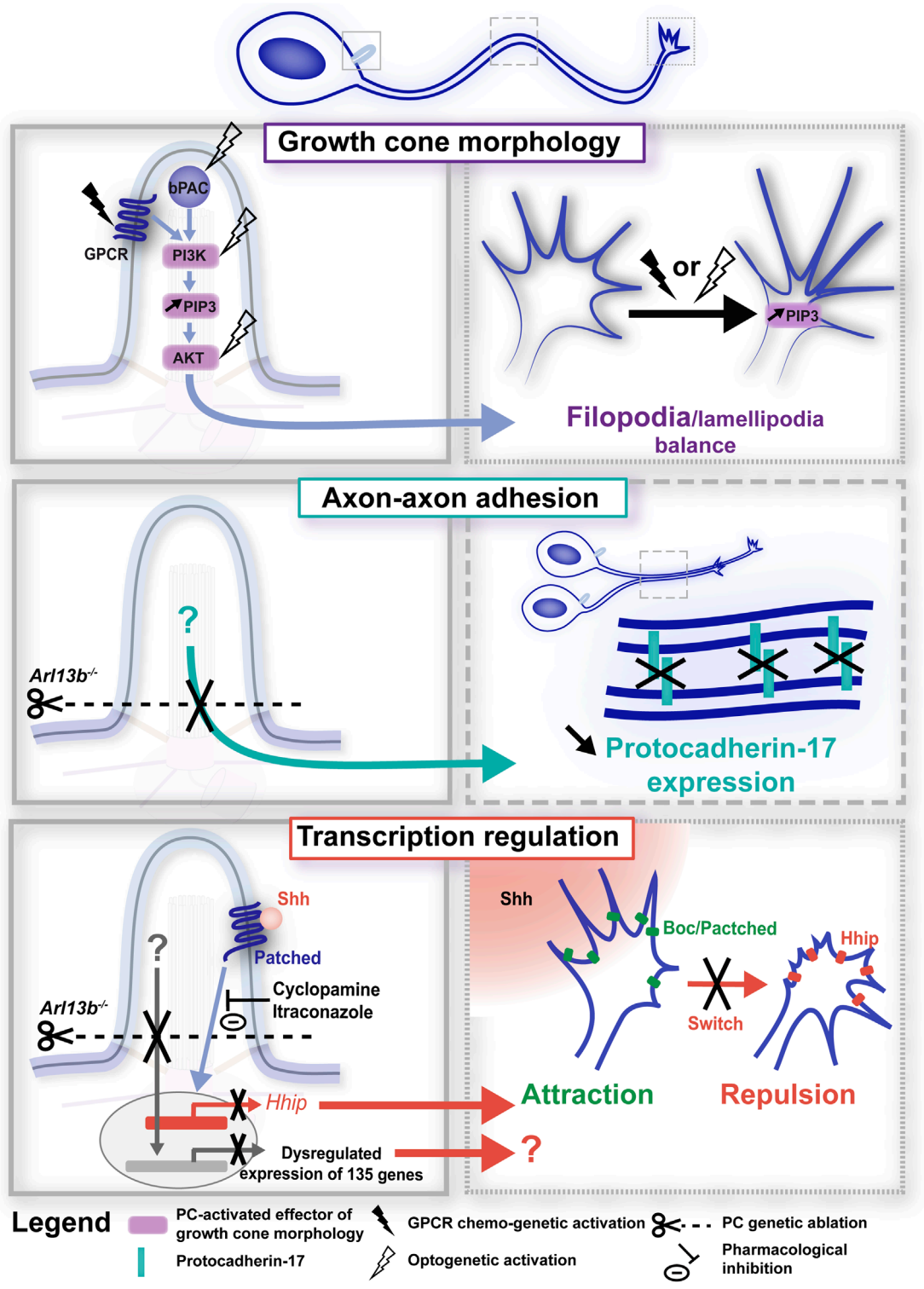

FIGURE 3
Primary cilium-elicited signalling pathways in axon pathfinding. Signalling pathways elicited at the PC (left-hand boxed regions, full line) induce phenotypic changes at the axonal and/or growth cone compartments (right-hand boxed region, large and small dotted lines, respectively). PC-elicited signalling pathways have been found to regulate axon pathfinding dynamics through the regulation of growth cone morphology (top; Guo et al., 2019), axon-axon adhesion (middle; Guo et al., 2019) and transcription (bottom; Guo et al., 2019; Dumoulin et al., 2024). In all panels, experimental manipulations (genetic, chemo-genetic, optogenetic or pharmacological) performed to alter ciliary signals, together with their phenotypic consequences, are colour-coded in black. Left- and right-hand boxed regions correspond to a higher magnification of the PC (full line), axon (large dotted line) or growth cone (small dotted line) compartments of the developing neuron depicted above. GPCR, G-protein coupled receptor; bPAC, bacterial (*Beggiatoa*) photoactivated adenylyl cyclase; Shh, Sonic hedgehog.

of the GPR161 negative regulator of canonical Shh signalling. Finally, pharmacological inhibition of the Src family kinases, which mediate the cytoskeletal rearrangements downstream of non-canonical Shh signalling, induced axon collapse, supporting a model in which the re-assembled PC is required for axonogenesis by mediating non-canonical Shh signalling. Whether the non-canonical Shh signalling is also required during growth cone turning events, in addition to axon extension, remains to be uncovered.

Taken together, these studies show to what extent guidance signalling pathways initiated in the ciliary compartment close to the soma influence the axon and growth cone behaviours required for accurate axon navigation. It is interesting to note that a long-distance influence of ciliary signals was also reported to regulate the branching behaviour of the leading process in the case of neuronal migration (Atkins et al., 2023b). Further studies will be required to precisely unravel the molecular effectors linking ciliary signals to axonal and growth cone behavioural remodelling.

5 Conclusion: Insights to be gained from ciliary guidance pathways for our understanding of the aetiology of neurodevelopmental disorders

The increasing interest for the once-neglected ciliary compartment initially arose from the discovery of its involvement in a wide range of disorders. Indeed, in addition to ciliopathies, a dysfunction of the PC has now been involved in different neurodevelopmental (*e.g.*, schizophrenia, autism spectrum disorder, bipolar disorder, intellectual disability …) and neurodegenerative disorders (Valente et al., 2014; Kaliszewski et al., 2015; Youn and Han, 2018; Park et al., 2019; Hasenpusch-Theil and Theil, 2021; Karalis et al., 2022; Ma et al., 2022; Volos et al., 2025), as well as in cancer cell migration/invasion (Eguether and Hahne, 2000; Higgins et al., 2019), including glioblastoma (Álvarez-Satta and Matheu, 2018). Conversely, studying the PC-elicited signalling pathways and molecular mechanisms regulating guidance processes in physiological conditions now appears as a key step to better understand the aetiology of such disorders. Interestingly, our increasing knowledge of PC-elicited guidance signalling and its functional and molecular versatility, both refines and complexifies our understanding of the role of this tiny organelle in pathology, at multiple levels.

First, the PC can regulate multiple aspects of a same neuronal guidance process. For example, during cell migration, the PC controls membrane dynamics, cytoskeletal dynamics but also focal adhesion dynamics. This occurs either through the activation of different ciliary membrane receptors (*e.g.*, PDGFR-α, P2Y11, CXCR4), or through the activation of a same ciliary receptor (*e.g.*, PDGFR-α) that can regulate multiple cellular mechanisms (*e.g.*, membrane and microtubule dynamics, see Figure 2), sequentially or concomitantly through the activation of several parallel downstream pathways.

Second, a same ciliary signalling molecule can be involved in different stages of neuronal guidance. For example, in the genetic screen performed by Guo and colleagues, silencing of the Bardet-Biedl Syndrome-associated *BBS7* gene led to a disrupted apical-basal polarity of radial glial cells, but also to a defective multipolar to bipolar transition of migrating principal neurons, and to altered axonal trajectory and fasciculation of cortical neurons (Guo et al., 2015). Likewise, while Shh appears to regulate the migration of developing cortical interneurons (Baudoin et al., 2012), it is also involved in the extension and navigation of developing axons, either through the transcriptional regulation of key guidance receptors (Dumoulin et al., 2024), or through a non-canonical pathway involving Src kinase activation (Toro-Tapia and Das, 2020).

Third, the presence of multiple guidance receptors both at the PC and growth cone surface highlights the importance of understanding the specific function of guidance receptor activation at each subcellular compartment. For example, guidance receptors such as Robo1/2 and ErBb4 have been linked to neurodevelopmental disorders, such as Autism Spectrum Disorder for Robo1/2 (Anitha et al., 2008) and schizophrenia and bipolar disorder for ErbB4 (Iwakura and Nawa, 2013; Mei and Nave, 2014). Identifying the specific contribution of the compartmentalised ciliary signalling of these receptors appears crucial in this context to better apprehend the complexity of such disorders and gain new insigths into their aetiology and treatment. Likewise, in cancer cell migration, while CXCL12 (Guo et al., 2016; Luo et al., 2019; Hayasaka et al., 2022) and Ephrin (Campbell et al., 2006; Wang, 2011; Cho et al., 2018) signalling have been linked to metastasis, the specific role on invasive behaviour of their local signalling at the ciliary compartment remains poorly characterised. Unravelling PC-elicited signalling pathways and downstream molecular effectors may therefore provide precious clues for future translational studies aiming to identify new therapeutic targets specific to PC signalling in order to selectively correct specific cell behaviours (*i.e.,* invasion).

Understanding the specific role of the identified ciliary guidance receptors (see Figure 1) in different steps of neuronal guidance is a crucial step of this complex process. The complexity of the task lies in the diversity of ciliary receptors, that are not always exclusive to the ciliary compartment. Rising to this challenge will critically rely on the use and development of new tools to selectively manipulate (*i.e.*, block/activate) specific membrane receptors located exclusively at the ciliary surface (without affecting the other ciliary receptors through PC genetic ablation, for example,) or their downstream second messenger signals. Such genetic, chemo-genetic and optogenetic tools are already starting to emerge to selectively buffer endogenous ciliary second messenger signals or trigger specific second messenger signalling within the ciliary compartment (Guo et al., 2019; Hansen et al., 2022; Atkins et al., 2023b).

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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The author(s) declare that no Generative AI was used in the creation of this manuscript.

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